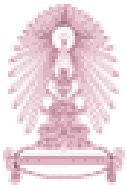

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BIOREMEDIATION OF SOIL CONTAMINATED WITH CARBOFURAN UNDER SATURATED CONDITION: A SOIL COLUMN STUDY

Mr. Wisarut Suphannafai

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Environmental Management
(Interdisciplinary Program)
Graduate School
Chulalongkorn University
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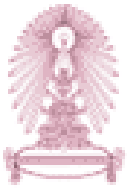

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การบำบัดทางชีวภาพของดินที่ปนเปื้อนคาร์โบฟูรานภายใต้สภาวะอิ่มตัวด้วยน้ำ: การศึกษาในคอลัมน์ดิน

นาย วิศรุต สุพรรณฝ่าย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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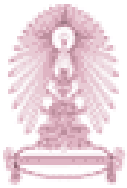

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Thesis Title	Bioremediation of Carbofuran Contaminated in Soil under Saturated Condition: A Soil Column Study
By	Mr. Wisarut Suphannafai
Field of Study	Environmental Management
Thesis Advisor	Associate Professor Alissara Reungsang, Ph.D.

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วิศรุต สุพรรณฝ่าย: การบำบัดทางชีวภาพของดินที่ปนเปื้อนคาร์โบฟูรานภายใต้สภาวะอิ่มตัวด้วยน้ำ: การศึกษาในคอลัมน์ดิน. (BIOREMEDIATION OF SOIL CONTAMINATED WITH CARBOFURAN UNDER SATURATED CONDITION: A SOIL COLUMN STUDY.)
อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. อลิศรา เรืองแสง, 99 หน้า

งานวิจัยนี้ได้ใช้คอลัมน์ดินเป็นแบบจำลองพื้นฐานเพื่อจำลองการเคลื่อนที่ของคาร์โบฟูรานในดินในนาข้าวภายใต้สภาวะอิ่มตัวด้วยน้ำ และศึกษาการประยุกต์ใช้เทคนิคการกู้ฟื้นฟูทางชีวภาพ ได้แก่ การเติมจุลินทรีย์ (bioaugmentation) และการกระตุ้นจุลินทรีย์ (biostimulation) เพื่อส่งเสริมการย่อยสลายคาร์โบฟูรานในดินนาข้าว และป้องกันการเคลื่อนที่ของคาร์โบฟูรานจากดินนาข้าวสู่แหล่งน้ำใต้ดิน ใช้ความเข้มข้นคาร์โบฟูรานในดินเท่ากับ 10 มิลลิกรัมต่อกิโลกรัมดิน ในชุดการทดลองที่มีการเติมจุลินทรีย์ ได้ใช้จุลินทรีย์ที่มีความสามารถในการย่อยสลายคาร์โบฟูราน ได้แก่ *Burkholderia* sp. PCL 3 ในรูปแบบเซลล์อิสระและเซลล์ตรึง สำหรับชุดการทดลองที่มีการกระตุ้นจุลินทรีย์ได้ใช้ฟางข้าวเป็นแหล่งสารอินทรีย์เสริม ผลการทดลองพบว่า ในชุดควบคุมที่ไม่มีกิจกรรมของจุลินทรีย์ และมีกิจกรรมของจุลินทรีย์ประจำถิ่นในดินเพียงอย่างเดียว มีปริมาณคาร์โบฟูรานที่พบในน้ำที่ไหลผ่านคอลัมน์ เท่ากับ 52.07 และ 22.5 เปอร์เซ็นต์ของปริมาณคาร์โบฟูรานที่เติมลงไปในดินตามลำดับ การใช้เทคนิคการเติมจุลินทรีย์หรือการกระตุ้นจุลินทรีย์อย่างใดอย่างหนึ่งช่วยเพิ่มประสิทธิภาพการย่อยสลายคาร์โบฟูรานในดิน และป้องกันการเคลื่อนที่ของคาร์โบฟูรานในดินได้ โดยพิจารณาจากการลดลงของปริมาณคาร์โบฟูรานที่พบในน้ำที่ไหลผ่านคอลัมน์ซึ่งมีค่า อยู่ในช่วง 14.62-15.54 เปอร์เซ็นต์ สำหรับชุดการทดลองที่มีการใช้เทคนิคการเติมจุลินทรีย์ที่ร่วมกับการกระตุ้นจุลินทรีย์พบว่ามีปริมาณคาร์โบฟูรานที่พบในน้ำที่ไหลผ่านคอลัมน์อยู่ในช่วง 22.08-22.57 เปอร์เซ็นต์ จากการศึกษาจำนวนจุลินทรีย์ที่มีความสามารถในการย่อยสลายคาร์โบฟูรานในน้ำที่ไหลผ่านคอลัมน์ในระหว่างการทดลอง และจำนวนจุลินทรีย์ที่มีความสามารถในการย่อยสลายคาร์โบฟูราน ในดินเมื่อสิ้นสุดการทดลอง พบว่าจำนวนจุลินทรีย์ในน้ำที่ไหลผ่านคอลัมน์มีค่าอยู่ในช่วง 10^5-10^8 CFU ต่อมิลลิลิตร และจำนวนจุลินทรีย์ในดินมีค่าอยู่ในช่วง 10^4-10^8 CFU ต่อกรัมดิน และพบว่าจำนวนจุลินทรีย์ในดินแต่ละส่วนไม่มีความแตกต่างกัน ผลทดลองแสดงให้เห็นว่าสามารถประยุกต์ใช้เทคนิคการฟื้นฟูทางชีวภาพ เพื่อเพิ่มประสิทธิภาพการย่อยสลายคาร์โบฟูรานในดิน และป้องกันการเคลื่อนที่ของคาร์โบฟูรานสู่น้ำใต้ดินได้ โดยเฉพาะในบริเวณที่มีการใช้คาร์โบฟูรานอย่างแพร่หลาย

สาขาวิชา การจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต .....
ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

Chulalongkorn University
จุฬาลงกรณ์มหาวิทยาลัย

5187581120: MAJOR ENVIRONMENTAL MANAGEMENT
KEYWORDS: SOIL COLUMN/ BIOREMEDIATION/ *Burkholderia* sp. PCL3/ CARBOFURAN/ IMMOBILIZATION/ SATURATED ZONE

WISARUT SUPHANNAFAI: BIOREMEDIATION OF SOIL CONTAMINATED WITH CARBOFURAN UNDER SATURATED CONDITION: A SOIL COLUMN STUDY. THESIS ADVISOR ASSOC. PROF. ALISSARA REUNGSANG, 99 pp.

In the present study, the disturbed soil columns were used as a basic model to simulate the movement of carbofuran in rice field soil under the saturated condition. Bioremediation technique i.e., bioaugmentation and biostimulation were applied to enhance the degradation of carbofuran in soil and prevent the movement of carbofuran to groundwater. The initial carbofuran concentration in soil was 10 mg kg^{-1}soil. The specific carbofuran degrader, *Burkholderia* sp. PCL3, in free and immobilized cells forms were used as seed inocula in bioaugmentation treatments. Rice straw was used as organic amendment in biostimulation treatments. In abiotic control and in the treatment with only indigenous microorganisms, the mass recovery percentage of carbofuran in the effluent was 52.07 and 22.54%, respectively. The application of bioaugmentation or biostimulation alone significantly enhanced carbofuran degradation and reduced the movement of carbofuran in soil as indicated by a low mass recovery in the effluent in the range of 14.62-15.54%. In the soil column with bioaugmentation together with biostimulation treatments, the mass recovery in the effluent were in the range of 22.08-22.57%. Number of carbofuran degraders in the effluent from each column was in the ranges of 10^5-10^8 CFU ml^{-1}. Number of carbofuran degraders 10^4-10^8 CFU $soil^{-1}$was not markedly different, among soil sections at the end of column operation. Results suggested that the bioremediation technique could be applied to enhance the degradation of carbofuran in soil and prevent the movement of carbofuran to groundwater especially in the area where carbofuran have been extensively used.

Field of Study: Environmental Management Student's Signature : ……
Academic Year: 2010 ..... Advisor's Signature: ……

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Ultimatley, I could not accomplish this thesis work without the inspiration from my relatives especially my parents and my grandmother throughout my thesis work.

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CHAPTER I

INTRODUCTION

1.1 Rationale

Carbofuran (2,3-dihydro-2,2 dimethylbenzofuran-7-yl methylcarbamate) is a broad-spectrum insecticide widely used in agriculture especially in rice fields to control insects and nematodes on contact or after ingestion. Carbofuran is of environmental concern because it can cause highly acute toxicity to mammals through cholinesterase inhibition, neurotoxicity and adverse reproductive effects (Lalah and Wandiga, 1996; EPA, 2006). In 2008, Thailand imported carbofuran in solid and liquid forms up to 5,000 tons for agriculture purposes (FMC corporation, 2009). Continuous use of carbofuran in agricultural areas may cause contamination risk to surface water and soil because carbofuran is soluble in water with a solubility of 351 mg l^{-1} (Paraíba et al., 2007). Thus, carbofuran is highly mobile (Tariq et al., 2006), resulting in a high potential for groundwater contamination. Understanding in the movement of carbofuran in soil and the removal of carbofuran from a contaminated area is necessary to prevent contamination of carbofuran into groundwater.

One of the effective routes for pesticide removal is microbial degradation by a specific degrader and/or indigenous microorganisms. Previous studies reported the discovery of microorganisms capable of degrading carbofuran and other pesticides from contaminated natural matrices (Bano et al., 2004; Yan et al., 2007; Mallick and Dutta, 2008). These degraders could use the pesticide as their energy source i.e. C- or N-, or C and N-sources. The addition of microbial cultures capable of degrading pesticide, or the so-called bioaugmentation technique, is reported to be an effective bioremediation approach for improving pesticide degradation in contaminated soils and water that lacks indigenous microbial activity (Plangklang and Reungsang, 2009, 2010). In addition to bioaugmentation, biostimulation is another bioremediation treatment to remove pesticides contamination in the environment. This treatment stimulates the activity of the indigenous microorganisms by the addition of organic and/or inorganic additives. The amendments added would be used by the indigenous microorganisms for cell growth, resulting in an increase in cell numbers as well as their activities to degrade the pesticides. Moreover, the

amendments could act as enzyme-inducers and/or co-metabolic substrates in the pesticide degradation pathways (Robles-Gonzalez, 2008).

The use of a soil column to simulate and monitor the movement of pesticide in soil have been reported (Abdullah et al., 2001; Farahani et al., 2008), however, the reports on the prevention of the movement of pesticides to soil is limited. In the present study, the soil columns were used to simulate the movement of carbofuran in rice field soil under saturated condition. The saturated condition represented the rice cultivation under flooded condition. The mobility of carbofuran in soil collected from rice fields was investigated to understand the possibility of carbofuran to contaminate groundwater. The bioremediation treatments i.e., the bioaugmentation technique using specific carbofuran degrader *Burkholderia* sp. PCL3 and biostimulation technique using rice straw as organic amendment were applied with the aim of enhancing the degradation of carbofuran in soil by preventing the movement of carbofuran to groundwater, especially in the area where carbofuran has been extensively used.

1.2 Thesis objectives

The main objective of this study is to investigate the effect of bioremediation techniques on carbofuran degradation and the movement of carbofuran to soil under saturated conditions. The soil column was used as a basic model to represent the carbofuran degradation in soil under saturated condition. A saturated condition is used to mimic a rice cultivation condition i.e., flooded soil. In order to achieve the major goal, the main objective was divided into 4 sub-objectives as follows:

1.2.1 To investigate the carbofuran degradation and movement of carbofuran in the soil column with the present of only indigenous microorganisms.

1.2.2 To study the effect of a bioaugmentation technique using *Burkholderia* sp. PCL3 in free and immobilized cells form on carbofuran degradation and its movement in the soil column.

1.2.3 To study the effect of biostimulation using rice straw as an organic amendment on carbofuran degradation and its movement in soil column.

1.2.4 To study the combined effect of bioaugmentation and biostimulation techniques on carbofuran degradation and its movement in soil column.

1.3 Research scope

This research covers the investigation of carbofuran degradation and its movement in rice field soil under saturated condition in soil column. Eight soil column treatments including control were studied to determine the effects of bioremediation technique i.e. bioaugmentation and biostimulation, natural attenuation as well as the effect of the abiotic degradation process on carbofuran degradation efficiency and its movement in soil. A carbofuran degrader, *Burkholderia* sp. PCL3, in free and immobilized cells form was used as the inoculum in bioaugmentation treatments. Rice straw was used as organic amendment in the biostimulation treatments. The soil column study was used to represent *in situ* bioremediation in a saturated zone. Carbofuran residues, the metabolite products, pH value and the number of carbofuran degraders in the soil and in effluent were analyzed throughout the experiment.

1.4 Hypotheses

Addition of microorganisms capable of degrading carbofuran and/or organic amendment can enhance the carbofuran degradation efficiency in soil under saturated condition and prevent the movement of carbofuran into groundwater.

CHAPTER II

LITERATURE REVIEW

2.1 Carbofuran

Carbofuran (2, 3-dihydro-2,2 dimethylbenzofuran-7-yl methylcarbamate) (Figure 2.1) is a broad-spectrum insecticide widely used in agriculture to control insects and nematodes on contact or after ingestion (EPA, 2006). 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).

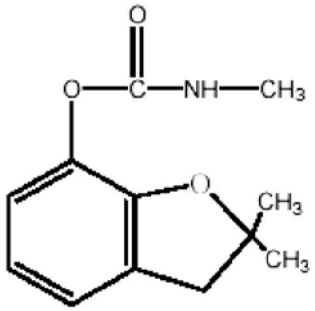

Figure 2.1 Structure of carbofuran (Evert, 2002)

2.1.1 Properties of carbofuran

Carbofuran is an odorless, crystalline solid varying from colorless to grey depending on the purity of the compound. It is soluble in water (320 mg l^{-1} at 25 ^{o}C) (DeVries and Evans, 1999) and has a low adsorption coefficient (K_{oc}= 22, Table 2.1) leading to a high potential for groundwater contamination (Howard, 1991). The physicochemical properties of carbofuran are shown in Table 2.1.

Table 2.1 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 (at 25 ^oC)	5X10^{-5} Pa m^3 mol^{-1}
Octanol/water partition coefficient (K_{ow})	17 for 1 mg l^{-1} at 20 ^oC 26 for 10 mg l^{-1} at 20 ^oC
Adsorption coefficient (K_{oc})	22 ml g^{-1}
Solubility at 25^oC - in water - in acetone - in xylene	0.07% (w/v) 15.0% (w/v) 1.0% (w/v)
Specific gravity (at 20 ^oC)	1.18 g cm^{-3}
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

2.1.2 Use of carbofuran

In 2008, Thailand imported carbofuran in form of Furadan 3G up to approximately 5,000 tons for agriculture purpose (FMC Cooperation, 2008). Carbofuran was widely applied in plants and crops such as rice, corn, sorghum, potato, tobacco, banana, cotton, vegetables etc. (Ngampongsai, 1990). In rice fields, Furadan granules 3% (3G) is occasionally applied to young plants after 10 d of seeding at the rates of 1.3 to 1.6 kg ha^{-1}. In crop fields, the granular formulations of carbofuran are applied to the soil at

the time of seeding. Furadan 5% (5G) can also be applied to potato, onions, turnip and carrot at the rate of 2-5 kg ha^{-1} (IDPID, 1993). Liquid carbofuran is 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 routes 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 and Aagard, 2003). Since carbofuran is 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 areas (Ferrell, 2003). Carbofuran is moderately persistent in soil. Its half-life in soil is approximately 30 to 120 d 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 highly mobile in soil and in surface runoff (Cohen, 1996). Carbofuran could be sorbed and less mobile in clay soil because of organic matter and clay content (Kumari et al., 1988). The half-lives of carbofuran in water at 25 ^{o}C are 690, 8.2, and 1.0 weeks at pH of 6.0, 7.0, and 8.0, respectively (EPA, 2006). Carbofuran was detected (1 to 5 μg l^{-1}) 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 2.1) resulting in a low tendency to volatilize from water or moist soils (Deul et al., 1979). Shibamoto et al. (1993) reported that 0.3 to 0.66 μg m^{-3} of carbofuran was detected after a 44-hours 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. The environmental fate of carbofuran is shown in Figure 2.2.

