BIOREMEDIATION OF CARBOFURAN CONTAMINATED SOIL AND WATER BY *Burkholderia* sp. PCL3

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การบำบัดทางชีวภาพของดินและน้ำที่ปนเปื้อนด้วยคาร์โบฟูรานโดย *Burkholderia* sp. PCL3

นางสาวเพ็ญศรี ปลั่งกลาง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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เพ็ญศรี ปลั่งกลาง : การบำบัดทางชีวภาพของดินและน้ำที่ปนเปื้อนด้วยคาร์โบฟูรานโดย *Burkholderia* sp. PCL3. (BIOREMEDIATION OF CARBOFURAN CONTAMINATED SOIL AND WATER BY *Burkholderia* sp. PCL3) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร. อลิศรา เรืองแสง, 184 หน้า.

แบคทีเรีย PCL3 มีความสามารถในการย่อยสลายคาร์โบฟูราน ถูกคัดแยกมาจากดินรอบรากพืชที่ใช้ในการบำบัดคาร์โบฟูราน งานวิจัยนี้ได้จำแนกชนิดของ PCL3 โดยใช้เทคนิคการวิเคราะห์ลำดับเบสของยีนบริเวณ 16s rDNA พบว่า PCL3 มีความคล้ายกับ *Burkholderia cepacia* 96% จากนั้นได้ศึกษาผลของความเข้มข้นคาร์โบฟูรานที่ 5-200 มก./ล. ต่อความสามารถในการย่อยสลายคาร์โบฟูรานของเซลล์ตรึง PCL3 บนซังข้าวโพดและชานอ้อยเปรียบเทียบกับเซลล์อิสระ ในอาหารเลี้ยงเชื้อ Basal Salt Medium (BSM) ผลการทดลองพบว่า ความสามารถในการย่อยสลายคาร์โบฟูรานของ PCL3 ในรูปเซลล์อิสระจะถูกยับยั้งที่ความเข้มข้นคาร์โบฟูรานสูงกว่า 100 มก./ล. ค่าพารามิเตอร์ทางจลนพลศาสตร์การย่อยสลายคาร์โบฟูรานจากการทำนายโดยใช้แบบจำลองการยับยั้งโดยสับเสตรท คือ q_{max} = 0.451 ต่อวัน K_s = 171.96 มก./ล. S_i = 114.86 มก./ล. และ S_m = 248.76 มก./ล. สำหรับชุดการทดลองที่ใช้ PCL3 ในรูปเซลล์ตรึง ไม่พบผลการยับยั้งโดยคาร์โบฟูราน ดังนั้นจึงใช้แบบจำลองของ Monod ในการทำนาย ซึ่งพบว่า มีค่า q_{max} = 0.124 และ 0.198 วัน และค่า K_s = 160.83 และ 113.95 มก./ล. สำหรับเซลล์ตรึงของ PCL3 บนซังข้าวโพดและชานอ้อยตามลำดับ ซึ่งผลการทดลองยืนยันว่าเทคนิคการตรึงเซลล์สามารถช่วยป้องกันการยับยั้งโดยสับเสตรทส่งผลให้ประสิทธิภาพการย่อยสลายเพิ่มขึ้นได้ จากนั้นได้ศึกษาการบำบัดคาร์โบฟูรานใน BSM และในดิน โดย PCL3 ในรูปเซลล์ตรึงเปรียบเทียบกับเซลล์อิสระที่ระดับความเข้มข้นคาร์โบฟูรานเท่ากับ 5 มก./ล. และ 5 มก./กก. ตามลำดับ ผลการทดลองพบว่าค่าครึ่งชีวิตของคาร์โบฟูรานใน BSM ที่เติมเซลล์ตรึงและเซลล์อิสระมีค่าไม่แตกต่างกัน คือ อยู่ในช่วง 3-4 วัน โดยเซลล์ตรึงสามารถอยู่รอดได้ตลอดระยะเวลา 30 วันในการบ่ม แต่เซลล์อิสระจะมีจำนวนลดลงหลังจากระยะเวลาการบ่มผ่านไป 10 วัน และเซลล์ตรึงสามารถนำกลับมาใช้ซ้ำได้ 2 รอบ ซึ่งถือเป็นข้อได้เปรียบเมื่อเปรียบกับการใช้เซลล์อิสระ นอกจากนี้ยังพบว่า เทคนิคการเติมจุลินทรีย์ PCL3 ช่วยเพิ่มประสิทธิภาพในการย่อยสลาย ให้ค่าครึ่งชีวิตของคาร์โบฟูรานในดินสั้นลง 5 เท่า เมื่อเปรียบเทียบกับดินที่ไม่มีการเติม PCL3 จากนั้นได้ศึกษาการบำบัดคาร์โบฟูรานในดิน ในถังปฏิกรณ์ชีวภาพแบบกึ่งแข็งกึ่งเหลวที่ระดับความเข้มข้นคาร์โบฟูราน 20 มก./กก. ผลการทดลองพบว่า ถังปฏิกรณ์ที่มีการเติมเซลล์ตรึง PCL3 บนซังข้าวโพดให้ค่าประสิทธิภาพการย่อยสลายคาร์โบฟูรานสูงที่สุด 96.97% รองลงมาคือถังปฏิกรณ์ที่มีการเติมเซลล์ตรึง PCL3 และโมลาส (82.23%) แสดงให้เห็นว่าวิธีการบำบัดทางชีวภาพเป็นวิธีที่มีประสิทธิภาพในการบำบัดคาร์โบฟูรานในดิน จากนั้นได้ศึกษาการย่อยสลายคาร์โบฟูรานใน BSM โดยใช้ถังปฏิกรณ์ชีวภาพแบบสลับเป็นกะ โดยศึกษาผลของระยะเวลากักเก็บ การเติมแหล่งอาหาร และความเข้มข้นของคาร์โบฟูรานต่อประสิทธิภาพการย่อยสลาย ผลการทดลองพบว่า ที่สภาวะที่เหมาะสมที่สุด คือ ระยะเวลากักเก็บ 6 วันและใช้รำข้าวเป็นแหล่งอาหารเสริม คาร์โบฟูรานที่ความเข้มข้น 40 มก./ล. จะถูกย่อยสลายได้ 100% และให้ค่าคงที่อัตราการสลาย (k_1) และค่าครึ่งชีวิต ($t_{1/2}$) ของคาร์โบฟูรานใน BSM เท่ากับ 0.044 ต่อชม. และ 16 ชม.ตามลำดับ

สาขาวิชาการจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต.....

ปีการศึกษา 2551 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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PENSRI PLANGKLANG : BIOREMEDIATION OF CARBOFURAN CONTAMINATED SOIL AND WATER BY *Burkholderia* sp. PCL3.
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The isolate PCL3, a carbofuran degrader, was previously isolated from carbofuran phytoremediated rhizosphere soil. In this study, the isolate PCL3 was characterized and studied on the potential use to bioremediate carbofuran. From 16s rRNA based identification, PCL3 showed the highest similarity of 96% to *Burkholderia cepacia*. The effects of carbofuran concentrations (5-200 mg l^{-1}) on biodegradation of carbofuran by the immobilized PCL3 in comparison to free cells were studied in Basal Salt Medium (BSM). The inhibitory effect of carbofuran at the concentration of greater than 100 mg l^{-1} on the isolate PCL3 in free cells form was observed. The estimated kinetic parameters for free cells of PCL3 from the proposed substrate inhibition model were q_{max} = 0.451 /d, K_s = 171.96 mg/l, S_i = 114.86 mg/l and S_m = 248.76 mg/l. The inhibitory effect was not found when using immobilized PCL3, therefore the Monod equation was used to model the biodegradation kinetic of carbofuran. The q_{max} values of 0.124 and 0.198 /d and the K_s values of 160.83 and 113.95 for the immobilized PCL3 on corncob and sugarcane bagasse, respectively was obtained. From the kinetic behavior of PCL3, it was confirmed that the immobilization technique could protect the PCL3 cell from substrate inhibition hence enhancing carbofuran degradation efficiency. Free and the immobilized PCL3 on corncob and sugarcane bagasse were further investigated for their abilities to degrade carbofuran in BSM and soil microcosm at the carbofuran concentration of 5 mg l^{-1} and 5 mg kg^{-1}, respectively. Short half-lives ($t_{1/2}$) of carbofuran of 3-4 d in BSM were obtained using the isolate PCL3 in both free and immobilized cell forms. Immobilized cells could survive through 30 d of incubation, while the number of free cells decreased continuously after 10 d. Immobilized PCL3 could be reused twice without loss in their abilities to degrade carbofuran in BSM. Free and immobilized cells of PCL3 showed an effective capability to remediate carbofuran residues, both of which indicated by 5-folds decrease in carbofuran half-lives in the augmented soil. The effects of bioremediation techniques, i.e. bioaugmentation by using the immobilized PCL3 on corncob and biostimulation by adding organic amendments together with bioreactors technology in to remove carbofuran from contaminated matrices, soil and aqueous phases, were examined. Soil slurry phase sequencing batch reactors were used to remove carbofuran in contaminated soil at the concentration of 20 mg kg^{-1} soil. The results indicated that bioaugmentation treatment in the soil slurry phase reactor (addition of PCL3) gave the highest percentage of carbofuran removal (96.97%), followed by bioaugmentation together with biostimulation (addition of molasses) treatment (88.23%), suggesting that bioremediation was an effective technology for removing carbofuran from contaminated soil. The sequencing batch reactors (SBRs) augmented with the immobilized PCL3 on corncob were applied to remove carbofuran in aqueous phase (BSM). The effects of hydraulic retention time (HRT), biostimulation, and carbofuran concentrations on the performance of SBRs were investigated. The optimum conditions for SBRs were achieved when it was operated at the HRT of 6 d with the initial carbofuran concentration of 40 mg l^{-1} by using 0.1 g l^{-1} of rice brand as a biostimulated amendment. The carbofuran degradation efficiency of SBR at the optimum condition was 100% with the k_1 value and $t_{1/2}$ of 0.044 h^{-1} and 15.57 h, respectively.

Field of Study : Environmental Management Student's Signature .....

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CHAPTER I

INTRODUCTION

1.1 General introduction

Carbofuran (2,3-dihydro-2,2 dimethylbenzofuran-7-yl methylcarbamate) is a broad-spectrum insecticide widely used in agriculture to control insects and nematodes on contact or after ingestion. Carbofuran is of environmental concern because it is soluble in water and highly mobile in soil resulting in a high potential for groundwater contamination and can cause acute toxicity to mammals through cholinesterase inhibition (EPA, 2006). In 2007, Thailand imported carbofuran in form of Furadan3G up to approximately 5,000 tons for agriculture purpose (FMC cooperation, 2008). Continuous use of carbofuran in rice field may subsequently cause contamination risk to soil and groundwater, thus a removal of carbofuran is necessary.

One of the effective routes for pesticide removal is microbial degradation by specific degrader and/or indigenous microorganisms. Previous research reported a discovery of microorganisms capable of degrading carbofuran and other pesticides from contaminated natural matrices (Yan et al., 2007; Bano and Musarrat, 2004). These degraders could use the pesticide as their energy sources, i.e., C- or N- or C and N-sources. Addition of microbial cultures capable of degrading pesticide, or so-called bioaugmentation technique, is reported as an effective bioremediation approach to improve pesticide degradation in contaminated soil and water that lack of the microbial activities (Dams et al., 2007). Not only bioaugmentation but also biostimulation, the addition of organic and/or inorganic amendments to stimulate the activity of indigenous microorganisms, has been reported to enhance a degradation rate of contaminants of interest (Robles-Gonzalezl et al., 2008).