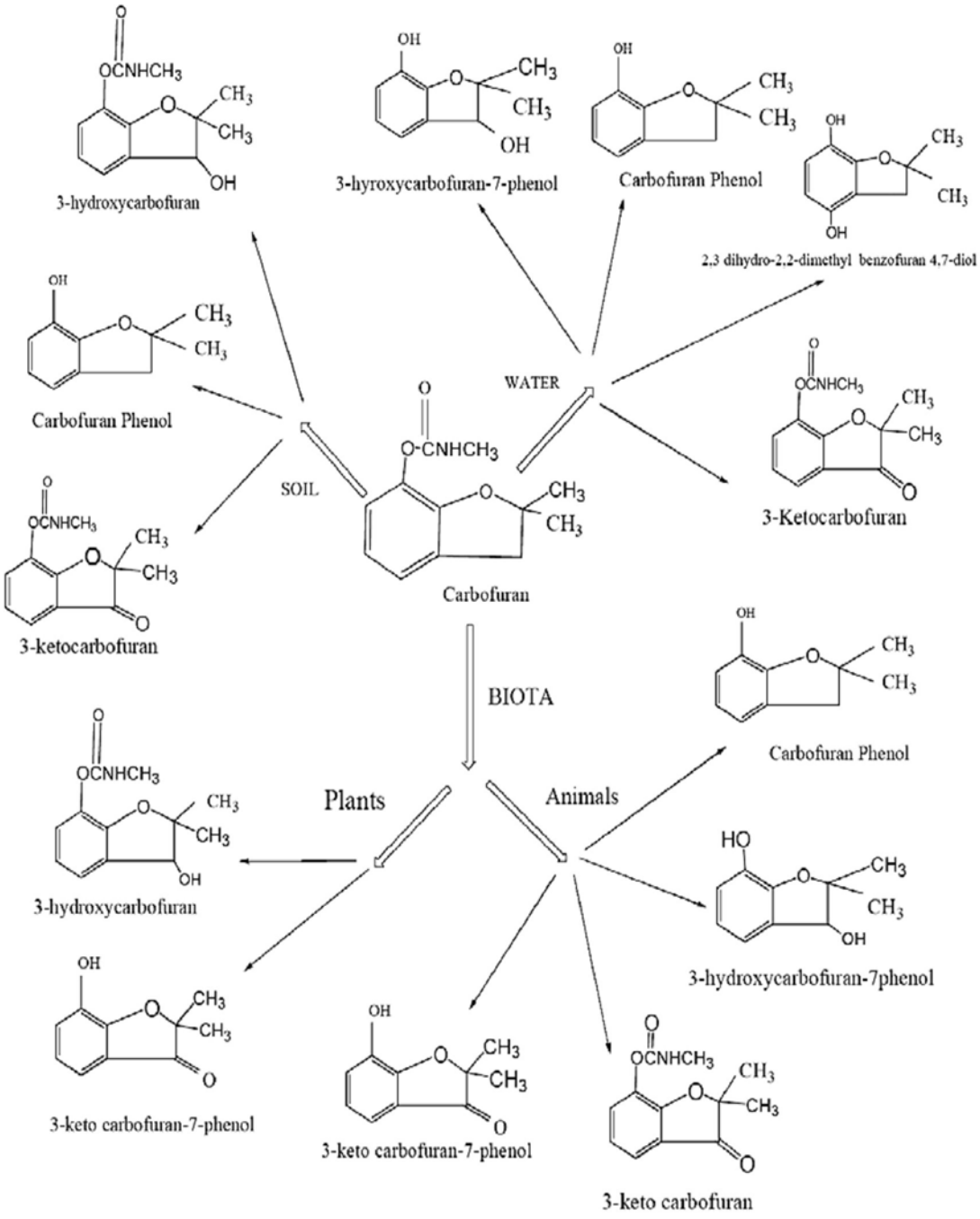

Figure 2.2 Environmental fate of carbofuran (Evert, 2002)

2.1.4 Health Effects

Carbofuran has a high toxicity through inhalation and ingestion with a moderate toxicity by dermal absorption (Baron, 1991). It can cause sub-lethal or chronic effects to aquatic organisms through cholinesterase inhibition, neurotoxicity and reproductive effects (De Melo Plese et al., 2005; EPA, 2006). Carbofuran causes a high toxicity to mammalian through cholinesterase inhibition (LD_{50} = 2 mg kg^{-1}) (EPA, 2006). Rats given very high doses of carbofuran (5 mg kg^{-1} 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. One granule was sufficient to kill a small bird ingested carbofuran granules (EPA, 2006). 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^{-1} in rainbow trout and 0.24 mg l^{-1} in bluegill sunfish (Kidd and James, 1991). The ecological toxicity of carbofuran was shown in Table 2.2.

Table 2.2 Ecological toxicity of carbofuran (EPA, 2006)

Species	Ecological toxicity	Data
Mallard Duck	LD_{50}	0.40 mg kg^{-1}
Mallard Duck	8-Day LC_{50}	190.00 mg kg^{-1}
Bobwhite Quail	LD_{50}	5.00 mg kg^{-1}
Bluegill Sunfish	96-hr LC_{50} (BCF 2-12X)	0.24 mg kg^{-1}
Rainbow Trout	96-hr LC_{50}	0.28 mg kg^{-1}
Daphnia Magna	48-hr LC_{50}	38.60 µg kg^{-1}
Honeybee	48-hr LD_{50}	0.16 µg bee^{-1}

2.1.5 Regulatory status

The used of carbofuran in agriculture is prohibited in some countries such as Canada due to its ecotoxicity (De Melo Plese et al., 2005). Since 1985, the U.S. Environmental Protection Agency (US 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 in the USA 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 μg l^{-1}. The EPA believes that this level of protection would not cause any of the potential health problems (EPA, 2006). In Thailand, carbofuran is categorized as a hazardous material type 3 in which its production, import, export and possession are strictly controlled (Ministry of Industry, 2003).

2.1.6 Degradation of Carbofuran

2.1.6.1 Degradation pathway and metabolites of carbofuran

Hydrolysis is the main metabolic degradation pathways of carbofuran. The degradation process of carbofuran exists when the carbofuran molecule (RX) reacts with water molecule to create a new C-N bond and break C-X bond in the original molecule. The final reaction is a direct displacement of X by OH (De Melo Plese et al., 2005; Seiber et al., 1978; Mabury and Crosby, 1996). The factors which can accelerate hydrolysis of carbofuran are high pH or alkaline condition, enzymatic activities of microorganisms and light intensity (Siddaramappa and Seiber, 1979; Chapman and Cole, 1982; Ramanand et al., 1991). The other processes involving in carbofuran degradation are oxidation, volatilization and photolysis which result in different degradation products (Deuel et al., 1979). The main carbofuran metabolites found from the degradation processes of carbofuran are 3-ketocarbofuran, 3-hydroxycarbofuran (Das et al., 2003), carbofuran-phenol, and 3-keto-7-phenol (Tejada and Magallona, 1985; Rouchaud et al., 1990). The molecular structures of carbofuran metabolites are shown in Figure 2.3.

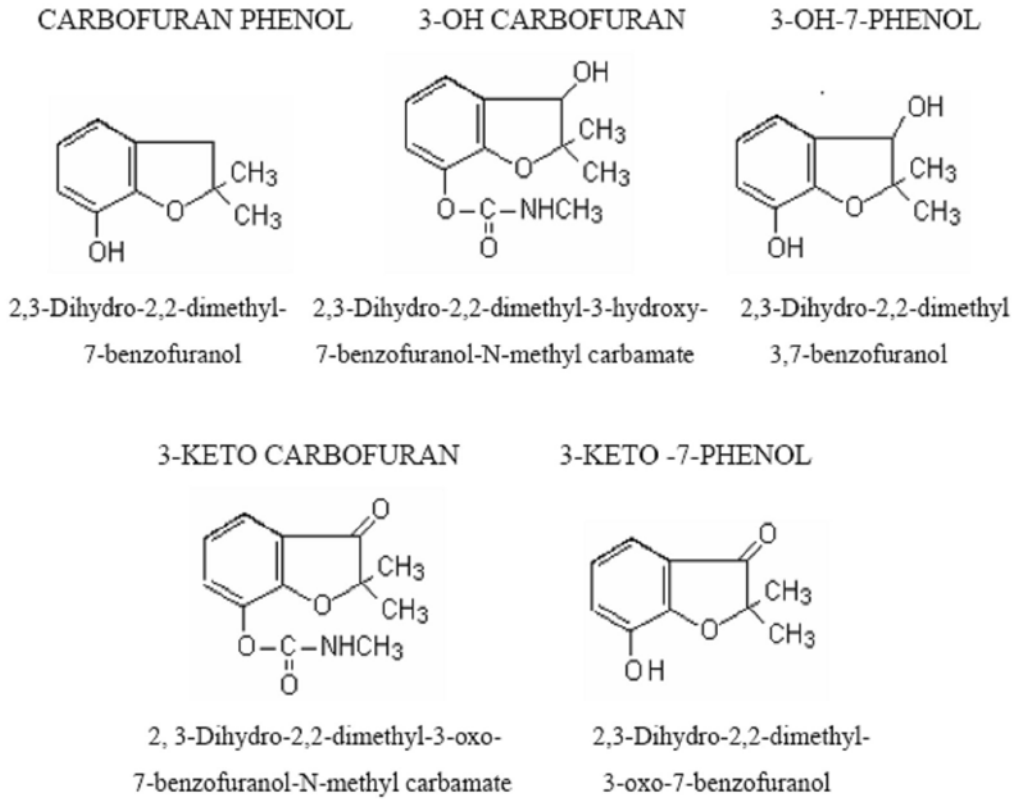

Figure 2.3 Molecular structures of carbofuran metabolites (Mora et al., 1996)

2.1.6.2 Biological degradation of carbofuran

Biological degradation is the main carbofuran degradation pathway in neutral and acidic conditions. Microorganisms which can produce differences enzymes responsible for metabolizing carbofuran as their energy sources, i.e. C- or N-, or C and N-sources are the key to succeed the carbofuran biodegradation. These microorganisms utilize carbofuran by hydrolysis the methylcarbamate likange, carbamate ester or carbamate side chain yielding different carbofuran metabolites such as 2-hydroxy-3-(3-methylpropane-2-ol) benzene-N-methylcarbamate (Yan et al., 2007), 2,3-dihydro-2,2-dimeth-yl-4-hydroxybenzofuran-7-yl methylcarbamate (Chaudhry et al., 2002), 2,2-dimethyl-7-hydroxy-2,3-dihydrobenzofuran (T.T. Lee, 2002), 2,3-dihydro-2,2-dimethyl-7-benzofuranol (Feng et al., 1997). Microorganisms capable of degrading carbofuran have been isolated from contaminated natural matrices and characterized for their carbofuran degradation abilities (Chaudhry et al., 2002; Karpouzas et al., 2000). *Pseudomonas* sp. (Felsot et al., 1981) and *Pseudomonas* sp. 50432 (Chaudhry et al., 2002) could degrade carbofuran to 4- hydroxycarbofuran or 7-phenol by their hydrolase

enzyme. *Arthrobacter, Pseudomonas, Bacillus* and *Actinomyces* were isolated from carbofuran treated soil. These microorganisms utilized carbofuran as their sole C-source (Ambrosoli et al., 1996). *Pseudomonas* sp. NJ-101 isolated from agricultural area metabolized carbofuran as sole C- and N- sources with the half-live of 20 d in soil microcosms (Bano and Musarrat, 2004). A gram-negative *Novosphingobium* sp. FND-3 was isolated from carbofuran contaminated sludge and showed a high carbofuran degradation rate of 28.6 mg l^{-1} h^{-1} in mineral salt medium containing 100 mg l^{-1} of carbofuran. Several metabolites included carbofuran phenol, 2-hydroxy-3-(3-methypropan-2-ol) phenol, 2-hydroxy-3-(3-methylpropan-2-ol) benzene-N methyl-carbamate and one unknown metabolite could be detected in culture medium by GC/MS (Yan et al., 2007). A study on the persistence of carbofuran and the effect 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. They also reported that with the activity of these microorganisms, carbofuran persistence in soil was only 9 d. 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 biological co-metabolic process. *Burkholderia* sp. PCL3 is a carbofuran degrader isolated from carbofuran phytoremediated rhizosphere soil of rice. PCL3 had an ability to degrade carbofuran yielding carbofuran phenol as the metabolite with the short half-lives of 3-4 d in Basal Salt Medium (BSM) and 12-14 d in soil (Plangklang and Reungsang, 2008; 2009).

A fungus capable of utilizing carbofuran had also been isolated and identified. *Mucor ramannianus* had capabilities for degrading both carbofuran and carbofuran phenol. Two unidentified degradation products from carbofuran could be found in its culture medium while the metabolites from carbofuran phenol were most likely to be 2-hydoxy-3-(3-methylpropan-2-ol)phenol or 7a-(hydroxymethyl)-2,2-dimethylhexahydro-6H-furo[2,3-b]pyran-6-one and 3-hydroxy-carbofuran-7-phenol (Seo et al., 2007). *Gliocladium* L_c capable of using carbofuran as sole C- and N-sources exhibited a high carbofuran degradation with the degradation efficiency of 81% within 48 h in synthetic salt medium supplemented with 200 mg l^{-1} of carbofuran (Slaoui et al., 2007).

2.1.6.3 Chemical degradation of carbofuran

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) 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) found that abiotic degradation by hydrolysis at the carbamate linkage, producing carbofuran phenol as the degradation product, was the important process involving in the disappearance of carbofuran from the soil suspension. The carbofuran degradation rate in water was strongly influenced by pH (Chapman and Cole, 1982). Hydrolysis was also observed to be much more rapid in natural paddy water than deionized (DI) water (Seiber et al., 1978). Chemical oxidation is one effective process for removing carbofuran from the contaminated aqueous solution. Various oxidizing agents have been successfully used for elimination of carbofuran such as Fenton reagents (Wang et al., 2003), $H_2O_2/S_2O_8^{2-}$ (Chu et al., 2006), degussa P-25, TiO_2 and ZnO (Mahalakshmi et al., 2007). In addition, the combination of chemical oxidation and photocatalytic process had been reported to improve the efficiency of carbofuran degradation (Wang and Lemley, 2003; Chu et al., 2006). Abdessalem et al. (2010) studied the remediation of three pesticides i.e., carbofuran, chlortoluron, and bentazon using advance oxidation process. The result showed that electro-Fenton process required 8 h which is longer than using photo-Fenton process which required 2 h to achieve the degradation percentage of more than 90%. The study of Pu Lumei et al. (2008) on the degradation of carbofuran in liquid solution by oxidation process using low temperature plasma showed that the increase in temperature, pH, and initial concentration of carbofuran could enhance carbofuran degradation. The 70% mineralization of carbofuran at the initial concentration of 10 mg l^{-1} by using multisolution Fe (III) $8x10^{-4}$ mg l^{-1} combined with photodegradation process could be achieved within 25 h at pH 2.8 (Katsumata et al., 2005).

2.1.6.4 Physical degradation

The degradation rate of carbofuran in soil could be affected by temperature and moisture content in which degradation could be enhanced at a high temperature and optimum moisture content (Ou et al., 1982). Yen et al. (1997) found that the half-lives of carbofuran in silty clay loam (pH 6, organic matter 2.9%) were 105 and

35 d at 15 and 35 ^{o}C, respectively. The dissipation of carbofuran in water could be influenced by photolysis and volatilization (Sieber et al., 1978; Deuel et al., 1979). Volatilization rates of carbofuran were more rapid in flooded soil than in non-flooded soil because of co-evaporation with the water on the surface of soil (Lalah et al., 1996). Sunlight and high temperature have increased the rate of carbofuran loss from water (Siddaramappa and Sieber, 1979). Photolysis is major route of physical degradation of carbofuran via photo-fries rearrangement, hydroxylation of the benzene ring, oxidation of the 2,3-dihydrobenzofuran ring, cleavage of the carbamate group, hydrolysis of the ether group, the radical coupling and decarboxylation processes (Detomaso et al., 2005). 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). In addition to the physical method, the sorption to the porous materials could effeciently remove carbofuran from the aqueous solution, however, without the degradation processes or degradation products. Sorption of carbofuran was strongly influenced by types and sizes of sorbent, temperature and pH. The decrease in particle size of the materials resulted in the increase in surface area, hence increasing the adsorption capacity (Gupta et al., 2006). However, a high temperature and pH could reduce the effectiveness of adsorption process to remove carbofuran (Gupta et al., 2006). Commercial activated carbon, Filtersorb 300 (F300), was used as the adsorbent to eliminate carbofuran contaminated in aqueous solution. The result indicated that the percentage of carbofuran removal using this activated carbon was up to 96.97-97.35% (Salman et al., 2010).

2.2. Bioremediation

Bioremediation is a promising process using natural biological activity to remediate the environmental contaminant until its concentration is below detectable limit or less than the maximum contaminant level (Vidali, 2001). In general, bioremediation uses indigenous microorganisms in the contaminated area as the degraders. In some instances, the indigenous microbes at contaminated sites may not be efficient to degrade the target contaminants because the microorganisms capable of degrading the contaminant present in very small number. In such cases, inoculation of the microorganisms capable of degrading specific contaminants or so-called bioaugmentation technique and/or the addition of amendments for stimulating microbial activity in the contaminated site or so-

called biostimulation technique might lead to the successful bioremediation (Fantroussi and Agathos, 2005).

2.2.1 Bioaugmentation

Bioaugmentation is the addition of microbial cultures into the contaminated areas to improve a specific biological activity (Fantroussi and Agathos, 2005). This technique has been practiced 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), physicochemical characteristic of the contamination matrices (e.g. water, organic matter 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 (Thompson et al., 2005). Traditional bioaugmentation has achieved its greatest remediation via specific contaminant degrading bacteria isolated from contaminated sites or pollutant repeated application sites. Researchers had isolated microorganisms capable of degrading carbofuran (Yan et al., 2007; Bano and Musarrat, 2004; Chaudhry et al., 2002; Plangklang and Reungsang, 2004; Karpouzas et al., 2000; Duquenne et al., 1996) and other pesticides (Rousseaux et al., 2003; Dams et al., 2007) from contaminated natural matrices. The effects of inoculums size, microbial distribution, and nutrient amendments to soil on the degradation of carbofuran by bacteria strain C28 were studied by Duquenne et al. (1996). In this study, soil was taken from a cultivated plot in Auxonne, France, and was a source of the carbofuran degraders used in the bioaugmentation treatment. Results indicated that an increase in the inoculums size and the distribution of C28 as microbial strain could enhance the carbofuran degradation. The carbofuran degrader, *Novosphingobium* sp. strain FND-3, gram-negative bacteria was isolated from sludge contaminated with carbofuran in the wastewater treatment system in a pesticide factory at Xuzhou, Jiangsu province, China. The inoculation of carbofuran degraders, *Novosphingobium* sp. strain FND-3, gram-negative bacteria demonstrated the maximum rate of carbofuran degradation at 28.6 mg l^{-1} h^{-1} in a mineral salts medium with initial carbofuran concentration of 100 mg l^{-1} (Yan et al., 2007). *Pseudomonas* sp. (NJ-101) isolated from sandy loam soil at a site contaminated with pesticide application in Aligarh, India, was found to be able to enhance carbofuran degradation. A carbofuran degradation

rate of 0.035 day^{-1} was found in carbofuran contaminated soil added with *Pseudomonas* sp. (NJ-101) (Bano and Musarrat, 2004).

Chelatobacter heintzii Cit1 was isolated from different areas in France i.e., Cîteaux, Dijon, Epoisses (Burgundy) and La Bouzule (Lorraine). These gram negative bacteria were inoculated into four sterile and four non-sterile soils in order to remediate antrazine in soil with various amounts of inoculums. The addition of *C. heintzii* Cit1 (10^4 CFU g^{-1} soil) resulted in 3 times increase in atrazine degradation efficiency when compared to soil without inoculation (Rousseaux et al., 2003). Soil, collected from Boyndie (Boyndie series), Northern Scotland and UK, was contaminated with pentachlorophenol (PCP). This soil was inoculated with *Sphingobium chlorophenolicum* ATCC 3972 to remediate PCP in the soil. The result revealed that the introduction of *S. chlorophenolicum* ATCC 3972 to polluted soil resulted in approximately four times faster degradation of PCP compared to the non-inoculated soil (Dam et al., 2007). *Pseudomonas aeruginosa* was isolated by an enrichment technique in a nonsulfur medium (NSM) mixed with endosulfan. The microbe was introduced to soil slurry to remove endosulfan. The results showed that the degradation of endosulfan (100 mg l^{-1}) in soil slurry by *P. aeruginosa* was the most effective to achieve 85% endosulfan removal within 16 d in comparison to soil without inoculation (16% removal) (Arshad et al., 2007). *Paracoccus* sp. strain HPD-2 was isolated from soil contaminated with polycyclic aromatic hydrocarbon (PAH) in Wuxi, Jiangsu province, East China. The soil had a long term history of PAH contamination over approximately 30 yr. The results suggested that bioaugmentation using *Paracoccus* sp. strain HPD-2 could significantly reduce the concentration of PAH in aged soil. The PAH concentration was reduced from 9,942 to 7,638 mg kg^{-1} dry soil by *Paracoccus* sp. strain HPD-2 (Teng et al., 2010). The isolation of *Streptomyces* sp M7 was obtained from wastewater sediment of a copper filter plant in agricultural land of Tucuman, Argentina. The inoculation of *Streptomyces* sp M7 into sterile soil could reduce various concentrations of lindane at 100, 150, 200, and 300 mg kg^{-1} whereas the percentages of lindane degradation in abiotic control soil were 29.1%, 78.03%, 38.81%, and 14.42%, respectively. The most effective bioaugmentation treatment was 56% degradation by *Streptomyces* sp M7 in autoclaved soil contaminated with 100 mg $kg^{-1}$of lindane. The inoculums of *Streptomyces* sp M7 were added at 2 g cells kg^{-1} soil (w/w) to remove lindane (Benimeli et al., 2008). Soil from a farm at Huajiachi campus, Zhejiang University, China, where the *Verticillium* sp. DSP was

isolated, had been exposed to chlorpyrifos for five years. The addition of *Verticillium* sp. DSP to soil contaminated with chlorpyrifos enhanced the degradation rate in comparison to autoclaved soil (Fanga et al., 2008). The microbial community i.e., *Enterobacter sakazakii* and *Serratia* sp. was isolated from contaminated soil with a history of Tebuconazole application at Santa Rosa, Rio Grande do Sul, Brazil. The results demonstrated that bioaugmentation using *E sakazakii* and *Serratia* sp. could remove 42.76 mg l^{-1} of tebuconazole. Tebuconazole removal from soil was up to 51 % (Nicole et al., 2010). *Serratia* spp. strain JC1 and JCN13 were isolated from activated sludge in the wastewater treatment plant of the Pesticide Corporation in Shandong, China. These microorganisms could remove Beta-cyper-methrin (beta-cp) in a minimal salt medium (MSM). The bioaugmentation technique was effective in reducing beta-cp in MSM by *Serratia* spp. strain JC1 and JCN13. These two microbes utilized beta-cp as sole carbon source in MSM. Results showed that the degradation of beta-CP achieved 92% for 10 d by *Serratia* spp. strain JC1 and JCN13 (Zhang et al., 2010). Organophosphorus pesticides in soil was effectively removed using the bioaugmentation technique. Organophosphorus pesticides in soil could be eliminated by up to 64.3–85.7% by *P. putida* DAK as the non-chemotaxic mutant (Guo et al., 2009). In the research of Cycon et al. (2009), a soil sample free from diazinon application for 5 years was taken from Upper Silesia, southern Poland. The bacterial strains i.e., *Serratia liquefaciens, S. marcescens,* and *Pseudomonas* sp. able to utilize the diazinon as a carbon source were isolated form polluted soil by an enrichment technique. The bioaugmentation technique was applied using *S. liquefaciens, S. marcescens,* and *Pseudomonas* sp. to eliminate the diazinon in MSM. The initial concentration of diazinon, 50 mg l^{-1}, in MSM was effectively eliminated over 14 d of incubation with approximate 80%-90% of the diazinon degraded. A consortium, namely AE (*Ochrobacterum* sp.), BE (*Arthrobacter* sp.), and CE (*Burkholderia* sp.), was isolated by an enrichment technique from soil supplemented with endosulfan as the sole carbon source for used in bioaugmentation treatment. A soil sample was taken from the contaminated site with many applications of agricultural pesticides. During the experiment, bioaugmentation using this consortium could eliminate γ-endosulfan at 57%, 88% and 91% as well as β-endosulfan at 4%, 60% and 67%, respectively, after 30 d of incubation (Kumar et al., 2008). Bioaugmentation of carbofuran using *Burkholderia* sp. PCL3 in free cell form was conducted to examine its ability to enhance carbofuran degradation in Fuzzy flatsedge rhizosphere soil and bulk soil. The degradation of

carbofuran in the Fuzzy flatsedge rhizosphere soil was not improved by PCL3. However, an ability of PCL3 to degrade carbofuran was evident in bulk soil ($t_{1/2}$ of 12 d) and autoclave soil ($t_{1/2}$ of 13-14 d) compared to soil without an inoculation ($t_{1/2}$ of 58 d) (Plangklang and Reungsang, 2008). Immobilized PCL3 on corncob and sugarcane bagasse were investigated for their abilities to degrade carbofuran in Basal Salt Medium (BSM) and soil microcosm. Short $t_{1/2}$ of carbofuran of 3–4 d in BSM were obtained. Immobilized cells also showed an effective capability to remediate carbofuran residues in soil which indicated by 5-folds decrease in carbofuran $t_{1/2}$ in augmented soil in comparison to soil with no inoculation (Plangklang and Reungsang, 2008). PCL3 was used to remove carbofuran from soil slurry in sequencing batch reactor (SBR). The result indicated that bioaugmentation of PCL3 in immobilized cells form to soil achieved the highest carbofuran removal up to 96.97% within 48 h at the initial carbofuran concentration of 20 mg kg^{-1} soil (Plangklang and Reungsang, 2010).

Table 2.3 Examples of bioaugmentation researches

Contaminated Matrices	Microorganism(s)	Pollutant	Reference
Soil	*Pseudomonas* sp. strain ADP	Atrazine	[Shapir et al., 1997]
	Agrobacterium radiobacter J14a	Atrazine	[Struthers et al., 1998]
	Escherichia coli pAtzA	Atrazine	[Strong et al., 2000]
	Consortia degrading atrazine	Atrazine	[Goux et al., 2003]
	Alcaligenes eutrophus TCP	2,4,6-Trichlorophenol	[Andreoni et al., 1998]
	Desulfitobacterium frappieri PCP-1	Pentachlorophenol	[Beaudet et al., 1998]
	Ralstonia eutropha (pJP4)	2,4-Dichlorophenoxyacetic acid	[Daane et al., 1999]
	R.. eutropha JMP134	2,4-Dichlorophenoxyacetic acid	[Roane et al., 2001]
	Pseudomonas sp. strain P51	1,2,4-Trichlorobenzene	[Tchelet et al., 1999]
	P. pseudoalcaligenes POB310	3-Phenoxybenzoic acid	[Halden et al., 1999]
	Desulfomonile tiedjei	3-Chlorobenzoate	[Fantroussi et al., 1999]
	Arthrobacter sp. B1B and *R. eutrophus* H850	Polychlorinated biphenyl	[Singer et al., 2000]
	A. RP17	Phenanthrene	[Schwartz et al., 2000]
	R. basilensis RK1	2,6-Dichlorophenol	[Steinle et al., 2000]
	Encapsulated consortium	Gasoline	[Moslemy et al., 2002]
	Burkholderia sp. PCL3	Carbofuran	[Plangklang and Reungsang, 2009]
Activated sludge	*Comamonas* sp. RN7(R503)	Phenol	[Watanabe et al., 2002]
	C. testosteroni I2	3-Chloroaniline	[Boon et al., 2003]
	Candidatus Accumulibacter phosphatis	Phosphorus	[Dabert et al., 2005]
	D. frappieri PCP-1	Pentachlorophenol	[Guiot et al., 2002 ; Lanthier et al., 2002]
Aquifer/ groundwater	Methanogenic consortia	BTEX	[Da Silva et al., 2004]
	P. stutzeri KC	Carbon tetrachloride	[Dybas et al., 2002]
	Consortium that contains *Dehalococcoides*	Chloroethenes	[Lendvay et al., 2003]
	Consortium that contains *Dehalococcoides*	Chloroethenes	[Adamson et al., 2003]
	Consortium that contains *Dehalococcoides*	Chloroethenes	[Major et al., 2002]
	P. putida GJ31, *P. aeruginosa* RHO1 and *P. putida* F1DCC	Chlorobenzenes	[Wenderoth et al., 2003]
	Butane-utilizing enrichment culture	1,1,1-Trichloroethane	[Jitnuyanont et al., 2001]
	Hydrogenophaga flava ENV735	Methyl tert-butyl ether	[Streger et al., 2002]
	β -proteobacterium strain PM1	Methyl tert-butyl ether	[Smith et al., 2005]

2.2.2 Biostimulation

Biostimulation is an addition of nutrients, air or oxygen into the contaminated systems in order to stimulate the intrinsic microbial population to degrade the contaminants of concern (Vidali, 2001). Advantages of this method are that it is simple to maintain, applicable over large areas, cost-effective, and leads to the complete destruction of the contaminant (Vidali, 2001). Previous research indicated that biostimulation by adding nutrients from agricultural residues is an effective tool to remediate various types of contaminant. For example, a removal of petroleum hydrocarbon in contaminated soil was increased 1.18 times compared to non-stimulated soil at day 15 (Perez et al., 2004). Diesel removal was increased to 67% compared with non-stimulated diesel-contaminated soils when corn and crop residues were added into diesel contaminated soil at C/N ratio of 100:10 (Barahona et al., 2004). Phenanthrene biodegradation was rapidly degraded from 40 mg kg^{-1} to below the detection limit when zinc was added into the contaminated soil at a concentration of 140 mg kg^{-1} (Wong et al., 2005). Selenate [Se (VI)], 1000 μg l^{-1}, contaminating agricultural drainage water was completely removed when high amounts of rice straw (3-4g) were added. 93-95% of selenate was removed within 5-7 d (Zhang and Frankenberger, 2002). Additions of farm manure, straw and nitrogen fertilizer stimulated microbial activity and accelerated atrazine degradation in soil (Hance, 1973). The combination of biostimulation and bioaugmentation gave 14 and 18% increase in the biodegradation rate of *cis*-DCE and *trans*-DCE, respectively, (Olaniran et al., 2005). The additional of rice straw accelerated the hydrolysis of carbofuran to carbofuran-phenol in anaerobic flooded soil. Nutrients in the form of indigenous microbial supplementation and sewage sludge were effective to stimulate the creosote removal from Mispah soil by increasing the total heterotrophic and creosote degrading microorganisms and increasing the degradation rate of creosote to 88.7 % and 86.1%, respectively (Atagana, 2004). The addition of wood chip could improve 20-30% of simazine, trifluralin, and dieldrin degradation in soil microcosom as compared to the soil without amendment (Fragoeiro and Magan, 2008). The *ex situ* biostimulation was achieved by adding the green tree waste to remove 1,2-dichlorobenzene (DCB) from soil to below detection limit (0.2 mg kg^{-1}). The percentage removal of DCB from soil was approximately 90% within 2-3 weeks of incubation (Guerin, 2008).

2.3. Immobilization

A successful bioaugmentation of pesticides by the isolated degraders had been reported (Piutti et al., 2003; Rousseaux et al., 2001; Franzmann et al., 2000; Struthers et al., 1998) in which most of pesticide degraders were used in free cell form. However, some limitations of applying free cell of the degraders in the bioremediations system could be found such as low survival ability in natural conditions, low recovery and low recycling capabilities (Bekatorou et al., 2004). These limitations might be overcome by immobilization technique which offers many advantages over free cells including regeneration and reuse of immobilized cells for extended period of works. The supporting materials used for immobilization may act as protective agents against the effects of pH, temperature, solvent, heavy metals or even substrate and product inhibition hence enhancement the cell survival (Bekatorou et al., 2004; Braud et al., 2007).

Immobilization techniques can be divided into 4 major categories based on the physical mechanisms (Figure 2.4) (Bekatorou et al., 2004).

2.3.1 Attachment or adsorption on carrier solid

Cell immobilization on a solid carrier is carried out by physical adsorption due to electrostatic forces or by covalent binding between the cell membrane and the carrier.

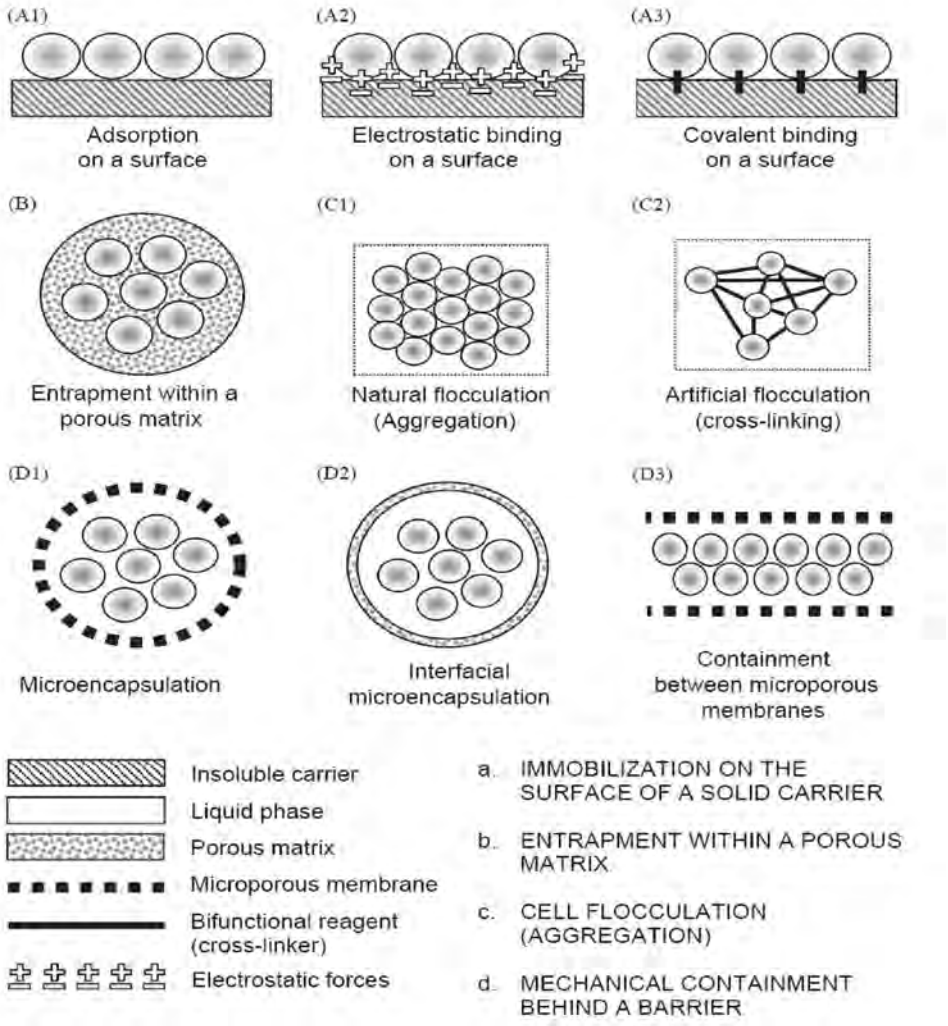

Figure 2.4 Basic methods of cell immobilization (Bekatorou et al., 2004)

2.3.2 Entrapment within a porous matrix

Cells are either allowed to penetrate into the porous matrix until their mobility is obstructed by the presence of other cells, or the porous material is formed *in situ* into a culture of cells.

2.3.3 Self-aggregation by flocculation (natural) or with cross-linking agents (artificially induced)

Self-aggregation is a combination of cells to form a larger unit or the property of cells in suspensions to adhere in clumps and sediment rapidly (Jinan and Speers, 1998). The ability to form aggregates is mainly observed in moulds, fungi and plant cells.

2.3.4 Cell containment behind barriers

Containment of cells behind a barrier can be attained either by use of microporous membrane filters or by entrapment of cells in a microcapsule or by cell immobilization onto an interaction surface of two immiscible liquids.

The support materials used for immobilization could be either synthetic polymers (Table 2.4) or natural materials like agricultural residues (Table 2.5). Natural polymeric gels such as agar, carrageenan and calcium alginate have been used as support materials for cell immobilization (Katzbauer et al., 1995). However, use of these materials is limited by their mechanical strength and the lack of open spaces to accommodate cell growth and cell release into the growth medium (Barbotin and Nava Saucedo, 1998; Kumar and Das, 2001). Calcium alginate gels were reported to be unstable when it contacts with complex anions such as phosphate and citrate which are usually used in media (Birnbaum et al., 1981). *Acenetobacter* sp. strain W-17, immobilized on porous sintered glass completely degraded 500 mg phenol l^{-1} in nutrient medium within 40 h, while free cells required 120 h for this to be achieved. In addition, these immobilized cells can be reused 6 times without losing their phenol degradation activities (Beshay et al., 2002).

The several advantages of synthetic polymer such as PVA, PUF and other support materials such as diatomaceous earth, activated carbon (Fennell et al., 1992; Fennell et al., 1993), glass (Phelps et al., 1990; Arvin, 1991) and ceramic packing material (Strandberg et al., 1989) including high mechanical strength, resistant to organic solvents and microbial attack, easy handling and regenerability have been established (Patil et al., 2006). The immobilized cells of genetically engineered *E. coli* on highly porous sintered glass beads were used for remediation of coumaphos in continuous-flow packed bed bioreactor. The complete degradation of coumaphos could be achieved by adjusting the feed-in rate and coumaphos and surfactant concentration to the optimum values (Mansee et al., 2000). Immobilized *Candida parapsilosis* and *Penicillium frequetans* on granular clay could degrade C-2 to C-8 alkanes greater than 70%, whereas using free cells resulted in only 15% of alkanes degradation (Omar and Rehm, 1988).