In our previous study, thirteen different bacterial strains were isolated from carbofuran phytoremediated rhizosphere soils by an enrichment technique (Plangklang, 2004). Their abilities to degrade carbofuran were investigated in Basal Salt Medium (BSM) containing 5 mg l^{-1} of carbofuran. The shortest half-life of carbofuran, 3 d, was found in C-limited BSM cultured with an isolate named PCL3

indicating that carbofuran was used as its sole C-source. In this present study, the isolate PCL3 would be characterized and used for bioremediation of carbofuran.

Addition of specific degrader in to the pesticide contaminated environmental matrices was reported to improve the remediation efficiency (Rousseaux et al., 2003; Yu et al., 2003). However, some limitations of applying the degraders in the bioremediations system have been reported 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 extend period of works. The supporting materials used for immobilization can act as protective agents against the effects of pH, temperature, or even substrate and product inhibitions hence enhancement the cell survival (Bekatorou et al., 2004; Braud et al., 2007). The support materials used for immobilization could be either synthetic polymers or natural materials. Disposal of synthetic polymer is of the concern due to its non-biodegradable characteristic, unlike the agricultural residues which are natural materials and biodegradable. Therefore, this study was designed to examine a degradation of carbofuran residues in synthetic medium and soil using PCL3 adsorbed on agricultural residues, i.e. corncob and sugarcane bagasse in comparison to free cell.

Though there are evidences on the microbial adaptation to metabolize pesticide as their energy source as described earlier, the high-strength of pesticide concentration may cause the inhibitory effects to microbial cell growth leading to a decrease of degradation capability of the degraders (Sahinkaya and Dilek, 2005; 2006, Lodha et al., 2007). Thus, in order to effectively use the isolate PCL3 in bioremediation of carbofuran, the kinetic information on microbial degradation of carbofuran were investigated in this study for optimal design and operation of carbofuran bioremediation systems.

Bioaugmentation and biostimulation can be applied both *in situ* by directly adding degraders and/or amendments to the contaminated areas and *ex situ* through biodegradation in bioreactor. Though *in situ* treatment possesses the advantages of simplicity and cost-effective, it requires long time to complete the degradation and can be restrained by some limit conditions such as low permeability and heterogeneous of the contaminated matrices (Prasanna et al., 2008). Therefore, the

bioreactor technology which can be specially designed in variety of configurations for maximizing the microbial activity has drawn our attention to bioremediate carbofuran.

Soil-slurry phase reactors comprising of 10-40% of soil in liquid (water) are relatively new application for soil and sediment bioremediation. It is an alternative technology for decontamination of soil and sediment by minimizing the limiting effect of mass transferring on biodegradation (Robles-Gonzalezl et al., 2008; Venkata Mohan et al., 2004). The soil-slurry treatment system can utilize both indigenous microorganisms in soil and inoculated specific degrader to degrade the contaminant which can partition itself to soil and liquid phases. Therefore, the degradation process can be taken place in both soil, water, and/or soil-water phases resulting in an enhancement of contaminant degradation (Venkata Mohan et al., 2008). Published data indicated a successful bioremediation of the hazardous substance using soil-slurry phase reactor especially pesticides such as pendimethalin (Ramakrishna et al., 2008), and hexachlorocyclohexane isomers (Quintero et al., 2005; 2006). In addition, they also reported that the remediation efficiency of the contaminated soil could be effectively improved by using bioslurry reactor with the augmentation of specific microorganisms capable of degrading pesticide of interest. However, to the best of our knowledge, there is no report on carbofuran remediation in bioaugmented soil-slurry phase reactor.

For bio-treatment systems of contaminated water, Sequencing Batch Reactor (SBR) are an attractive alternative to continuous system, mainly because of their simplicity and flexibility of operation, better solid retention and cost effective in comparison to continuous process (Ratusznei et al., 2000). The operation of SBR mainly consists of four steps, i.e. feeding, reaction, settling and liquid withdrawal (Zaiat et al., 2001), in which exposure time between microorganisms and contaminant, frequency of exposure and level of the respective concentration can be set independently of any inflow conditions. The contaminant degradation efficiency as well as the quality of the effluent from SBR can be easily controlled by adjusting Hydraulic Retention Time (HRT) or time scale of each operation steps and accelerating mass transfer and microbial activities (Zaiat et al., 2001). SBR had been developed by many researchers for treating various types of pesticide such as atrazine (Protzman et al., 1998), 2,4-dichlorophenoxyacetic acid (2,4-D) (Mangat and Elefsiniotis, 1999) and 2,4-dichlorophenol (2,4-DCP) (Wang et al., 2007), in which

their results evidenced the enhancement of pesticides biodegradation in SBR system. Therefore, SBR was used as a tool for optimizing the carbofuran degradation ability of PCL3.

According to the findings from the previous research, this present study was designed to investigate (1) a phylogenetic analysis and kinetic characterization of PCL3 in free and immobilized cell forms (2) an application of PCL3 in bioremediation of carbofuran contaminated matrices in soil microcosms and bioreactors including soil slurry phase reactor and SBR. Results from this study would provide the information on the ability of PCL3 to bioremediate carbofuran in contaminated matrices.

1.2 Objectives

The main objective of this study is to determine the ability of isolated carbofuran degrader, PCL3, in remediating carbofuran contaminated matrices. In order to achieve the goal, we have divided the main objective into 4 sub-objectives as follows:

1.2.1 To identify and characterize the carbofuran degrader isolated from carbofuran phytoremediated rhizosphere soil by using 16s rDNA gene sequencing and phylogenetic analysis technique.

1.2.2 To determine the carbofuran degradation ability and growth kinetic of free and immobilized cells of PCL3 in synthetic medium.

1.2.3 To investigate the ability of the isolate PCL3 in removal of carbofuran from soil using stirred aerobic sequencing batch reactor and bioslurry phase sequencing batch reactor.

1.3 Research Plan

Research plan of this study was divided into 2 parts. Part I was the characterization of carbofuran degrader isolated from carbofuran phytoremediated rhizosphere soil (Figure 1-1 and 1-2) and Part II was the application of PCL3 in bioremediation of carbofuran from soil and synthetic medium (Figure 1-3). The entire plan is summarized as follows:

PART I: Characterization of carbofuran degrader isolated from carbofuran phytoremediated rhizosphere soil

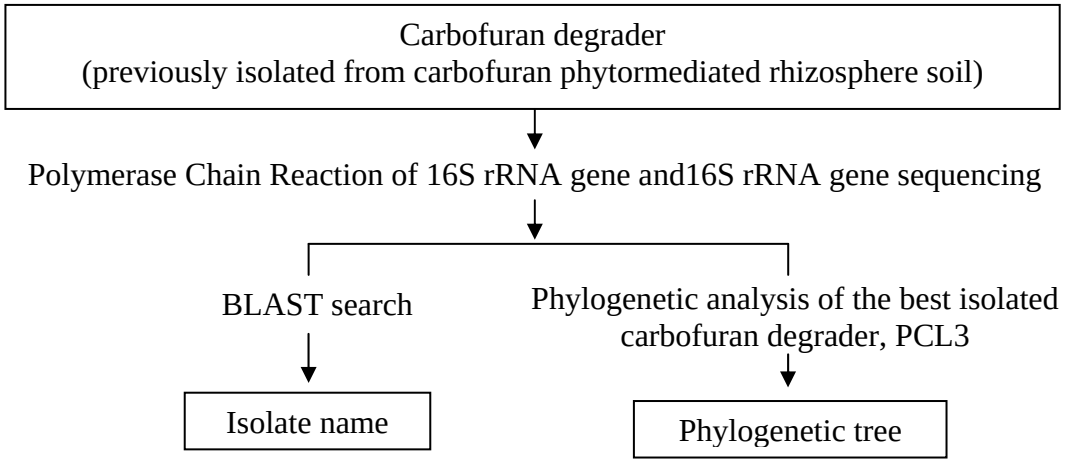
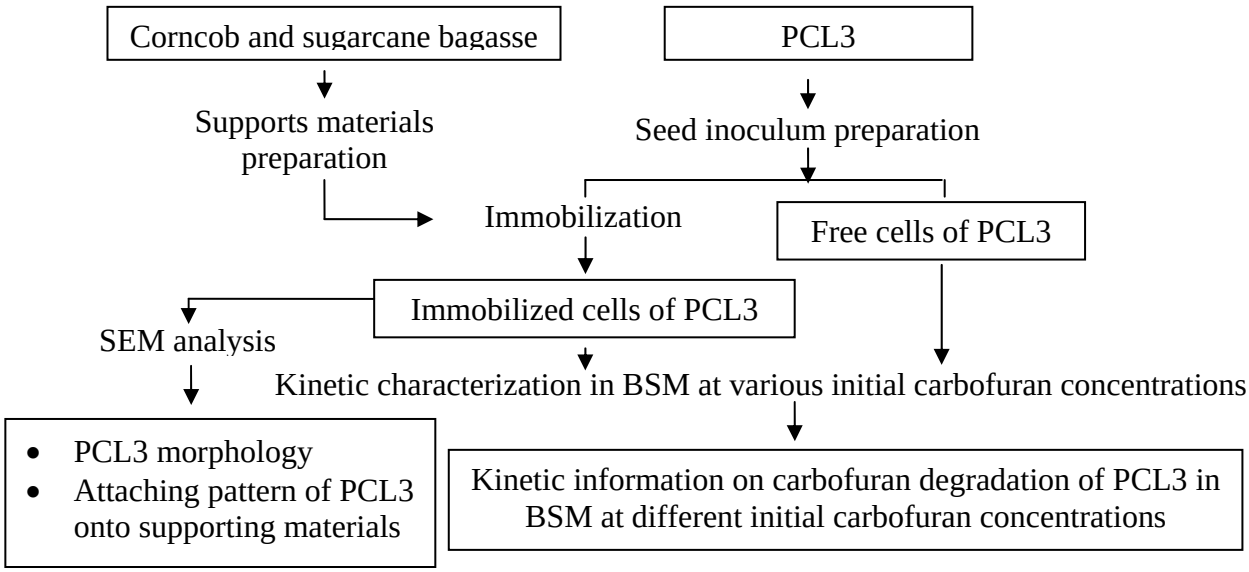


Figure 1-1 Flow diagram of the experiment on phylogenetic analysis of carbofuran degraders



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PART II: Application of PCL3 in bioremediation of carbofuran from soil and synthetic medium