Table 2.4 Examples of immobilized cell used in bioremediation of toxic substances

Compounds degraded or reactions	Microorganisms	Carriers	References
4-Chloro-2-nitrophenol	Mixed culture	granulated Lecaton-particles	[Overmeyer and Rehm. 1995]
2-Chloroethanol	*P. putida* US2	alginate	[Lee et al., 1994]
Chlorophenols	Mixed culture	polyurethane	[Valo et al., 1990]
Chlorophenol	*Rhodococcus* spp	celite R-633 microcarriers	[Shieh et al., 1990]
Chlorophenols	Activated sludge	glass, cellulose, chitin	[Portier and Fujisaki 1986]
Chlorinated phenols	Several strains	alginate	[Westmeier and Rehm.1985,
4-Chlorophenol	*Alcaligenes* sp A 7-2	granulated Lecaton particles	Westmeier and Rehm.1987]
4-Chiorophenol	*Alcaligenes* sp A 7-2	granular clay	[Balfanz and Rehm.1991]
p-Cresol	*Pseudomonas* sp	alginate	[O'Reilly et al., 1988]
p-Cresol	*Pesudomonas* sp	alginate	[O'Reilly and Crawford. 1989]
Cyanuric acid	*Pseudomonas* sp NRRL B-12228	polyurethane	[Ernst and Rehm. 1995]
DDT	Mixed culture	granular clay	[Beunink and Rehm. 1988]
Dechlorination of spent	*Streptomycetes* spp	alginate	[Zhon et al., 1993]
sulphite	*Xanthobacter autotrophicus*	polyurethane	[Heinze and Rehm. 1993]
Hydrocarbons	Mixed culture	agar	[Heitkamp et al., 1990]
Inorganic cyanides	*(Pseudomonas* spp)	alginate	[Wiesel et al., 1993]
p-Nitrophenol	Mixed culture	carrageenan	[O'Reilly et al., 1988]
PAHs	*Flavobacterium* sp	diatomaceous earth biocarrie	[O'Reilly and Crawford . 1989]
Pentachlorophenol	*Flavobacterium* sp	granular clay	[Lin and Wang. 1991]
Pentachlorophenol	*Arthrobacter* sp ATCC 33790	slag of lava	[Siahpush et al., 1991]
Pentachlorophenol	*Phanerochaete chrysosporium*	alginate	[Hu et al., 1994]
Pentachlorophenol	*Arthrobacter* sp ATCC 33790	polyurethane	[Stormo and Crawford. 1994]
Pentachlorophenol	*Flavobacterium* sp	alginate	[Wu et al., 1993]
Pentachlorophenol	*Flavobacterium* spp	alginate and activated carbon	[Hackel et al., 1975]
Pentachlorophenol	Mixed culture	alginate	[Bettmann and Rehm.1984]
Pentachlorophenol	*Candida*	alginate and activated carbon	[Babu et al., 1992]

Table 2.5 Example of agricultural materials used as supporting materials for cell immobilization

Reactions	Microorganisms	Carriers	References
Carbendazim and 2,4	Microbial consortium from paddy soil sample	Loofa sponge	[Pattanasupong et al., 2004]
Dichlorophenoxyacetatic Acid degradation	*Rhizopus cohnii*	Sawdust	[Huidong et al., 2007]
Hexavalent chromium degradation	*Aspergillus terreus*	Papaya wood	[Iqbal et al., 2004]
Itaconic acid production	*Candida guilliermondii*	Sugarcane bagasse	[Santo et al., 2005]
Xylitol production	*Saccharomyces cerevisiae* strain AXAZ-1	Apple pieces	[Kourkoutas et al., 2006]
Ethanol production	*Phanerochaete chrysosporium*	Sugarcane bagasse	[Mohammadi et al., 2007]
Polycyclic aromatic hydrocarbons (PAHs) degradation	*Acinetobacter haemolyticus*	Wood husk	[Zakaria et al., 2007]
Cr(VI) degradation	*Chlorella sorokiniana*	Loofa sponge	[Akhtar et al., 2004]
Removal of nickel(II)	*Streptomyces clavuligerus*	Loofa sponge	[Saudagar et al., 2008]
Clavulanic acid production	*S. cerevisiae* 101	Watermelon rind pieces	[Reddy et al., 2008]
Wine production	*S. cerevisiae*	Grape skins	[Malloucho et al., 2002]
Carbofuran degradation	*Burkholderia* sp. PCL3	Corncob	[Plangklang and Reungsang, 2009; 2010]

Though the synthetic materials are efficient to be used as support materials, they were considered to be expensive for use in large-scale. In addition, the disposal of the synthetic polymers is of the concern due to its non-biodegradable characteristic, unlike the natural materials such as agricultural residues which are biodegradable. Therefore, collateral research on cell immobilization using natural supporting material has been developed. Agricultural matrices are alternative support materials for cell immobilization because it is environmental friendly, locally available and cheaper than synthetic polymer. Previous research indicated a successful use of various agricultural residues to immobilize the specific degraders in bioremediation work. The microbial consortium isolated from agricultural soil was immobilized on loofa sponge and the coconut fiber and used to degrade 0.2 μM carbendazim and 2,4-dichlorophenoxyacetic acid (2,4-D) in

synthetic medium. After 4 d of incubation, carbendazim was degraded 95% and 80% by immobilized consortium on a loofa sponge and the coconut fiber, respectively. Both of the immobilized consortiums showed approximately 7.5 times greater than carbendazine removal by the free cells consortium. The consortia immobilized on the loofa sponge and the coconut fiber completely degraded 2, 4-D within 1 d while the treatment with free-living cells took 2 days for complete degradation of 2, 4-D (Pattanasupong et al., 2004). Chitin and chitosan flakes obtained from shrimps wastes were used to immobilize *Rhodococcus corynebacterioides* QBTo (hydrocarbon-degrading bacterial strain) for removal of crude oil from polluted seawater. The percentage of hydrocarbon removal obtained in the microcosm inoculated with immobilized cells was two times higher than microcosm inoculated with free cell. The results also indicated that chitin and chitosan flakes improved the survival and the activity of immobilized cells (Gentili et al., 2006). Corncob and sugarcane bagasse were used to immobilize *B. cepacia* PCL3 for bioremediation of carbofuran in synthetic medium and soil in comparison to free cells. The carbofuran degradation ability between free and immobilized cell were not significantly different. However, immobilized cell could survive longer than free cells and it could be reused twice without the loss in carbofuran degradation ability (Plangklang and Reungsang, 2009). In addition, PCL3 immobilized on corncob was used as inoculums to remove carbofuran in contaminated soil at the carbofuran concentration of 20 mg kg^{-1} soil in a bioslurry phase seguencing batch reactor (SBR). The highest percentage of carbofuran removal of 96.97% was achieved in the treatment bioaugmented with PCL3 immobilized on corncob (Plangklang and Reungsang, 2010).

2.4. Bioremediation in soil column studies

The soil column is applied as a model to study the biodegradation of contaminants in soil in saturated and unsaturated zones. The study of a soil column is used to determine the feasibility of application of the bioremediation technique at contaminated sites. For example, *Ex situ* bioremediation of soil contaminated with lindane was conducted using the soil column. Various concentrations of sugarcane bagasse (10%, 20%, 30%, 40% and 50%; w/w) were supplied to a soil column at concentration of 50 mg kg^{-1} in order to increase biological activity and prevent lindane movement by adsorption. The optimal concentration of sugarcane bagasse was 50% (w/w) which was able to eliminate lindane in the upper soil column at 53% biodegradation (Abhilash et al., 2008). Molasses and

succinate were added to improve 2, 4, 6-TNT degradation in soil when the initial concentration of 2, 4, 6-TNT was varied from 3,179-4,176 mg kg^{-1} in each column experiment. Soil columns were operated for 371 d of incubation period as *in situ* bioremediation. The most effective procedure via adding molasses gave 97.5% biodegradation of 2, 4, 6-TNT. The addition of succinate to the soil column contaminated with 2, 4, 6-TNT could also achieve 20% biodegradation. The biostimulation technique by introducing molasses to contaminated soil is better than the addition of succinate (Boopathy et al., 1996).

A soil column, used with a bioaugmentaion technique, was also able to remove several pollutants from soil apart from the biostimulation technique. *P. putida* KP 201, for example, was inoculated into a soil column contaminated with 2, 4, 6-TNT at a concentration of 1,000 mg kg^{-1} TNT. The 2, 4, 6-TNT removal eliminated approximately 50% after 25 d in soil. After 60 d of incubation, 99% of 2, 4, 6-TNT could be removed (Park et al., 2003). The *in situ* bioremediation was used over 330 d to remove petroleum hydrocarbon-diesel (TPHd) in the soil column. The soil column was inoculated with the bacterial consortia *C. testosteroni* CC-CF3, *G. alkanivorans* CC-JG39, and *P. aeruginosa* CC-RS1. The optimal range of petroleum hydrocarbon-diesel (TPHd) concentration was effectively reduced from 10,290 mg TPHd dry $soil^{-1}$ to 1,851 mg TPHd kg dry $soil^{-1}$ using the bioaugmentation technique which was equivalent to 80% removal efficiency. (Grace Liu et al., 2008). The consortium of atrazine degraders was categorized as *Klebsiella ornithinolytica* ND2 and *Agrobacterium tumefaciens* ND4, which were immobilized on phosphorylated-polyvinyl alcohol. These immobilized mixed-atrazine degraders were inoculated in a sand column study to remove the atrazine soluble infiltrate at the initial atrazine concentration of 1.5 mg l^{-1}. The immobilized bacterial consortiums could remove atrazine at 65–80% compared to the mixed-atrazine degraders in free cells at 42–80% biodegradation. This sand column study was constructed as *ex situ* bioremediation (Siripattanakul et al., 2009).

CHAPTER III

RESEARCH METHODOLOGY

3.1 Chemicals and reagents

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

3.2 Soil

The soil sample was collected from a rice field at Ban Nonmuang, A. Muang, Khon Kaen. Soil was passed through a 2 mm sieve and kept in a plastic bag at 4 °C until usage. The soil sample was classified as sandy loam soil with percentages of sand, silt, and clay of 58.66, 31.09, and 10.05, respectively. Organic matter in the soil was 1.43%.

3.3 Microorganism preparation

The carbofuran degrader, identified by 16s rRNA as *Burkholderia* sp. PCL3 (GenBank accession number EF990634) was used in this experiment. This microorganism is capable of using carbofuran as a sole C-source (Plangklang and Reungsang, 2004). It was grown in 100 ml Nutrient Broth (NB) (Criterion, USA) containing 5 mg l^{-1} of carbofuran at 30 °C and 150 rpm for 36 h and used as seed inoculum for cell immobilization. In addition, the cells were pelleted by centrifugation at 4 ^{o}C, 5,000 rpm for 10 min and further washed and resuspended in 0.85% NaCl solution prior the usage as seed inoculum for bioaugmentation treatment.

3.4 Immobilization of *Burkholderia* sp. PCL3

3.4.1 Supporting materials preparation

Natural supporting materials i.e. corncob were used to immobilize *Burkholderia* sp. PCL3 because it has high matrix porosity and a pore size that can enhance the cell adsorption capability. Corncob was cut into 0.7x0.7x0.7 cm pieces and then boiled in alkaline solution (1% NaOH) with the ratio of corncob to alkaline solution of 100 g: 3 l for 3 h to remove lignin and fibers inside the materials which might react with the cells (Bardi and Koutinas 1994). The alkaline-boiled corncob was washed three times with 3 l of distilled water, soaked in distilled water overnight and then sterilized by autoclaving at 121 ^{o}C for 15 min and kept at 4 ^{o}C prior to usage.

3.4.2 Cell immobilization

Adsorption was used as the immobilization method in this study. This method was typically performed when the porous media were used as support materials with the advantage of ease of operation (Bickerstaff, 1997). The immobilization technique was implemented by adding 75 g of sterile corncob into the sterile 250 ml NB containing 5 mg l^{-1} of carbofuran before inoculating with PCL3 (10^{6} CFU ml^{-1}) at 10 % inoculums size. The flask was then incubated at 150 rpm, room temperature (30±2^{o}C), for 48 h. After incubation, support materials were transferred to a fresh NB containing 5 mg l^{-1} of carbofuran and incubated, as previously described, before harvesting by filtration through Buchner filter funnel and washing with 0.85% NaCl by aseptic technique. This process was repeated twice. Immobilized cells were kept at 4 ^{o}C until being used in the bioaugmentation experiments. The internal cell density on the corncob was approximately 6.48x10^{7} CFU g^{-1} support material. The procedures of cell immobilization followed the methods described by Plangklang and Reungsang (2009).

3.5 Synthetic surface water

A modified Basal Salt Medium (BSM) (pH 6.8) was used as synthetic surface water. It consists of (in g l^{-1}) 5.57, Na_2HPO_4; 2.44, KH_2PO_4; 2.00, NH_4Cl; 0.20, $MgCl_2.6H_2O$; 0.0004, $MnCl_2.4H_2O$; 0.001, $FeCl_3.6H_2O$ and 0.001, $CaCl_2$.

3.6 Sorption of carbofuran

3.6.1 Sorption of carbofuran to soil

Adsorption isotherms of carbofuran into the soil were determined by conducting a batch equilibrium experiment at carbofuran concentrations of 0.1, 1.0, 5.0, 10.0, and 20.0 mg l^{-1}. All solutions were prepared in 0.01 M $CaCl_2$. A total of 3-g of air-dried soil was put into 15 ml glass tubes and mixed with 9 ml of 0.01 M $CaCl_2$ solution containing carbofuran. Tubes were shaken on a horizontal shaker for 48 h at 90 cycles per minute. After centrifugation at 5,000 g, the supernatant was filtered through a 0.45 µm nylon membrane filter and analyzed for carbofuran concentration using HPLC. The data was fitted to the Freundlich equation (eq. 1) (Sposito, 1980) to describe the kinetics of carbofuran sorption to the soil.

$$\mathbf{C_s = K_f C_{eq}^{1/n}} \qquad)$$

where C_s is the carbofuran concentration in the soil (mg kg^{-1}soil), C_{eq} is the equilibrium solution concentration of carbofuran (mg l^{-1}), K_f is an index of adsorption capacity and 1/n is an empirical constant.

3.6.2 Sorption of carbofuran to corncob and rice straw

Corncob and rice straw were air-dried overnight and grinded into small pieces using a blender and passed through a 2 mm sieve. A 0.25 g of air-dried or de-lignified corncob or rice straw were put into a 250 ml conical flask and mixed with 50 ml of 0.01 M $CaCl_2$ solution containing carbofuran at various concentrations of 0.05, 0.1, 1.0, 5.0, 10.0, and 20.0 mg l^{-1}. Flasks were horizontally shaken at a constant speed of 100 cycles per minute for 48 h at an average room temperature of 29 ± 2 ^{0}C. After 48 h, the solution was passed through Whatmann filter paper No. 1 and the filtrate was extracted by the liquid–liquid partitioning method and quantified for carbofuran concentration by HPLC. The data was fitted to the Freundlich equation (eq. 1) (Sposito, 1980) to describe the kinetics of carbofuran sorption to corncob and rice straw.

3.7 Soil column experiment

The soil columns were made up of glass with inner diameter of 5.4-cm and height of 30-cm with the inlet and outlet ports arranged at the bottom and 5-cm from the top of the column, respectively (Figure 3.1). The columns were firstly packed with 0.5 g of glass wool followed by 2 g of gravel and 3 g of sand at the bottom of the column to prevent soil loss and provided a homogenous distribution for flow-through. Eight different column treatments (Table 3.1) were conducted. The compositions in each treatment are presented in Table 3.2. Soil was well mixed with carbofuran to achieve a final concentration of approximately 10 mg kg^{-1} dry soil before being packed into the columns. In the bioaugmentation treatment, PCL3 in free or immobilized cell form were inoculated into the carbofuran contaminated soil at the final cell concentration of 10^6 CFU g^{-1}dry soil (10% inoculums size). In the biostimulation treatments, rice straw 1.5% (w/w) (Sittijanda and Reungsang, 2006) was mixed with the carbofuran contaminated soil before being packed into the columns. The synthetic surface water stored in the plastic bottle was fed into each column through silicone tube at the inlet port at the bottom of the column with a flow rate of 50 ml d^{-1}. The columns were set upright to allow vertical flow of the influent in the bottom-up direction. The flow rate was adjusted and controlled using the manual valve. Carbofuran contaminated soil in the column was saturated with synthetic surface water for 24 h before being continuously fed by synthetic surface water. The overflow effluent was collected every 2 d for 45 d. Carbofuran in the effluent was extracted by the liquid-liquid partitioning method. pH and number of carbofuran degraders in the effluent were determined using a pH meter. At the end of column operation, each 5-cm depth of the soil was sectioned. Each section was passed through 1 mm sieve to separate the soil from rice straw and/or corncob. The soil and amendments from each section were air-dried at room temperature, weighed and analyzed for carbofuran and its metabolite concentrations. Carbofuran was extracted from soil and amendments by Accelerated Solvent Extractor (ASE100). The concentration of carbofuran in all extracts was further analyzed by HPLC. The number of carbofuran degraders in the soil from section was determined by drop plate technique.