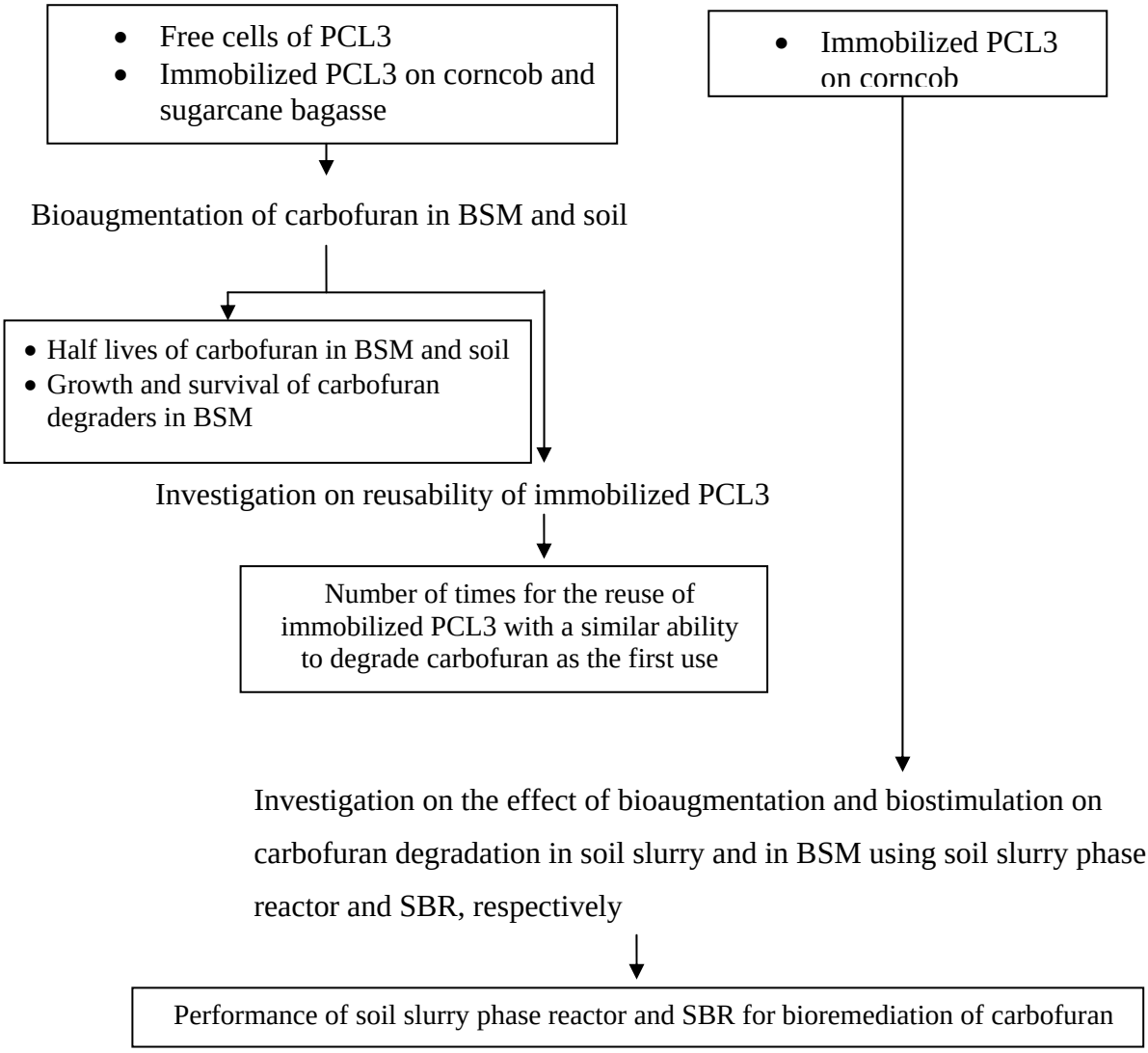

Figure 1-3 Flow diagram of the experiments on the application of isolate PCL3 for bioremediation of carbofuran from soil and synthetic medium

1.4 Dissertation Organization

This research is divided into 7 chapters. Chapter 1 includes general introduction, plans and organization of the dissertation. Chapter 2 contains the literature review for the dissertation. Chapters 3, 4, 5 and 6 are the work as shown in research plan which were prepared based on manuscript for publication. Chapter 3 is a manuscript entitled "Isolation and Kinetic Characterization of Carbofuran Degrading Bacterium from Carbofuran Phytoremediated Rhizosphere Soil". This manuscript is prepared for

submission to International Biodeterioration and Biodegradation. Chapter 4 is the manuscript entitled “Bioaugmentation of carbofuran residues in soil using *Burkholderia cepacia* PCL3 adsorbed on agricultural residues”. This manuscript had been accepted to be published in International Biodeterioration and Biodegradation. Chapter 5 is a manuscript entitled “Bioaugmentation of Carbofuran by *Burkholderia cepacia* PCL3 in Bioslurry Phase Sequencing Batch Reactor”. This manuscript has been submitted to Process Biochemistry. Chapter 6 is a manuscript entitled of “Bioremediation of carbofuran immobilized *Burkholderia cepacia* PCL3 on corncob in a stirred aerobic sequencing batch reactor”. This manuscript is prepared for submitting to Journal of Hazardous Materials. Lastly, the conclusions and the recommendations for future works are presented in Chapter 7.

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. It is used against soil dwelling and foliar feeding insects of field, fruit, vegetable and forest crops (EPA, 2006). Products containing the active ingredient carbofuran include Furadan, Curaterr, Yaltox, Bay 70143, Carbodan and ENT 27164 (Trotter et al., 1991).

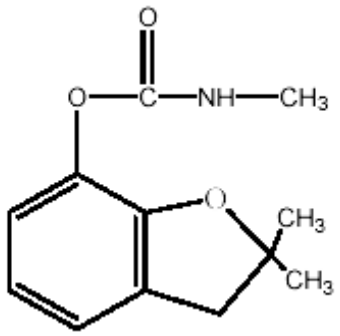

Figure 2-1 Structure of carbofuran (Evert, 2002)

2.1.1 Properties of carbofuran

Carbofuran is an odorless, crystalline solid varying from colorless to gray depending on the purity of the compound. It is soluble in water (320 mg/L at 25 ^{o}C) and has a low adsorption coefficient (K_{oc}= 22, Table 2-1) resulting in a high potential for groundwater contamination (Howard, 1991; EPA, 2006). The physicochemical properties of carbofuran were shown in Table 2-1.