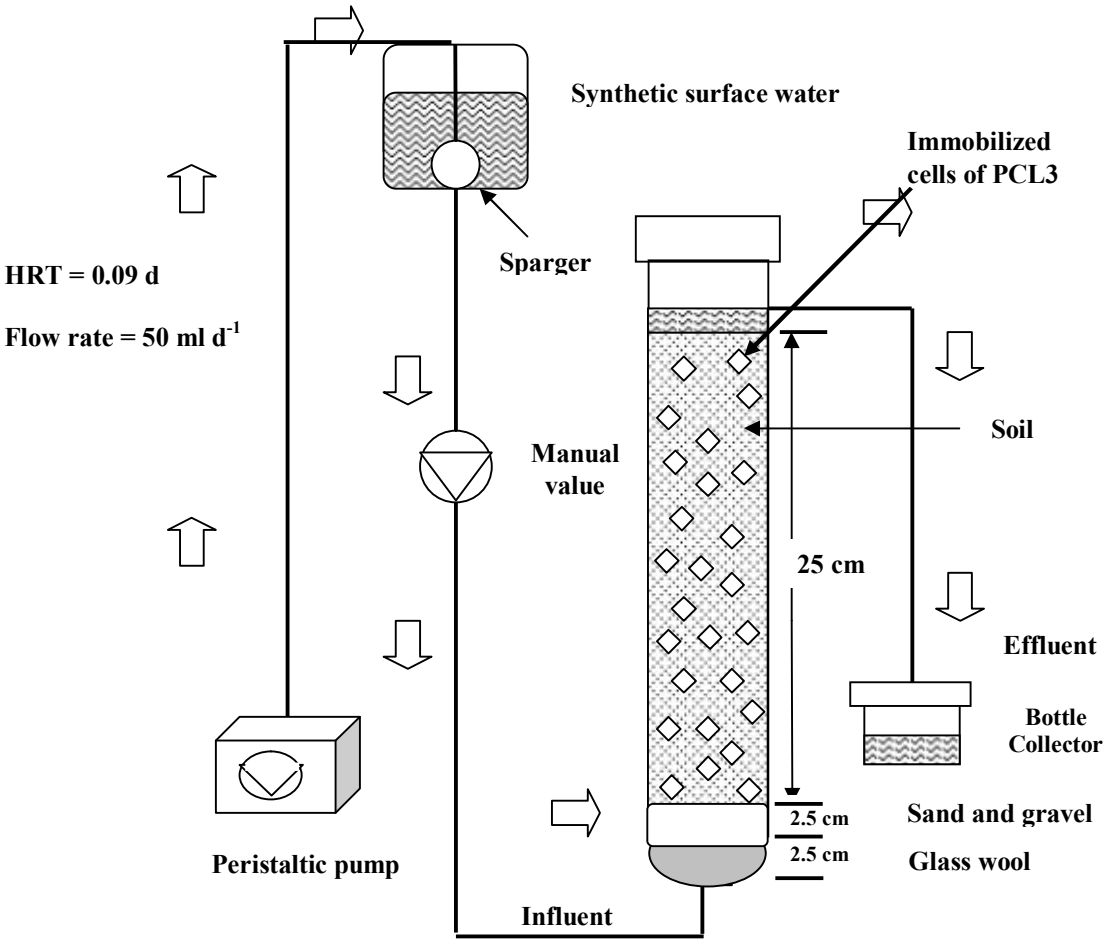

Figure 3.1 Soil column schematic diagram (not subject to scale)

Table 3.1 Experimental setup

Treatment	Experimental Setup	Objective
A	Soil	To study the biodegradation of carbofuran by indigenous microorganisms present in soil.
B	Soil sterilized using sodium azide	Abiotic control: To study the degradation of carbofuran without the biological activity.
C	Soil + free cells of PCL3	To study the effect of free cells of PCL3 on carbofuran degradation efficiency (Bioaugmentation).
D	Soil + immobilized PCL3	To study the effect of immobilized PCL3 on carbofuran degradation efficiency (Bioaugmentation).
E	Soil + rice straw	To study the effect of rice straw as organic amendment on carbofuran degradation efficiency (Biostimulation).
F	Soil + rice straw + free cells of PCL3	To study the effect of biostimulation together with bioaugmentation using free cells of PCL3 on the efficiency of carbofuran degradation.
G	Soil + rice straw + immobilized PCL3	To study the effect of biostimulation together with bioaugmentation using the immobilized PCL3 on the efficiency of carbofuran degradation.
H	Soil + corncob	Control of immobilized cell treatment: To study the effect of corncob on carbofuran degradation efficiency.

Table 3.2 Experimental setup

Treatment	Soil (g)	Carbofuran (mg)	Inoculums:amount (CFU/g soil)	Autoclaved corncob (g)	Rice straw (g)
A	838.26	8.66	-	-	-
B	792.40	9.61	-	-	-
C	801.00	9.24	free cells:10^6	-	-
D	650.77	7.22	immobilized cells:10^6 (60 g kg-soil^{-1})	-	-
E	861.08	9.76	-	-	12.92
F	752.79	8.69	free cells:10^6	-	11.29
G	650.06	7.21	immobilized cells:10^6 (60 g kg-soil^{-1})	-	9.75
H	715.89	7.94	-	40.62 (60 g kg-soil^{-1})	-

3.8 Analytical method

3.8.1 Extraction of carbofuran and its metabolites from synthetic surface water

Extraction of carbofuran from synthetic surface water using the liquid-liquid partitioning method was conducted by adding 2-ml of methanol into 2-ml of the synthetic surface water samples and then sonicating for 10 min, 50/60 voltage cycle, twice. After sonication, carbofuran and its metabolites were extracted from the liquid mixtures using dichloromethane in a separation funnel. This extraction was done 3 times. The first, second and third times of carbofuran extraction were added by 4, 2 and 2 ml of dichloromethane, respectively into the liquid mixtures and hand shaken for 30 sec. The organic fraction of the samples from each extraction was pooled and evaporated to dryness in the fume hood and then re-dissolved in 4 ml of 60% methanol and passed through a 0.45 μm nylon membrane syringe filter prior to being analyzed by HPLC. Percentage recoveries of this extraction procedure were 98.4, 96.2 and 97.0% for carbofuran, carbofuran phenol and 3-keto carbofuran, respectively.

3.8.2 Extraction of carbofuran and its metabolites from soil

Carbofuran and its metabolites were extracted from 13-g (dry weight) air-dried soil samples using an Accelerated Solvent Extractor ASE 100 (Dionex, USA) equipped with an 11-ml stainless-steel extraction cell. The samples were extracted under the conditions which were 100 ^{o}C extraction temperature, 5 min static extraction time, 60% methanol as the extraction solvent and two extraction cycles. The ASE parameters were used according to the default settings. The maximum extraction pressure was set not to exceed 1500 psi. The flush volume was 60% of the extraction cell volume. The nitrogen-purge time was set to 1 min. After 2 static extractions, the raw extracts were collected in 200-ml glass bottle. The volume of the extract was adjusted to 25 ml by 60% methanol (AR grade) and passed through a 0.45 μm nylon membrane syringe filter prior to being analyzed by HPLC (Plangklang and Reungsang, 2009). Percentage recoveries of this extraction procedure were 97.2, 95.7 and 95.4% for carbofuran, carbofuran phenol and 3-keto carbofuran, respectively.

3.8.3 Extraction of carbofuran and its metabolites from rice straw and corncob

The procedure for carbofuran and its metabolites extraction from rice straw and corncob was modified from Mrlina et al. (1994). Air dried rice straw or corncob (10 g dry weight) was cut into small pieces and blended with a Blendor for 3 min in 50 mL of a 9:1 (v/v) mixture of methanol/pH 8 phosphate buffer. The mixture was filtered through Buchners funnel lined with filter paper (Whatman No.1, Whatman Tnternational Ltd., England). The filter cake and the filter paper were again blended for 3 min with a second portion of the extraction mixture. The blending jars and the filter cakes were rinsed with 25 mL of blending solvent. The volume of the sample was adjusted to 150 mL with the blending solvent. Aliquot of 50 mL were transferred in a 250-mL separatory funnel and diluted with 50 mL of borax buffer (0.0052 M) containing 15 g of sodium chloride. The sample was extracted three times with 25 ml dichloromethane. The portion of dichloromethane was pooled, evaporated to dryness in fume hood and then re-dissolved in 4 ml of 60% methanol and passed through a 0.45 μm nylon membrane syringe filter prior to being analyzed by HPLC. Percentage recoveries of this extraction procedure were 87.1, 88.3 and 84.1% for carbofuran, carbofuran phenol and 3-keto carbofuran, respectively.

3.8.3 HPLC analysis of carbofuran

The carbofuran and its metabolite concentrations in the 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 HPLC operating parameters were: mobile phase, methanol-water (65:35); flow rate, 0.8 ml min^{-1} at the ambient temperature (Plangklang and Reungsang, 2008). 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 height. The detection limits of carbofuran, carbofuran phenol and 3-keto carbofuran were 0.05, 0.05 and 0.01 mg l^{-1}, respectively.

3.8.4 Enumeration of carbofuran degraders

The number of PCL3 and indigenous carbofuran degraders in the synthetic surface water and soil was determined by the drop plate technique on BSM agar coated with 5 mg l^{-1} of carbofuran. For the BSM agar, 1.5% of bactoagarose was added into BSM before sterilization by autoclaving at 121 °C for 15 min. 100 µl of carbofuran solution in sterile distilled water at a concentration of 5 mg l^{-1} was coated onto the BSM agar as a C-source prior to usage. The 20 µl of serial diluted synthetic surface water sample or soil sample was dropped on the BSM agar and incubated at 30 ^{o}C until the colonies formed.

To determine the number of PCL3 in corncob, the immobilized cells (5g) were blended into small particles using a blender and then added to 50 ml sterile 0.85% NaCl solution and shaken at 250 rpm for 5 min in order to dislodge cells from corncob. The number of PCL3 in the liquid phase was determined by plate count on the carbofuran-coated BSM agar as described above.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Sorption of carbofuran to soil, corncob, and rice straw

The sorption isotherm of carbofuran onto soil, corncob, and rice straw was investigated as shown in Figure 4.1. The data was fairly fitted to the Freundlich isotherm with the regression coefficient between 0.82-0.99 (Table 4.1). The K_f value was calculated from the intercept of the linear plot and the value of 1/n was computed from the slope of the linear plot. Results indicated that the sorption isotherm were not linear ($1/n \neq 1$). The 1/n value was less than 1.0 which indicates less effect of concentration change on the adsorptive capacity of the matrices (Faust and Aly, 1987). The K_f value was used to describe the extent of sorption between carbofuran and soil. The K_f value of 0.899, 0.634 and 0.028 l kg^{-1} were obtained for rice straw, soil and corncob, respectively (Table 4.1) which suggested that carbofuran might be adsorbed on soil more than on rice straw and corncob, respectively. However, the K_f values obtained are very low which implied that sorption phenomena might not has significant effect on the dissipation of carbofuran in soil column system. Carbofuran might not be adsorbed well in the matrices added into soil column and might be dissolved easily into the water phase.

4.2 Distribution of carbofuran in column effluent and soil

Carbofuran mass in column effluent during column operation is shown in Table 4.2. The percentage recovery of carbofuran mass in the effluent and carbofuran residues in the soil were examined at the end of column operation (Table 4.3). The assumed amounts of carbofuran degraded (Table 4.3) were calculated from subtraction of carbofuran mass added to soil by mass of carbofuran detected in soil plus in column effluent.

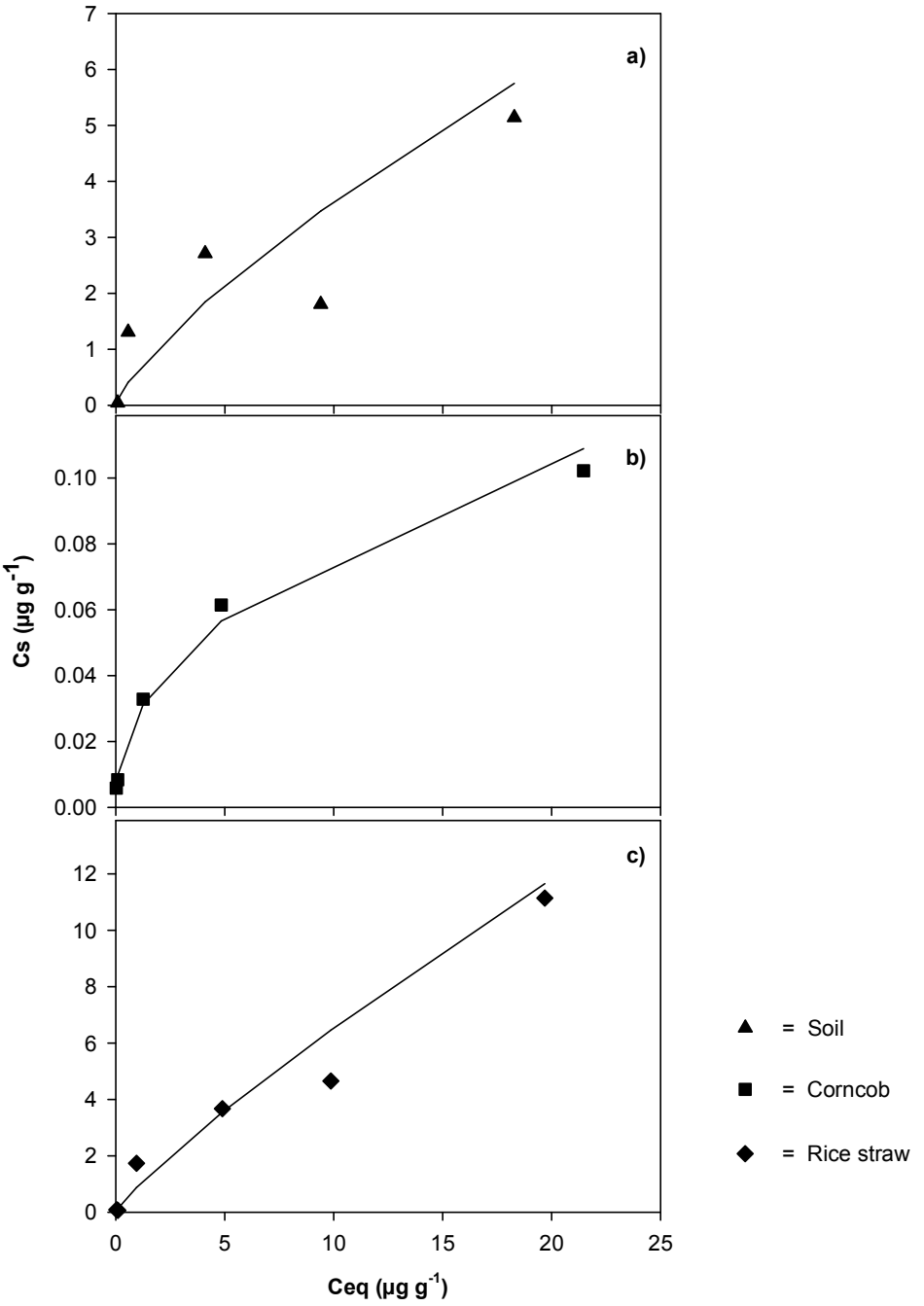

Figure 4.1 Sorption isotherms of carbofuran to soil (a), corncob (b), and rice straw (c); line indicates the sorption isotherm fitted to the Freundlich equation

Table 4.1 Sorption isotherms of carbofuran to corncob, rice straw, and soil

Material	K_f (l kg^{-1})	1/n	r^2
Corncob	0.028	0.440	0.993
Rice straw	0.899	0.855	0.953
Soil	0.634	0.759	0.815

Table 4.2 Carbofuran mass in the effluent approximately 100 ml collected every 2 d

Time (d)	Carbofuran mass in effluent (µg) ± SD			
	A	B	C	D
0-5	1937.20 (±0.85)	1923.90 (±0.14)	1068.00 (±1.41)	533.00 (±0.35)
6-7	224.12 (±0.4)	1181.80 (±0.42)	67.04 (±0.06)	50.52 (±0.04)
8-9	74.47 (±0.04)	748.92 (±0.59)	39.80 (±0.42)	50.70 (±0.71)
10-11	29.76 (±0.28)	274.46 (±0.47)	74.56 (±0.78)	44.18 (±0.03)
12-13	14.77 (±0.17)	72.87 (±0.96)	83.31 (±0.44)	84.39 (±0.54)
14-15	13.03 (±0.66)	61.91 (±0.18)	18.49 (±1.33)	71.82 (±0.44)
16-17	11.18 (±0.11)	61.34 (±0.17)	10.55 (±0.14)	83.87 (±0.59)
18-19	9.08 (±0.89)	30.78 (±0.34)	8.06 (±0.11)	42.39 (±0.41)
20-21	8.00 (±0.71)	41.12 (±0.20)	7.53 (±0.13)	19.47 (±0.03)
22-23	4.96 (±1.07)	43.99 (±0.17)	12.49 (±0.70)	24.69 (±0.20)
24-25	5.75 (±0.11)	29.02 (±0.49)	15.36 (±0.20)	8.24 (±0.01)
26-27	4.47 (±0.24)	29.29 (±0.14)	12.76 (±0.51)	6.15 (±0.20)
28-29	3.62 (±0.91)	26.31 (±0.16)	8.45 (±0.07)	8.67 (±0.89)
30-31	2.76 (±0.37)	15.53 (±0.16)	3.13 (±0.03)	14.73 (±0.89)
32-33	3.02 (±0.07)	14.42 (±0.28)	4.26 (±0.23)	9.79 (±0.47)
34-35	2.43 (±0.59)	8.51 (±0.14)	2.82 (±0.04)	2.30 (±0.44)
36-37	1.74 (±0.07)	2.88 (±0.04)	nd	0.44 (±0.01)
38-39	0.47 (±0.03)	2.51 (±0.17)	nd	nd
40-41	nd	2.29 (±0.13)	nd	nd
42-45	nd	1.80 (±0.01)	nd	nd
Total	2350.83 (±6.70)	4573.65 (±5.37)	1436.61 (±6.60)	1055.35 (±6.25)

nd = not detectable

A= soil; B=abiotic control; C=soil+free cells of PCL3; D=soil+immobilized PCL3; E=soil+rice straw; F=soil+free cells of PCL3+rice straw; G=soil+immobilized PCL3+rice straw; H=soil+autoclaved corncob

Table 4.2 (cont.) Carbofuran mass in the effluent approximately 100 ml collected every 2 d

Time (d)	Carbofuran mass in effluent (µg) ± SD			
	E	F	G	H
0-5	1248.00 (±0.65)	1660.40 (±1.2)	1356.00 (±1.41)	878.60 (±0.28)
6-7	54.83 (±0.47)	70.75 (±1.00)	80.08 (±0.16)	58.02 (±0.06)
8-9	36.30 (±1.70)	16.94 (±0.80)	16.56 (±0.71)	169.80 (±2.26)
10-11	32.24 (±1.74)	14.92 (±1.15)	15.03 (±0.25)	73.27 (±0.03)
12-13	45.09 (±0.31)	123.36 (±0.41)	31.34 (±0.34)	29.83 (±0.30)
14-15	17.68 (±0.18)	23.63 (±1.54)	6.23 (±0.04)	23.09 (±0.11)
16-17	20.02 (±0.37)	16.84 (±1.06)	10.65 (±0.07)	3.00 (±0.01)
18-19	16.76 (±0.16)	17.85 (±1.05)	22.73 (±0.03)	13.21 (±0.31)
20-21	7.30 (±0.40)	4.09 (±0.10)	20.99 (±1.39)	14.06 (±0.11)
22-23	4.23 (±0.31)	10.35 (±0.07)	2.76 (±1.00)	9.60 (±1.00)
24-25	2.01 (±0.03)	1.80 (±0.18)	4.49 (±0.41)	6.62 (±0.01)
26-27	2.39 (±0.52)	nd	6.88 (±0.17)	3.90 (±1.05)
28-29	1.91 (±0.10)	nd	7.92 (±0.13)	2.92 (±0.16)
30-31	1.00 (±0.3)	nd	1.99 (±0.92)	1.70 (±0.71)
32-33	0.70 (±0.14)	nd	2.96 (±1.07)	2.34 (±0.03)
34-35	nd	nd	5.74 (±0.89)	1.79 (±0.62)
36-37	nd	nd	nd	1.25 (±0.04)
38-39	nd	nd	nd	0.33 (±0.04)
40-41	nd	nd	nd	nd
42-45	nd	nd	nd	nd
Total	1490.46 (±7.38)	1960.93 (±8.56)	1592.35 (±8.99)	1293.33 (±7.13)

nd = not detectable

A= soil; B=abiotic control; C=soil+free cells of PCL3; D=soil+immobilized PCL3; E=soil+rice straw; F=soil+free cells of PCL3+rice straw; G=soil+immobilized PCL3+rice straw; H=soil+autoclaved corncob

Table 4.3 Percent recovery of carbofuran from d 5 until d 45

Parameter	Column							
	A	B	C	D	E	F	G	H
% mass recovery of carbofuran in the effluent	22.54b (± 1.33)	52.07c (± 2.15)	15.52a (±1.55)	14.62a (± 2.30)	15.29a (± 1.16)	22.57b (± 1.02)	22.08b (±1.76)	16.22a (±2.54)
% mass of carbofuran residues in soil at d 45	nd	2.00 (± 0.75)	nd	nd	nd	nd	nd	nd
% mass of carbofuran assumably degraded	77.46 (± 1.85)	47.93 (± 1.53)	84.48 (±1.21)	85.38 (± 2.14)	84.71 (±1.96)	77.43 (±1.78)	77.92 (± 2.08)	83.78 (±1.89)
% mass of carbofuran residues in corncob	-	-	-	nd	-	-	nd	nd
% mass of carbofuran residues in rice straw	-	-	-	-	nd	nd	nd	-

nd = not detectable

A= soil; B=abiotic control; C=soil+free cells of PCL3; D=soil+immobilized PCL3; E=soil+rice straw; F=soil+free cells of PCL3+rice straw; G=soil+immobilized PCL3+rice straw; H=soil+autoclaved corncob

Remarks: The assumed amounts of carbofuran degraded (Table 4.3) was calculated from subtraction of carbofuran mass added to soil by mass of carbofuran detected in soil plus in column effluent and in organic amendment.