2.1.2 Use of carbofuran

In 2007, Thailand imported carbofuran in form of Furadan3G up to approximately 5,000 tons for agriculture purpose (FMC cooperation 2008).

Table 2-1 Physicochemical properties of carbofuran (EPA, 2006).

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

Carbofuran was widely applied in plants and crops growing such as rice, corn, sorghum, potato, tobacco, banana, cotton and vegetables (Ngampongsai, 1990). In rice fields, Furadan granules 3% (3G) is occasionally applied into young plants after 10 days of seeding at the rates of 8 to 10 kg/rai. 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

(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 to improve an agricultural productivity provided many possible sources of the pesticide to enter the environment. The pesticides contamination in the environment can be resulted not only from direct use of pesticide in agriculture but also from the runoff of pesticides from croplands and rinsate from cleaning pesticides containers and application equipments (Ferrell and Aagard, 2003). Due to carbofuran was applied directly to the soil, it may be washed off from the soil into nearby bodies of surface water or may percolate through the soil to lower soil layers and groundwater (EPA, 2006). These resulted in the adverse effects to human and animals exposed to those contaminated areas (Ferrell and Aagard, 2003). Carbofuran is moderately persistent in soil. Its half-life in soil is approximately 30 to 120 days depending on temperature, moisture content, pH and numbers of microorganism (DeVries and Evans, 1999). Due to its high water solubility and low adsorption coefficient, carbofuran is relatively mobile in soil and in surface runoff (Cohen, 1996). Carbofuran could be sorbed and less mobile in clay soil because of organic matter and clay content (Kumari et at., 1987). The half-lives of carbofuran in water at 25 ^{o}C were 690, 8.2, and 1.0 weeks at pH of 6.0, 7.0, and 8.0, respectively. Carbofuran was detected (1 to 5 μg/L) in water table aquifers beneath sandy soils in New York and Wisconsin (Howard, 1991). Carbofuran has low vapor pressure and low Henry's Law constant (Table 2-1) resulting in a low tendency to volatilize from water or moist soils (Duel et al., 1979). Shibamoto et al. (1993) reported that 0.3 to 0.66 μg/m^3 of carbofuran was detected after a 44-hour sampling period following an application of 44% active ingredient carbofuran.

Carbofuran and its metabolites have not been observed to accumulate significantly in any biota (Evert, 2002). In the bluegill sunfish, carbofuran and its metabolites all become conjugated and excreted in the urine and bile (Eisler, 1985). Caro et al. (1976) reported that carbofuran was absorbed by roots and transported via plant fluids to other areas such as leaves. Approximately 14% of the applied carbofuran was taken up by the crop (Caro et al., 1976). Carbofuran does not

volatilize from water nor does it adsorb to sediment or suspended particles. The environmental fate of carbofuran was shown in Figure 2-2.