A relatively high desorption of carbofuran from soil into the effluent could be found at the early state, 5-9 d, of column operation in all treatments. The hydroponic nature of carbofuran, i.e. a high water solubility of 351 mg l^{-1} at 25 ^{o}C (Tomaševi et al., 2007) and low soil adsorption coefficient of 0.63 l kg^{-1} (Table 1) might be responsible for this trend.

In abiotic control, column B, carbofuran could be detected in effluent until the end of column operation (45 d) (Table 4.2) with the mass recovery of carbofuran of 52.07% (Table 4.3). With the presence of only indigenous microorganism (column A), the percent recovery of carbofuran in the effluent was 22.54% compared to 52.07% in the abiotic control treatment (column B) (Table 4.3).

A detectable amount of carbofuran in the effluent from column A was not observed after 39 d of column operation (Table 4.2.1). These results indicate that biological activity of indigenous carbofuran degraders could prevent the movement of carbofuran along with the effluent. Naturally, indigenous carbofuran degraders are prevalent in soil or rice field soil with previous applications of carbofuran, but present in a small amount as can be seen in column A ($2.07x10^5$ CFU g^{-1} soil). In this study, soil samples packed in the columns were taken from a rice field of Ban Nonmuang, Khon Kaen. This soil sample has a history of carbofuran application so that carbofuran degraders could be adapted to use carbofuran as an energy source and it could be detected in rice field soil.

Bioaugmentation of PCL3 in free and immobilized cell forms (columns C and D, respectively) significantly reduced the movement of carbofuran in soil as indicated by low percentage recovery of carbofuran mass in the effluent of 15.52 and 14.62 %, respectively (Table 4.3). The detectable amounts of carbofuran in columns C and D was observed until 35 and 37 d of column operation (Table 4.2.1), respectively. Biostimulation using rice straw as organic amendment (column E) provided a similar result to bioaugmentation treatments (columns C and D). The percentage of carbofuran recovery in the effluent from column E was 15.29% (Table 4.3) and the carbofuran residues in effluent were not detected after 33 d of column operation (Table 4.2.1 and 4.2.2). These results suggest an increase in the number of carbofuran degraders in the soil by adding specific carbofuran degraders, i.e. PCL3, or stimulating the activity of indigenous carbofuran degraders in the soil could improve the carbofuran degradation efficiency, hence preventing the movement of carbofuran along with the effluent.

The addition of PCL3 in free and immobilized cell forms together with rice straw (columns F and G, respectively) decreased the carbofuran degradation efficiency in soil columns. The percentage recoveries of carbofuran mass in the effluent of 22.57 and 22.08% were obtained from columns F and G, respectively (Table 4.3) which were higher than that observed in the treatments with bioaugmentation or biostimulation alone (columns C, D, and E). This is because PCL3 and/or indigenous carbofuran degraders might prefer to use by-products such as carbon, nitrogen, and phosphorus from rice straw degradation (Fores et al., 1988), than carbofuran. These phenomena could result in the decrease in carbofuran degradation efficiency and increase the movement of carbofuran along with the effluent.

Carbofuran residues in the soil could not be detected in all treatments with the biological activity, whereas in the abiotic control, the percentage recovery of carbofuran mass in the soil was 2% detected at the end of column operation (Table 4.3). The results confirm that biological treatments are an effective tool to enhance carbofuran degradation in soil and prevent the movement of carbofuran in soil along with the effluent. However, the abiotic control demonstrated the relatively high percentage of carbofuran degradation of 47.93%. This might have resulted from oxidation processes due to the continuous aeration of water before feeding to the column. Although the abiotic degradation processes such as oxidation and volatilization are not as important as microbial degradation, they also were contributing to dissipation processes which could be found in abiotic control as reported in previous study (Lalah and Wandiga, 1996).

4.3 Carbofuran metabolites production during column operation

Carbofuran phenol and 3-keto carbofuran were observed as metabolites in effluent from the columns (Tables 4.4 and 4.5) but no metabolite was detected in the soil (data not shown). Carbofuran phenol was found in the effluent from columns with the presence of biological activity (columns A, C, D, E, F, G, and H), but could not be found in effluent from the abiotic control (column B) (Table 4.4). The results suggest that carbofuran phenol is a metabolite from the biodegradation process.

Table 4.4 Carbofuran phenol detected in column effluent

Time (d)	Carbofuran phenol (mg l-1)			
	A	B	C	D
0-5	0.806 (±0.01)	nd	0.079 (±0.07)	0.294 (±0.13)
6-7	0.905 (±0.08)	nd	nd	nd
8-9	0.555 (±0.01)	nd	0.049 (±0.04)	0.427 (±0.04)
10-11	0.441 (±0.04)	nd	nd	nd
12-13	0.245 (±0.00)	nd	0.39 (±0.37)	nd
14-15	0.064 (±0.00)	nd	nd	nd
16-17	0.014 (±0.00)	nd	0.038 (±0.02)	0.216 (±0.02)
18-19	0.16 (±0.04)	nd	0.021 (±0.00)	nd
20-21	0.031 (±0.02)	nd	nd	nd
22-23	0.037 (±0.02)	nd	nd	nd
24-25	nd	nd	nd	nd
26-27	nd	nd	nd	nd
28-29	nd	nd	nd	nd
30-31	nd	nd	nd	nd
32-33	nd	nd	nd	nd
34-35	nd	nd	nd	nd
36-37	0.008 (±0.00)	nd	nd	nd
38-39	0.012 (±0.00)	nd	nd	nd
40-41	0.01 (±0.01)	nd	nd	nd
42-45	nd	nd	nd	nd

nd = not detectable

A= soil; B=abiotic control; C=soil+free cells of PCL3; D=soil+immobilized PCL3; E=soil+rice straw; F=soil+free cells of PCL3+rice straw; G=soil+immobilized PCL3+rice straw; H=soil+autoclaved corncob

Table 4.4 (cont.) Carbofuran phenol detected in column effluent

Time (d)	Carbofuran phenol (mg l-1)			
	E	F	G	H
0-5	nd	nd	0.42 (±0.07)	nd
6-7	nd	nd	0.232 (±0.04)	0.509 (±0.00)
8-9	nd	nd	0.243 (±0.05)	0.187 (±0.00)
10-11	nd	nd	0.159 (±0.01)	0.025 (±0.01)
12-13	nd	0.09 (±0.04)	0.171 (±0.06)	nd
14-15	0.051 (±0.02)	0.068 (±0.02)	0.067 (±0.03)	nd
16-17	nd	nd	0.021 (±0.01)	nd
18-19	nd	nd	nd	nd
20-21	0.019 (±0.01)	nd	nd	nd
22-23	nd	nd	nd	nd
24-25	nd	nd	nd	nd
26-27	nd	nd	nd	nd
28-29	nd	nd	nd	nd
30-31	nd	nd	nd	nd
32-33	nd	nd	nd	nd
34-35	nd	nd	nd	nd
36-37	nd	nd	nd	nd
38-39	nd	nd	nd	nd
40-41	nd	nd	nd	nd
42-45	nd	nd	nd	nd

nd = not detectable

A= soil; B=abiotic control; C=soil+free cells of PCL3; D=soil+immobilized PCL3; E=soil+rice straw; F=soil+free cells of PCL3+rice straw; G=soil+immobilized PCL3 +rice straw; H=soil+autoclaved corncob

Table 4.5 3-Keto carbofuran detected in column effluent

Time (d)	3-keto carbofuran (mg l-1)			
	A	B	C	D
0-5	0.052 (±0.02)	nd	0.04 (±0.03)	0.054 (±0.02)
6-7	0.021 (±0.00)	nd	nd	nd
8-9	0.013 (±0.01)	nd	nd	nd
10-11	0.013 (±0.00)	nd	0.043 (±0.03)	nd
12-13	0.02 (±0.01)	nd	nd	nd
14-15	0.071 (±0.01)	nd	nd	nd
16-17	0.046 (±0.02)	nd	0.03 (±0.02)	0.036 (±0.01)
18-19	0.056 (±0.01)	nd	0.018 (±0.00)	nd
20-21	0.052 (±0.02)	nd	nd	nd
22-23	0.079 (±0.02)	0.013 (±0.01)	0.012 (±0.00)	0.046 (±0.01)
24-25	nd	nd	nd	nd
26-27	0.05 (±0.01)	0.004 (±0.00)	nd	nd
28-29	0.002 (±0.00)	nd	nd	0.011 (±0.00)
30-31	nd	nd	nd	nd
32-33	0.024 (±0.01)	nd	0.02 (±0.02)	nd
34-35	nd	nd	nd	nd
36-37	0.006 (±0.00)	nd	nd	nd
38-39	nd	nd	nd	nd
40-41	nd	nd	nd	nd
42-43	nd	nd	nd	nd
44-45	nd	nd	nd	nd

nd = not detectable

A= soil; B=abiotic control; C=soil+free cells of PCL3; D=soil+immobilized PCL3; E=soil+rice straw; F=soil+free cells of PCL3+rice straw; G=soil+immobilized PCL3+rice straw; H=soil+autoclaved corncob

Table 4.5 (cont.) 3-Keto carbofuran detected in column effluent

Time (d)	3-keto carbofuran (mg l-1)			
	E	F	G	H
0-5	nd	0.046 (±0.03)	0.08 (±0.03)	0.06 (±0.01)
6-7	nd	nd	0.022 (±0.01)	0.022 (±0.00)
8-9	0.008 (±0.00)	nd	0.008 (±0.00)	0.022 (±0.01)
10-11	nd	0.023 (±0.02)	nd	0.028 (±0.00)
12-13	0.018 (±0.01)	0.003 (±0.00)	nd	0.149 (±0.00)
14-15	nd	nd	nd	nd
16-17	0.002 (±0.00)	0.022 (±0.02)	nd	nd
18-19	nd	0.006 (±0.01)	nd	nd
20-21	nd	nd	nd	nd
22-23	0.007 (±0.00)	0.012 (±0.00)	0.019 (±0.02)	0.395 (±0.05)
24-25	nd	nd	nd	nd
26-27	nd	nd	0.031 (±0.01)	0.032 (±0.00)
28-29	nd	nd	nd	nd
30-31	nd	nd	nd	0.029 (±0.01)
32-33	nd	nd	nd	nd
34-35	nd	nd	0.022 (±0.01)	nd
36-37	nd	nd	nd	nd
38-39	nd	nd	nd	nd
40-41	nd	nd	nd	nd
42-43	nd	nd	nd	nd
44-45	nd	nd	nd	nd

nd = not detectable

A= soil; B=abiotic control; C=soil+free cells of PCL3; D=soil+immobilized PCL3; E=soil+rice straw; F=soil+free cells of PCL3+rice straw; G=soil+immobilized PCL3+rice straw; H=soil+autoclaved corncob

The concentration of carbofuran phenol tended to decrease over time and could not be detected at the end of column operation, which implies that carbofuran phenol could be metabolized by PCL3 and/or indigenous microorganisms. Methyl carbamate degrading (*mcd*) gene coding the hydrolase enzyme catalyzes the degradation of carbofuran to carbofuran phenol. The *mcd* gene coding for hydrolase enzyme is responsible for carbofuran degradation, which was detected in PCL3 (Plangklang and Reungsang, 2008) and various types of carbofuran degrader isolated from the carbamate pesticides contaminated matrices such as *Achromobacter* sp. WM111 (Tomasek and Karns, 1989), *Sphingomonas* sp. 5 (Ryeol et al., 2006; Liu et al., 2006), *Novosphingobium* sp. FND-3 (Yan et al., 2007) and *Paracoccus* sp. YM3 (Peng et al., 2008). Some of the indigenous microorganisms have the capability to metabolize carbofuran phenol produced during the degradation of carbofuran (Plangklang and Reungsang, 2009), hence, carbofuran phenol could not be observed in the system (column B) at the end of the bioremediation process.

The 3-keto carbofuran was observed in the effluent from all column treatments (Table 4.5). In general, 3-keto carbofuran was detected as a key by-product of carbofuran degradation prevalence in water (Evert, 2002) mostly flooded condition (Kale et al., 2001). Since PCL3 has not been reported to produce 3-keto carbofuran (Plangklang and Reungsang, 2004; 2008; 2009), we speculated that 3-keto carbofuran was the metabolite from indigenous microorganism activity and/or abiotic degradation processes. Indigenous microorganisms can oxidize carbofuran into 3-keto carbofuran via oxidation reaction.

A smaller amount of carbofuran phenol and 3-keto carbofuran was observed in the effluent from bioaugmented (columns C and D) and biostimulated columns (column E) in comparison to the column with only indigenous microorganisms (column A) (Tables 4.4 and 4.5). This result suggests that introducing the bioremediation technique i.e., bioaugmentation (the addition of immobilized and free cells of PCL3) and biostimulation (rice straw supplementation) to the carbofuran contaminated soil in the columns could improve the degradation of carbofuran metabolites produced during the time of incubation.

4.4 CFU variation during column operation

4.4.1 Number of carbofuran degraders in soil

The initial number of carbofuran degraders in the soil without inoculation (column A) and soil augmented with the immobilized PCL3 (columns D and G) was $2.07x10^5$, $4.78x10^5$ and, $7.59x10^5$ CFU g^{-1}soil, respectively. The initial number of carbofuran degraders in soil inoculated with free cells of PCL3 (columns C and F) was $2.76x10^6$ and, $3.65x10^6$ CFU g^{-1}soil (Table 4.6).

Table 4.6 Initial number of carbofuran degraders in the soil

Treatment	Number of carbofuran degraders (CFU g^{-1}soil)±SD
A (soil)	$2.07x10^5$ ($\pm 0.57x10^3$)
C (soil + free cells of PCL3)	$2.76x10^6$($\pm 5.67x10^3$)
D (soil + immobilized PCL3)	$4.78x10^5$($\pm 1.41x10^3$)
F (soil + free cells of PCL3+rice straw)	$3.65x10^6$($\pm 7.07x10^3$)
G (soil + immobilized PCL3 + rice straw)	$7.59x10^5$($\pm 0.71x10^3$)

The number of carbofuran degraders in soil sections at the end of column operation is shown in Figure 4.2. Results indicate that the number of carbofuran degraders was not markedly different among soil sections. In column A (with only indigenous microorganisms), the number of carbofuran degraders was not markedly changed as compared to the initial value. The addition of rice straw (column E) or corncob (column H) resulted in an increase in the number of carbofuran degraders (Figure 4.2) to approximately 10^6-10^7 and 10^7-10^8 CFU g^{-1} soil, respectively, at the end of column operation. The results indicate that indigenous microorganisms could use rice straw and corncob as the energy source for their growth, resulting in an increase in their population which could give an improvement of carbofuran degradation in soil.

The addition of PCL3 (free cells) into soil results in a higher number of carbofuran degraders which could be responsible for enhanced degradation of carbofuran in soil in comparison to the treatment with no bioaugmentation. A slight decrease in the number of carbofuran degraders in the soil in the bioaugmentation treatment using free cells of PCL3 (column C) was observed (Figure 4.2). The results indicate that PCL3 in

free cells form might not have a capability to survive in soil for long term column operation. Meanwhile, the number of immobilized cells of PCL3 inoculated in the soil column G was stable throughout the experiment might be due to the supporting material acting as a protective agent. In other words, this supporting material could protect PCL3 from the diverse environments such as substrate and product inhibition or shear forces during the incubation period.

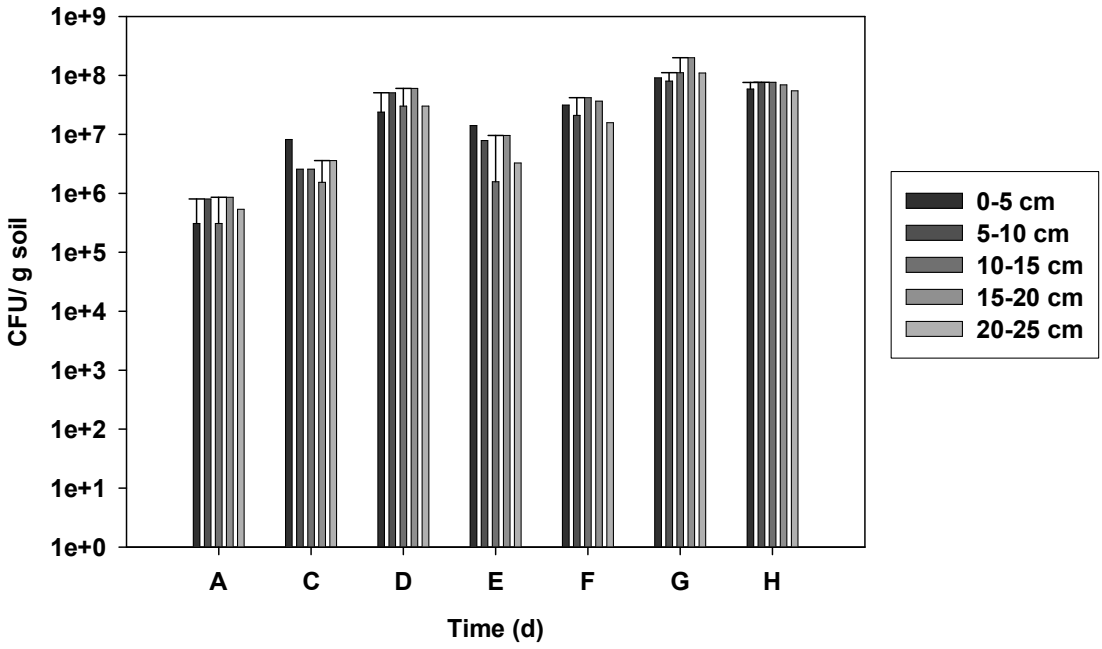

Figure 4.2 Number of cabofuran degraders in each soil section

The addition of PCL3 in immobilized cells form (column D) obviously increased the number of carbofuran degraders in the soil to be 10^7 CFU g^{-1} soil at the end of column operation (Figure 4.2). The increase in number of carbofuran degraders in the soil might be a result of the leaking of PCL3 from support material to soil and/or the stimulation of indigenous microorganisms by corncob. The number of carbofuran degraders in soil augmented with immobilized cells of PCL3 (column D) was greater than free cells (column C). This indicates that the immobilization technique could improve the growth and survival of PCL3 in the soil.

The addition of PCL3 together with rice straw (column G and H) gave the high number of carbofuran degraders in the soil of approximately 10^8 CFU g^{-1} soil at the end of

column operation. However, the carbofuran degradation efficiency was not improved in comparison to non-treated soil (column A) (Table 4.2). This might be because rice straw and/or by-products from rice straw degradation contain the preferred substrate for PCL3 and some indigenous microorganisms. Thus, they could metabolize rice straw and its degradation products for their growth without the degradation of carbofuran.

4.4.2 Numbers of carbofuran degraders in the column effluent

The number of carbofuran degraders in the effluent collected from the columns is shown in Figure 4.3. The results are in correlation with the number of

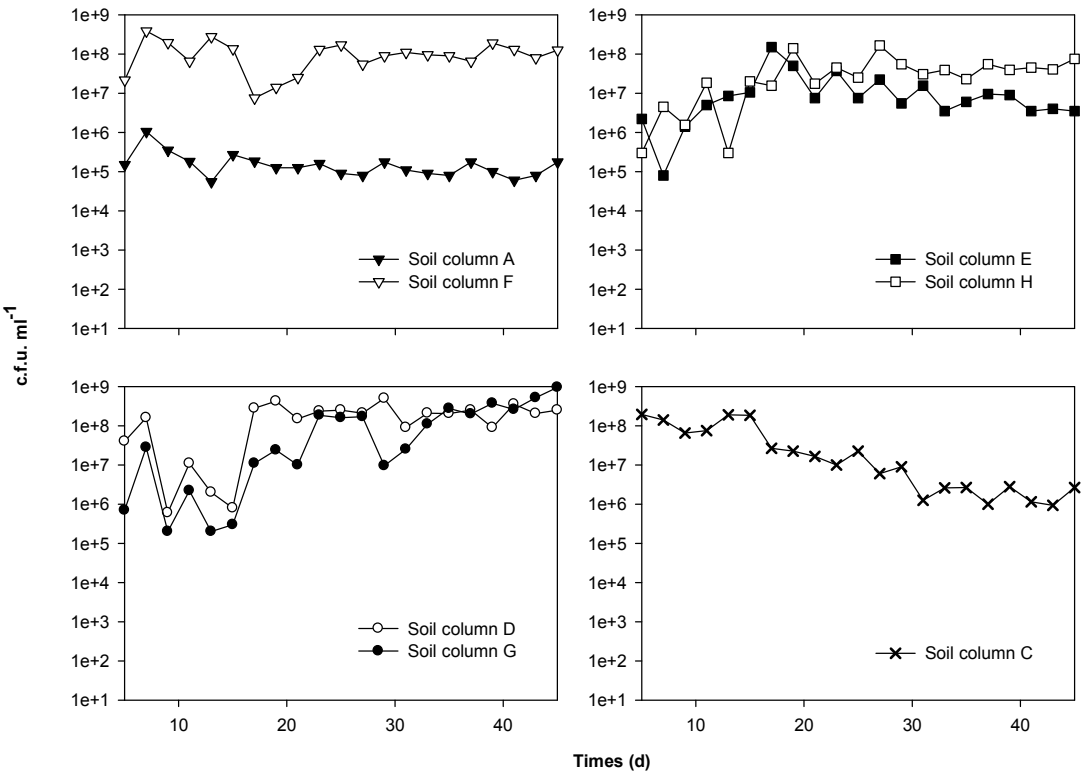

Figure 4.3 Number of carbofuran degraders in column effluent (A: soil, C: soil + free cells of PCL3, D: soil + immobilized PCL3, E: soil + rice straw, F: soil + free cells of PCL3 and rice straw, G: soil + immobilized PCL3 and rice straw, H: soil + autoclaved corncob)

carbofuran degraders in the soil. The number of carbofuran degraders in the effluent from column A (with only indigenous microorganisms) was observed to be stable at approximately 10^5 CFU ml^{-1}. The higher number of carbofuran degraders in soil in treatment bioaugmented with free cells of PCL3 (column C and F) resulted in the greater

number of carbofuran degraders in the effluent (10^6-10^8 CFU ml^{-1}). The amount of carbofuran in the effluent from column C (free cells of PCL3 bioaugmented column) decreased over time, suggesting that PCL3 in free cells form could not survive during column operation. The number of carbofuran degraders in column F (with free cells and rice straw added) remained above 10^7 CFU ml^{-1} throughout the experiment. This result indicates that rice straw might be used by indigenous microorganisms/or PCL3 for their growth, resulting in non-reduction of the number of carbofuran degraders in soil and effluent. The number of carbofuran degraders in effluent from the column with added rice straw (column E) and corncob (column H) slightly increased at the early stage of column operation and was stable at above 10^6 CFU ml^{-1}. Thus, the addition of organic amendments i.e. rice straw and corncob could stimulate and increase the number of carbofuran degraders in soil and effluent. The tendency to increase in the number of carbofuran degraders in the column effluent could also be observed in the treatment augmented with immobilized cells (column D and G) which could result from the leaking of the growing cells from the support materials and/or the biostimulation effect of corncob. A similar result was noted by Plangklang and Reungsang (2009) who observed that cell leakage from corncob could increase the number of PCL3 in Basal Salt Medium (BSM). Moreover, the finding of Kumar and Das (2001) reported that *Enterobactor cloacae* II-BT 08 leaked from the supporting material to the culture media because of lack of space. These findings indicate that the high matrix porosity and pore size of supporting material might enhance the adsorption capacity and substrate transfer to the immobilized cells (Gu et al., 1994; Jimoh, 2004). Immobilization by adsorption mechanism of supporting material should give not only good packing materials for bacterial growth, but they should also be effective for physical adsorption of the contaminant to support a transfer of the contaminant to the cells (Ma et al., 2006). This physical adsorption employs an electrostatic force or a covalent binding to the support material (Bekatorou et al., 2004).

4.5 pH variation in column effluent

pH of the effluent from the columns was monitored during column operation and is depicted in Figure 4.4. The pH of the effluent from the abiotic column (B) was observed to be stable at approximately 6.9 throughout the experiment which might be due to the absence of microbial activity. The pH of effluent with biological activity (columns A, C,

D, E, F, G, and H) was more varied than the abiotic control (column B). With the presence of only indigenous microorganisms (column A and H) and indigenous microorganisms together with PCL3 (column F), the pH value increased until 15, 11, and 9 d, respectively, and decreased thereafter until the end of column operation. The pH of the effluent from the columns augmented with PCL3 (columns C, D, F, and G) tended to increase continuously during column operation. The variation in effluent pH could result from the products i.e., 3-keto carbofuran and carbofuran phenol obtained from biodegradation activity. The increase in effluent pH might be caused by the alkaline nature of by-products i.e., 3-keto carbofuran and carbofuran phenol obtained during the metabolism of carbofuran and organic matter by microorganisms present in the soil (Prasanna et al., 2008). The decrease in effluent pH might be due to the formation of CO_2 from mineralization of carbofuran and organic matter in the soil (Venkata-Mohan et al., 2006). The similar results of Chapman and Cole (1982) indicate that the rate of carbofuran degradation in water has a strong impact on pH. The increase in pH could increase the rate of hydrolysis able to facilitate a degradation process of carbofuran (Seiber et al., 1978). This chemical hydrolysis is the mechanism to give carbofuran phenol (Getzin, 1973; Seiber et al., 1978; Yu et al., 1974) produced by *Pseudomonas* sp., *Arthrobacter* and *Bacillus* sp. after metabolizing carbofuran under neutral conditions (Cain and Head, 1991), or ring cleavage given CO_2 and H_2O by carbofuran degraders (Trabue et al., 1997).

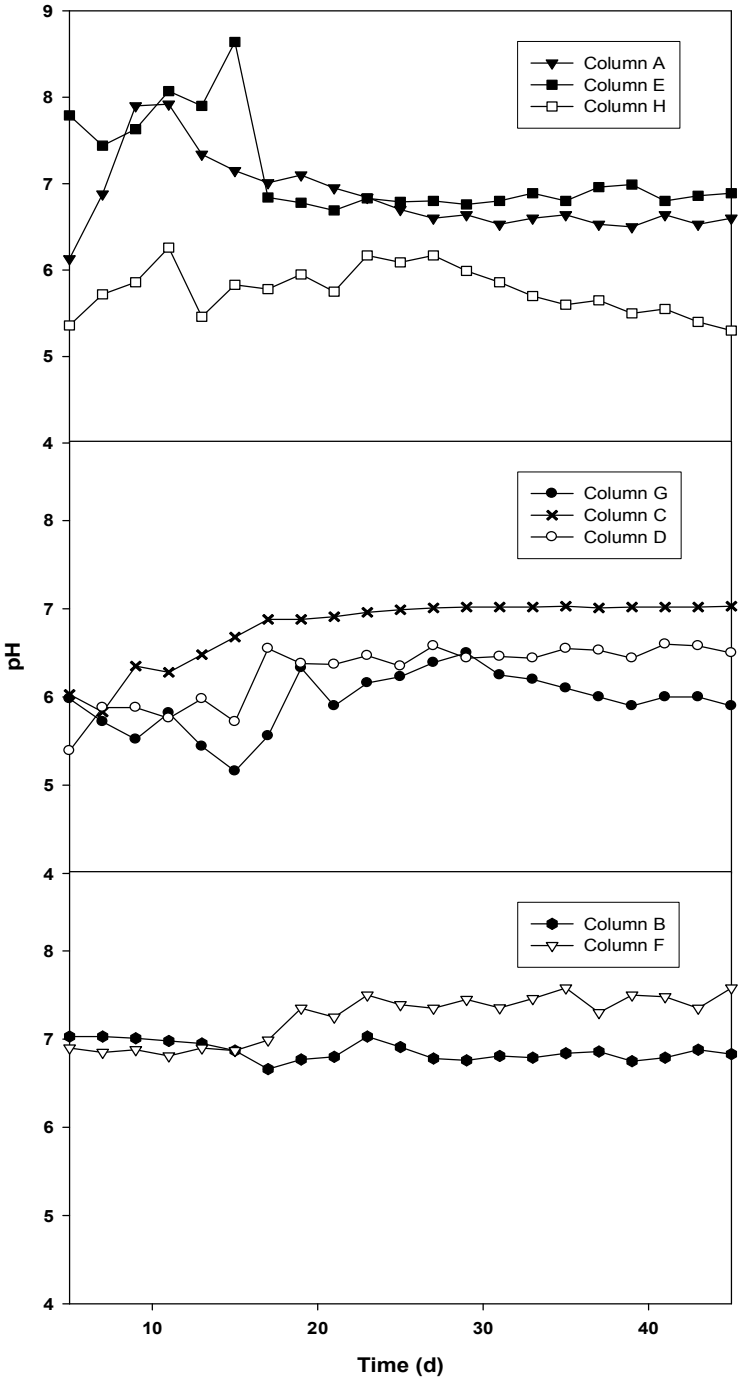

Figure 4.4 pH variation in columns effluent (A: soil, B: abiotic control, C: soil+free cells of PCL3, D: soil+immobilized cells of PCL3, E: soil+rice straw, F: soil+free cells of PCL3 and rice straw, G: soil+immobilized cells of PCL3 and rice straw, and H: soil+ autoclaved corncob)

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APPENDICES

APPENDIX A

CONTENTS IN SOIL COLUMN

Table A-1 Contents in soil column

Treatment	Soil (g)	Carbofuran (mg)	Inoculums:amount (CFU/g soil)	Autoclaved corncob (g)	Rice straw (g)
A	838.26	8.66	-	-	-
B	792.40	9.61	-	-	-
C	801.00	9.24	free cells:10^6	-	-
D	650.77	7.22	immobilized cells:10^6 (60 g kg-soil^{-1})	-	-
E	861.08	9.76	-	-	12.92
F	752.79	8.69	free cells:10^6	-	11.29
G	650.06	7.21	immobilized cells:10^6 (60 g kg-soil^{-1})	-	9.75
H	715.89	7.94	-	40.62 (60 g kg-soil^{-1})	-

APPENDIX B

SORPTION ISOTHERM OF CARBOFURAN TO CORNCOB, RICE STRAW, AND SOIL

Table B-1 Sorption isotherms of carbofuran to corncob, rice straw, and soil

Material	K_f ($l\,kg^{-1}$)	1/n	r^2
Corncob	0.028	0.440	0.993
Rice straw	0.899	0.855	0.953
Soil	0.634	0.759	0.815

APPENDIX C

(CARBOFURAN MASS IN THE EFFLUENT)

Table C-1 Carbofuran mass in the effluent approximately 100 ml, collected sample every 2 d

Time (d)	Carbofuran mass in effluent (µg) ± SD			
	A	B	C	D
0-5	1937.20 (±0.85)	1923.90 (±0.14)	1068.00 (±1.41)	533.00 (±0.35)
6-7	224.12 (±0.4)	1181.80 (±0.42)	67.04 (±0.06)	50.52 (±0.04)
8-9	74.47 (±0.04)	748.92 (±0.59)	39.80 (±0.42)	50.70 (±0.71)
10-11	29.76 (±0.28)	274.46 (±0.47)	74.56 (±0.78)	44.18 (±0.03)
12-13	14.77 (±0.17)	72.87 (±0.96)	83.31 (±0.44)	84.39 (±0.54)
14-15	13.03 (±0.66)	61.91 (±0.18)	18.49 (±1.33)	71.82 (±0.44)
16-17	11.18 (±0.11)	61.34 (±0.17)	10.55 (±0.14)	83.87 (±0.59)
18-19	9.08 (±0.89)	30.78 (±0.34)	8.06 (±0.11)	42.39 (±0.41)
20-21	8.00 (±0.71)	41.12 (±0.20)	7.53 (±0.13)	19.47 (±0.03)
22-23	4.96 (±1.07)	43.99 (±0.17)	12.49 (±0.70)	24.69 (±0.20)
24-25	5.75 (±0.11)	29.02 (±0.49)	15.36 (±0.20)	8.24 (±0.01)
26-27	4.47 (±0.24)	29.29 (±0.14)	12.76 (±0.51)	6.15 (±0.20)
28-29	3.62 (±0.91)	26.31 (±0.16)	8.45 (±0.07)	8.67 (±0.89)
30-31	2.76 (±0.37)	15.53 (±0.16)	3.13 (±0.03)	14.73 (±0.89)
32-33	3.02 (±0.07)	14.42 (±0.28)	4.26 (±0.23)	9.79 (±0.47)
34-35	2.43 (±0.59)	8.51 (±0.14)	2.82 (±0.04)	2.30 (±0.44)
36-37	1.74 (±0.07)	2.88 (±0.04)	nd	0.44 (±0.01)
38-39	0.47 (±0.03)	2.51 (±0.17)	nd	nd
40-41	nd	2.29 (±0.13)	nd	nd
42-45	nd	1.80 (±0.01)	nd	nd
Total	2350.83 (±6.70)	4573.65 (±5.37)	1436.61 (±6.60)	1055.35 (±6.25)

nd = not detectable

A= soil; B=abiotic control; C=soil+free cells of PCL3; D=soil+immobilized PCL3; E=soil+rice straw; F=soil+free cells of PCL3+rice straw; G=soil+immobilized PCL3+rice straw; H=soil+autoclaved corncob

Table C-1 Carbofuran mass in the effluent approximately 100 ml, collected sample every 2 d (cont.)