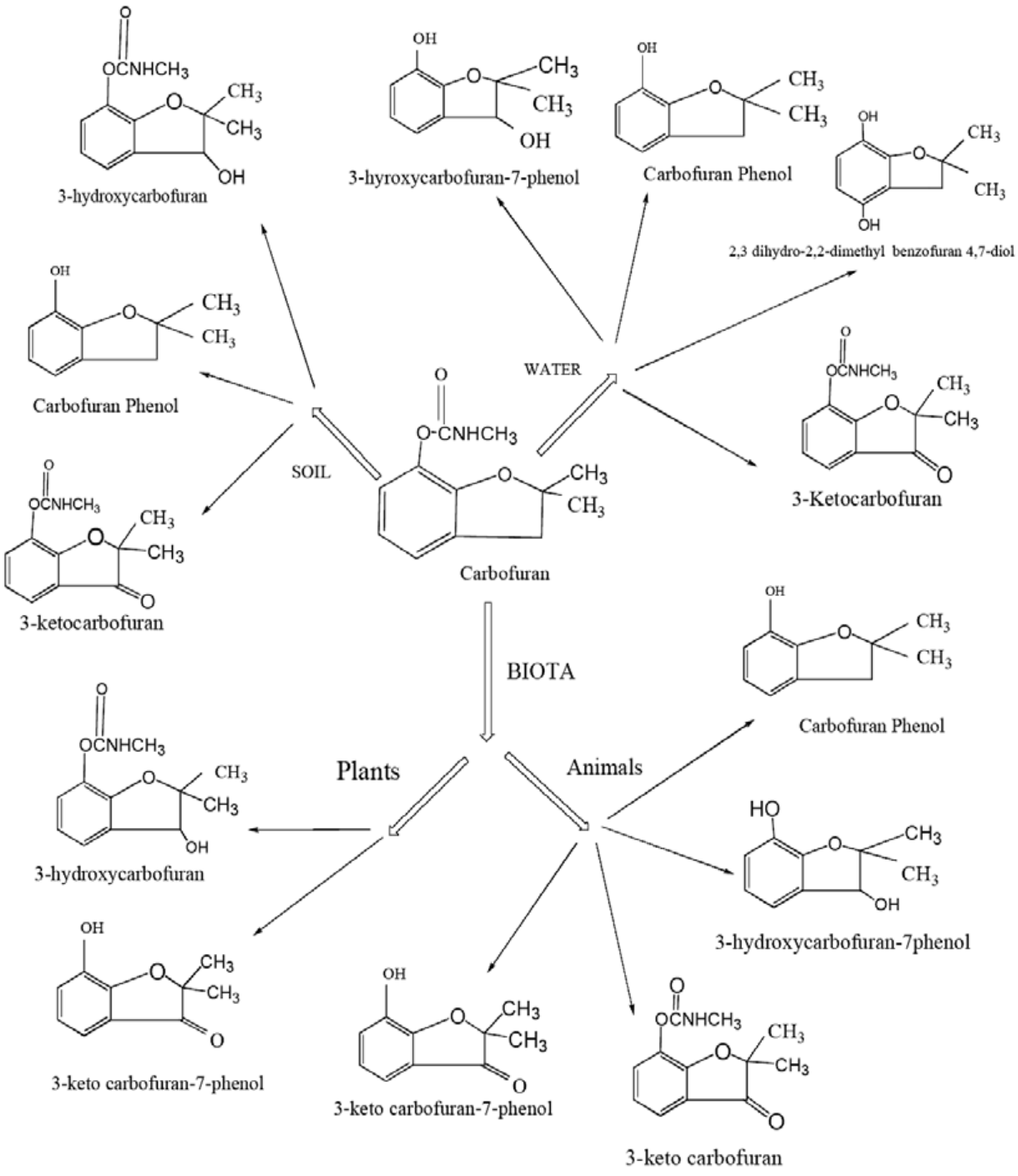

Figure 2-2 Environmental fate of carbofuran (Evert, 2002)

2.1.4 Health Effects

Carbofuran is of environmental importance because of its high toxicity through inhalation and ingestion and its moderate toxicity by dermal absorption (Baron, 1991). It causes cholinesterase inhibition in both humans and animals and affects nervous system function (EPA, 2006) with a high toxicity to mammalian (LD_{50} = 2 mg/kg) (EPA, 2006). Rats given very high doses of carbofuran (5 mg/kg·day) for two years showed decreases in weight. Similar tests with mice gave the same results (Baron, 1991). Carbofuran is highly toxic to birds, fish and invertebrates. 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 in rainbow trout and 0.24 mg/L in bluegill sunfish (Kidd and James, 1991). The ecological toxicity of carbofuran was shown in Table 2-2.

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

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

2.1.5 Regulatory status

In 1985, the U.S. Environmental Protection Agency (EPA) announced a special review for using of granular carbofuran in the United States because of concerns regarding negative impacts on bird species (IDPID, 1993). A ban on all granular formulation of carbofuran became effective on September 1, 1994. The ban was established to protect birds and was not related to human health concerns (Extoxnet, 1996). There is no ban on liquid formulations of carbofuran. The formulations of carbofuran are in toxicity class I (highly toxic) or class II (moderately

toxic). According to the Safe Drinking Water Act set by EPA, the Maximum Contaminant Level Goal (MCLG) and the Maximum Contaminant Level (MCL) for carbofuran were set at 40 parts per billion (ppb). The EPA believes that this level of protection would not cause any of the potential health problems (EPA, 2003).

2.1.6 Degradation of Carbofuran

2.1.6.1 Degradation pathway and metabolites of carbofuran

Hydrolysis is the main metabolic degradation pathways of carbofuran. The degradation process of carbofuran takes place when the carbofuran molecule (RX) react 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, enzymatic activities of microorganisms and light intensity (Siddaramappa and Seiber, 1979; Chapman and Cole, 1983). 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 carbofuran-phenol, 3-ketocarbofuran 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.

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 a successful carbofuran biodegradation. These microorganisms utilize carbofuran by hydrolysis the methylcarbamate likange, carbamate ester or carbamate side chain yielding different carbofuran metabolites (Yan et al., 2007; Chaudhry et al., 2002; Chaudhry and Ali, 1998; Feng et al., 1997; Karns et al., 1986). Microorganisms capable of degrading carbofuran (Table 2-3) 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.