Time (d)	Carbofuran mass in effluent (µg) ± SD			
	E	F	G	H
0-5	1248.00 (±0.65)	1660.40 (±1.2)	1356.00 (±1.41)	878.60 (±0.28)
6-7	54.83 (±0.47)	70.75 (±1.00)	80.08 (±0.16)	58.02 (±0.06)
8-9	36.30 (±1.70)	16.94 (±0.80)	16.56 (±0.71)	169.80 (±2.26)
10-11	32.24 (±1.74)	14.92 (±1.15)	15.03 (±0.25)	73.27 (±0.03)
12-13	45.09 (±0.31)	123.36 (±0.41)	31.34 (±0.34)	29.83 (±0.30)
14-15	17.68 (±0.18)	23.63 (±1.54)	6.23 (±0.04)	23.09 (±0.11)
16-17	20.02 (±0.37)	16.84 (±1.06)	10.65 (±0.07)	3.00 (±0.01)
18-19	16.76 (±0.16)	17.85 (±1.05)	22.73 (±0.03)	13.21 (±0.31)
20-21	7.30 (±0.40)	4.09 (±0.10)	20.99 (±1.39)	14.06 (±0.11)
22-23	4.23 (±0.31)	10.35 (±0.07)	2.76 (±1.00)	9.60 (±1.00)
24-25	2.01 (±0.03)	1.80 (±0.18)	4.49 (±0.41)	6.62 (±0.01)
26-27	2.39 (±0.52)	nd	6.88 (±0.17)	3.90 (±1.05)
28-29	1.91 (±0.10)	nd	7.92 (±0.13)	2.92 (±0.16)
30-31	1.00 (±0.3)	nd	1.99 (±0.92)	1.70 (±0.71)
32-33	0.70 (±0.14)	nd	2.96 (±1.07)	2.34 (±0.03)
34-35	nd	nd	5.74 (±0.89)	1.79 (±0.62)
36-37	nd	nd	nd	1.25 (±0.04)
38-39	nd	nd	nd	0.33 (±0.04)
40-41	nd	nd	nd	nd
42-45	nd	nd	nd	nd
Total	1490.46 (±7.38)	1960.93 (±8.56)	1592.35 (±8.99)	1293.33 (±7.13)

nd = not detectable

A= soil; B=abiotic control; C=soil+free cells of PCL3; D=soil+immobilized PCL3; E=soil+rice straw; F=soil+free cells of PCL3+rice straw; G=soil+immobilized PCL3+rice straw; H=soil+autoclaved corncob

APPENDIX D

PERCENT RECOVERY OF CARBOFURAN FROM D 5 UNTIL D 45

Table D-1 Percent recovery of carbofuran from d 5 until d 45

Parameter	Column							
	A	B	C	D	E	F	G	H
% mass recovery of carbofuran in the effluent	22.54 (± 1.33) b	52.07 (± 2.15) c	15.52(±1 .55) a	14.62 (± 2.30) a	15.29 (± 1.16) a	22.57 (± 1.02) b	22.08± (1.76) b	16.22 (±2.54) a
% mass of carbofuran residues in soil at d 45	nd	2.00 (± 0.75)	nd	nd	nd	nd	nd	nd
% mass of carbofuran assumably degraded	77.46 (± 1.85)	47.93 (± 1.53)	84.48 (±1.21)	85.38 (± 2.14)	84.71 (±1.96)	77.43 (±1.78)	77.92 (± 2.08)	83.78 (±1.89)
% mass of carbofuran residues in corncob	-	-	-	nd	-	-	nd	nd
% mass of carbofuran residues in rice straw	-	-	-	-	nd	nd	nd	-

nd = not detectable

A= soil; B=abiotic control; C=soil+free cells of PCL3; D=soil+immobilized PCL3; E=soil+rice straw; F=soil+free cells of PCL3+rice straw; G=soil+immobilized PCL3+rice straw; H=soil+autoclaved corncob

APPENDIX E

(CARBOFURAN METABOLITES SUCH AS CARBOFURAN PHENOL AND 3-KETO CARBOFURAN IN THE EFFLUENT)

Table E-1 Carbofuran metabolites from the effluent such as carbofuran phenol

Time (d)	Carbofuran phenol (mg l-1)			
	A	B	C	D
0-5	0.806 (±0.01)	nd	0.079 (±0.07)	0.294 (±0.13)
6-7	0.905 (±0.08)	nd	nd	nd
8-9	0.555 (±0.01)	nd	0.049 (±0.04)	0.427 (±0.04)
10-11	0.441 (±0.04)	nd	nd	nd
12-13	0.245 (±0.00)	nd	0.39 (±0.37)	nd
14-15	0.064 (±0.00)	nd	nd	nd
16-17	0.014 (±0.00)	nd	0.038 (±0.02)	0.216 (±0.02)
18-19	0.16 (±0.04)	nd	0.021 (±0.00)	nd
20-21	0.031 (±0.02)	nd	nd	nd
22-23	0.037 (±0.02)	nd	nd	nd
24-25	nd	nd	nd	nd
26-27	nd	nd	nd	nd
28-29	nd	nd	nd	nd
30-31	nd	nd	nd	nd
32-33	nd	nd	nd	nd
34-35	nd	nd	nd	nd
36-37	0.008 (±0.00)	nd	nd	nd
38-39	0.012 (±0.00)	nd	nd	nd
40-41	0.01 (±0.01)	nd	nd	nd
42-45	nd	nd	nd	nd

nd = not detectable

A= soil; B=abiotic control; C=soil+free cells of PCL3; D=soil+immobilized PCL3; E=soil+rice straw; F=soil+free cells of PCL3+rice straw; G=soil+immobilized PCL3+rice straw; H=soil+autoclaved corncob

Table E-1 Carbofuran metabolites from the effluent such as carbofuran phenol (cont.)

Time (d)	Carbofuran phenol (mg l-1)			
	E	F	G	H
0-5	nd	nd	0.42 (±0.07)	nd
6-7	nd	nd	0.232 (±0.04)	0.509 (±0.00)
8-9	nd	nd	0.243 (±0.05)	0.187 (±0.00)
10-11	nd	nd	0.159 (±0.01)	0.025 (±0.01)
12-13	nd	0.09 (±0.04)	0.171 (±0.06)	nd
14-15	0.051 (±0.02)	0.068 (±0.02)	0.067 (±0.03)	nd
16-17	nd	nd	0.021 (±0.01)	nd
18-19	nd	nd	nd	nd
20-21	0.019 (±0.01)	nd	nd	nd
22-23	nd	nd	nd	nd
24-25	nd	nd	nd	nd
26-27	nd	nd	nd	nd
28-29	nd	nd	nd	nd
30-31	nd	nd	nd	nd
32-33	nd	nd	nd	nd
34-35	nd	nd	nd	nd
36-37	nd	nd	nd	nd
38-39	nd	nd	nd	nd
40-41	nd	nd	nd	nd
42-45	nd	nd	nd	nd

nd = not detectable

A= soil; B=abiotic control; C=soil+free cells of PCL3; D=soil+immobilized PCL3; E=soil+rice straw; F=soil+free cells of PCL3+rice straw; G=soil+immobilized PCL3+rice straw; H=soil+autoclaved corncob

Table E-2 3-Keto carbofuran detected in column effluent

Time (d)	3-keto carbofuran (mg l-1)			
	A	B	C	D
0-5	0.052 (±0.02)	nd	0.04 (±0.03)	0.054 (±0.02)
6-7	0.021 (±0.00)	nd	nd	nd
8-9	0.013 (±0.01)	nd	nd	nd
10-11	0.013 (±0.00)	nd	0.043 (±0.03)	nd
12-13	0.02 (±0.01)	nd	nd	nd
14-15	0.071 (±0.01)	nd	nd	nd
16-17	0.046 (±0.02)	nd	0.03 (±0.02)	0.036 (±0.01)
18-19	0.056 (±0.01)	nd	0.018 (±0.00)	nd
20-21	0.052 (±0.02)	nd	nd	nd
22-23	0.079 (±0.02)	0.013 (±0.01)	0.012 (±0.00)	0.046 (±0.01)
24-25	nd	nd	nd	nd
26-27	0.05 (±0.01)	0.004 (±0.00)	nd	nd
28-29	0.002 (±0.00)	nd	nd	0.011 (±0.00)
30-31	nd	nd	nd	nd
32-33	0.024 (±0.01)	nd	0.02 (±0.02)	nd
34-35	nd	nd	nd	nd
36-37	0.006 (±0.00)	nd	nd	nd
38-39	nd	nd	nd	nd
40-41	nd	nd	nd	nd
42-43	nd	nd	nd	nd
44-45	nd	nd	nd	nd

nd = not detectable

A= soil; B=abiotic control; C=soil+free cells of PCL3; D=soil+immobilized PCL3; E=soil+rice straw; F=soil+free cells of PCL3+rice straw; G=soil+immobilized PCL3+rice straw; H=soil+autoclaved corncob

Table E-2 3-Keto carbofuran detected in column effluent (cont.)

Time (d)	3-keto carbofuran (mg l-1)			
	E	F	G	H
0-5	nd	0.046 (±0.03)	0.08 (±0.03)	0.06 (±0.01)
6-7	nd	nd	0.022 (±0.01)	0.022 (±0.00)
8-9	0.008 (±0.00)	nd	0.008 (±0.00)	0.022 (±0.01)
10-11	nd	0.023 (±0.02)	nd	0.028 (±0.00)
12-13	0.018 (±0.01)	0.003 (±0.00)	nd	0.149 (±0.00)
14-15	nd	nd	nd	nd
16-17	0.002 (±0.00)	0.022 (±0.02)	nd	nd
18-19	nd	0.006 (±0.01)	nd	nd
20-21	nd	nd	nd	nd
22-23	0.007 (±0.00)	0.012 (±0.00)	0.019 (±0.02)	0.395 (±0.05)
24-25	nd	nd	nd	nd
26-27	nd	nd	0.031 (±0.01)	0.032 (±0.00)
28-29	nd	nd	nd	nd
30-31	nd	nd	nd	0.029 (±0.01)
32-33	nd	nd	nd	nd
34-35	nd	nd	0.022 (±0.01)	nd
36-37	nd	nd	nd	nd
38-39	nd	nd	nd	nd
40-41	nd	nd	nd	nd
42-43	nd	nd	nd	nd
44-45	nd	nd	nd	nd

nd = not detectable

A= soil; B=abiotic control; C=soil+free cells of PCL3; D=soil+immobilized PCL3; E=soil+rice straw; F=soil+free cells of PCL3+rice straw; G=soil+immobilized PCL3+rice straw; H=soil+autoclaved corncob

APPENDIX F

INITIAL NUMBER OF CARBOFURAN DEGRADERS IN THE SOIL

Table F-1 Initial number of carbofuran degraders in the soil

Treatment	Number of carbofuran degraders (CFU g^{-1}soil)±SD
A (soil)	$2.07x10^5$ ($\pm 0.57x10^3$)
C (soil + free cells of PCL3)	$2.76x10^6$($\pm 5.67x10^3$)
D (soil + immobilized PCL3)	$4.78x10^5$($\pm 1.41x10^3$)
F (soil + free cells of PCL3+rice straw)	$3.65x10^6$($\pm 7.07x10^3$)
G (soil + immobilized PCL3 + rice straw)	$7.59x10^5$($\pm 0.71x10^3$)

APPENDIX G

(NUMBER OF CARBOFURAN DEGRADERS IN THE COLUMN EFFLUENT)

Table G-1 Number of carbofuran degrader in each column effluent

Time (d)	Number of microorganism in each column						
	A (CFU ml^{-1})	C (CFU ml^{-1})	D (CFU ml^{-1})	E (CFU ml^{-1})	F (CFU ml^{-1})	G (CFU ml^{-1})	H (CFU ml^{-1})
5	150000	195000000	40000000	2200000	21500000	700000	300000
7	1050000	140000000	160000000	80000	385000000	28000000	4500000
9	350000	65000000	600000	1400000	195000000	200000	1550000
11	180000	75000000	11000000	5000000	65000000	2200000	18500000
13	55000	190000000	2000000	8500000	275000000	200000	300000
15	270000	185000000	800000	10500000	135000000	300000	20000000
17	185000	26500000	280000000	150000000	7500000	11000000	15500000
19	125000	22500000	425000000	50000000	14000000	24000000	140000000
21	125000	16500000	150000000	7500000	25000000	10000000	17500000
23	160000	10000000	235000000	37000000	130000000	185000000	45000000
25	90000	22500000	250000000	7500000	170000000	160000000	25000000
27	80000	6000000	210000000	22200000	55000000	170000000	165000000
29	175000	9000000	500000000	5500000	90000000	9500000	55000000
31	110000	1250000	90000000	15500000	110000000	25100000	30500000
33	90000	2600000	210000000	3500000	95000000	110000000	39100000
35	80000	2650000	205000000	6000000	90000000	275000000	22900000
37	175000	1000000	251000000	9500000	65000000	199000000	55000000

APPENDIX H

(NUMBER OF CARBOFURAN DEGRADERS IN SOIL IN EACH SECTION OF COLUMN STUDY)

Table H-1 Number of cabofuran degraders in soil column in each soil section

	0-5cm	5-10cm	10-15cm	15-20cm	20-25cm
A	3.07E+05	8.04E+05	3.07E+05	8.57E+05	5.36E+05
C	8.21E+06	2.56E+06	2.56E+06	1.54E+06	3.59E+06
D	2.39E+07	5.08E+07	3.02E+07	6.00E+07	3.02E+07
E	1.41E+07	7.88E+06	1.58E+06	9.57E+06	3.26E+06
F	3.15E+07	2.10E+07	4.20E+07	3.67E+07	1.57E+07
G	9.10E+07	8.02E+07	1.11E+08	2.00E+08	1.10E+08
H	5.87E+07	7.62E+07	7.67E+07	6.94E+07	5.47E+07

APPENDIX I

(PH VARIATION IN COLUMN EFFLUENT)

Table I-1 pH variation in column effluent

Time (d)	pH							
	A	B	C	D	E	F	G	H
0-5	6.13	7.03	6.03	5.39	7.79	6.9	5.98	5.36
6-7	6.88	7.03	5.83	5.88	7.44	6.85	5.72	5.72
8-9	7.9	7.01	6.35	5.88	7.63	6.88	5.52	5.86
10-11	7.92	6.98	6.28	5.76	8.07	6.81	5.82	6.26
12-13	7.34	6.95	6.48	5.98	7.9	6.9	5.44	5.46
14-15	7.15	6.87	6.68	5.72	8.64	6.87	5.16	5.83
16-17	7.01	6.66	6.88	6.55	6.84	6.9	5.56	5.78
18-19	7.1	6.77	6.88	6.38	6.78	7.14	6.33	5.95
20-21	6.95	6.8	6.91	6.37	6.69	7.2	5.9	5.75
22-23	6.84	7.03	6.96	6.47	6.83	7.2	6.16	6.17
24-25	6.7	6.91	6.99	6.35	6.79	7.1	6.23	6.09
26-27	6.6	6.78	7.01	6.58	6.8	7.25	6.39	6.17
28-29	6.64	6.76	7.02	6.44	6.76	7.15	6.5	5.99
30-31	6.53	6.81	7.02	6.46	6.8	7.255	6.25	5.86
32-33	6.6	6.79	7.02	6.44	6.89	7.25	6.2	5.7
34-35	6.64	6.84	7.03	6.55	6.8	7.35	6.1	5.6
36-37	6.53	6.86	7.01	6.53	6.96	7.25	6	5.65
38-39	6.5	6.75	7.02	6.44	6.99	7.25	5.9	5.5
40-41	6.64	6.79	7.02	6.6	6.8	7.3	6	5.55
42-43	6.53	6.88	7.02	6.58	6.86	7.35	6	5.4
44-45	6.6	6.83	7.03	6.5	6.89	7.25	5.9	5.3

APPENDIX J

CALIBRATION CURVE

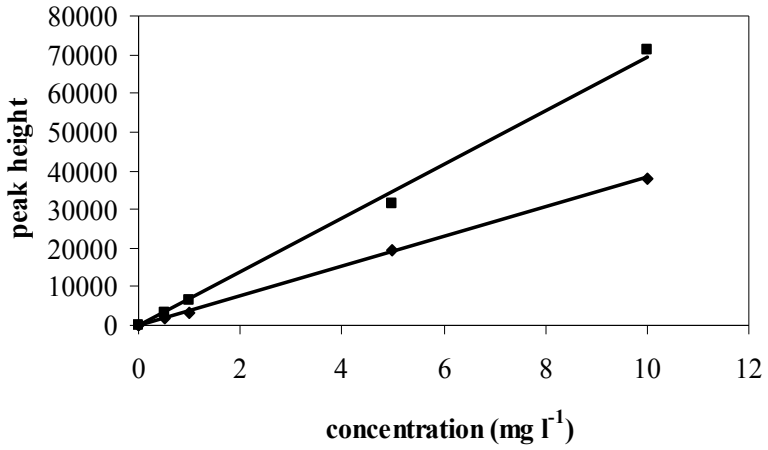

Figure J-1 Standard curve of carbofuran (◆) and carbofuran phenol (■)

Linear equation of carbofuran standard

$y = 3816x$ _____(1)

$R^2 = 0.9995$

Linear equation of carbofuran phenol standard

$y = 6953.9x$ _____(2)

$R^2 = 0.9959$

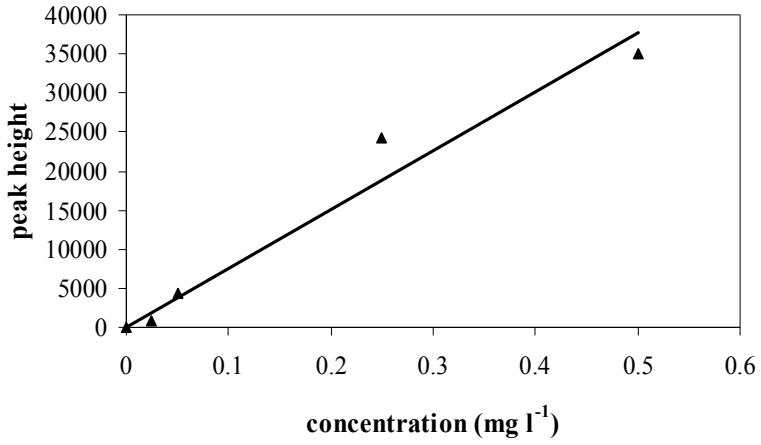

Figure J-2 Standard curve of 3-keto carbofuran (▲)

Linear equation of 3-keto carbofuran standard

$y = 75490x$_____(3)

$R^2 = 0.9615$

BIOGRAPHY

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