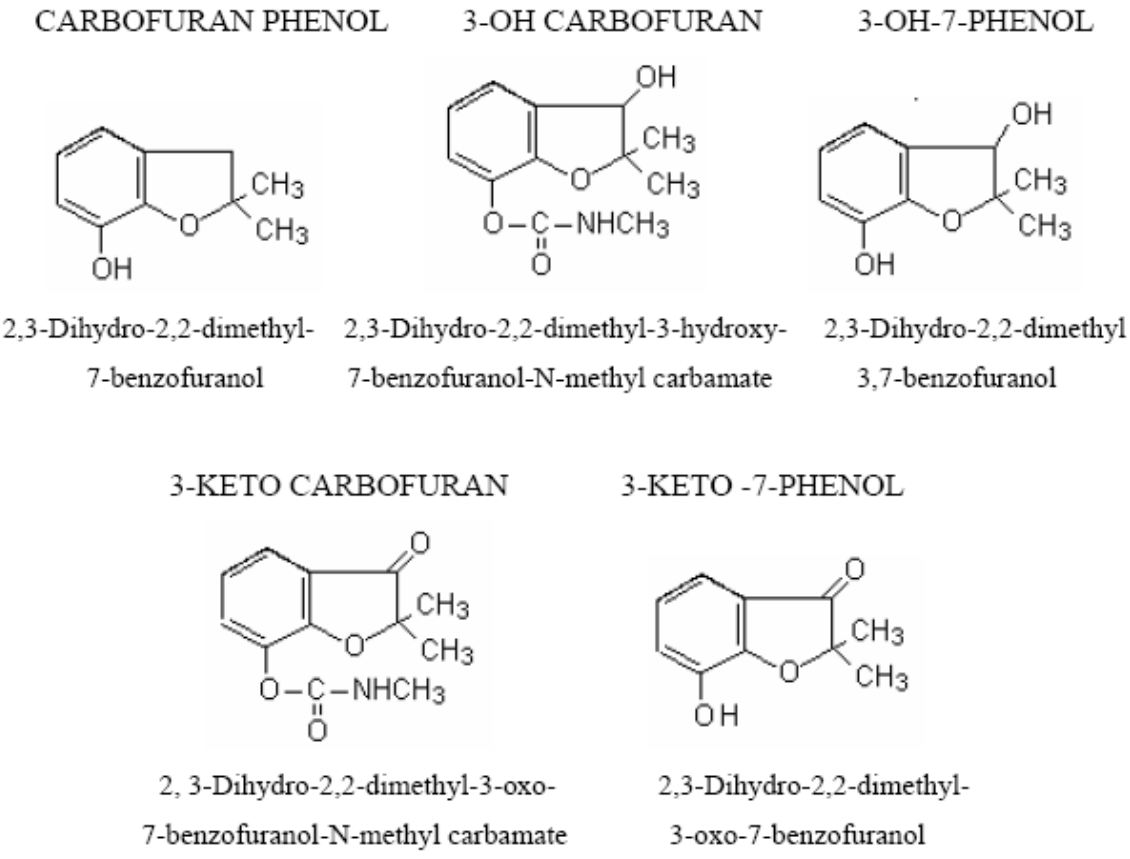

Figure 2-3 Molecular structures of carbofuran metabolites (Mora et al., 1996)

50432 (Chaudhry et al., 2002) could degrade carbofuran to 4-hydroxycarbofuran and carbofuran to 7-phenol by their hydrolase enzyme. *Artheobater, Arthrobacter, Pseudomonas, Bacillus* and *Actinomyces* were isolated from carbofuran treated soil. These microorganisms used 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 days 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 cultured medium by GC/MS (Yan et al., 2007). *Paracoccus* YM3 could completely degrade 50 mg l^{-1} of

carbofuran within 6 days yielding carbofuran phenol as metabolite (Peng et al., 2008). A study on the persistence of carbofuran and the effects of carbofuran on microorganisms in soil from paddy fields by Amal et al. (2003) indicated that *Bacillus, Corynebacterium, Aspergillus* and *Phytophthora* could grow in the carbofuran contaminated soil from paddy fields. However, *Pseudomonas, Staphylococcus, Micrococcus, Klepsiella, Humicola* and *Rhizopus* were inhibited. They also reported that with the activity of these microorganisms, carbofuran persistence in soil was only 9 days. The dynamics of carbofuran degraders in soil during three annual applications of carbofuran was studied by Trabue et al. (2001). The result indicated that the carbofuran hydrolysis in treated surface soil after the second application of carbofuran was greater than the first application and that the degradation of carbofuran in soil was a biological co-metabolic process.

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).

Table 2-3 Carbofuran degradation in synthetic medium by different isolated degraders

Carbofuran degrader	Carbofuran conc. (mg/l)	Degradation efficiency	Degradation time	Metabolite	Reference
Paracoccus YM3	50	100%	6 d	Carbofuran phenol	[Peng et al., 2008]
Novoshingobium sp. FND-3	100	100% 28.6 mg/l/h	9 d	2-hydroxy-3-phenol 2-hycroxy-3-benzene-N-methyl carbamate	[Yan et al., 2007]
Sphingomonas sp. CDS-1	200	100%	20 h	Carbofuran phenol	[Lui et al., 2006]
Sphingomonas sp. TA	50	85%	22 h	Carbofuran phenol	[Orgram et al., 2000]
Shingomonas sp. CD	50	90%	22 h	Carbofuran phenol	
B. cepacia PCL3	5	90%	10 d	Carbofuran phenol	[Plangklang, 2004]

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), hydroxy-7-phenolcarbofuran (Chiron et al. 1996), carbofuran phenol and *N*-methylcarbamic acid via the hydroxylation of the benzofuranyl moiety. 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 form 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 the 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 TiO2 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 Lemey, 2003; Chu et al., 2006).

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 enhance 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 days and 35 days at 15 ^{o}C and 35 ^{o}C, respectively. The dissipation of carbofuran in water could be influenced by photolysis and volatilization (Sieber et al., 1978). 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 temperatures 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 to 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 study of Gupta et al. (2006) fond that adsorption was found to be decreased when carbon slurry, blast furnace sludge, dust and slag, respectively were used as adsorbent. As a particle size of the materials decreased, their surface area increased resulting in a high adsorption capacity as well as by the enhancement of cabofuran penetrating to some of the interior pores of the particles. Moreover, high temperature and pH could reduce the effectiveness of adsorption process to remove carbofuran (Gupta et al., 2006).

2.2 Bioremediation

Bioremediation is a promising process using natural biological activity to remediate the environmental contaminant until its concentration is below detectable limits or less than the maximum contaminant level. In general, bioremediation uses indigenous microorganisms in the contaminated area as the degraders. In some instances, the indigenous microbes at a contaminaed sites may not have the appropriate metabolic potential for degradation and complete mineralization of the target contaminants due to the presence of very small numbers of microorganisms contaminating the enzymes responsible for degrading the contaminants. In such cases, inoculation of the microorganisms capable of degrading specific contaminants-bioaugmentation and/or the addition of amendments for stimulating microbial activity in the contaminated site-biostimulation might be the solution for successful bioremediation (Fantroussi and Agathos, 2005).

2.2.1 Bioaugmentation

Bioaugmentation is the addition of microbial cultures into the contaminated areas to increase microbial populations and improve a specific biological activity (Fantroussi and Agathos, 2005). This technique has been practiced intentionally in many areas including wastewater (Rittman and Whiteman, 1994), forestry and agricultural areas (Jasper, 1994). The parameter affecting the effectiveness of bioaugmentation included the contaminants characteristics (e.g. bioavailability, concentration and microbial toxicity), soil physicochemical characteristic (e.g. water, organic 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 repeated pollutant application sites. Researchers had isolated microorganisms capable of degrading carbofuran (Yan et al., 2007; Bano and Musarrat, 2004; Chaudhry et al., 2002; Karpouzas et al., 2000) and other pesticides (Goux et al., 2006) from contaminated natural matrices and then used in bioaugmentation of pesticides. This technique, is reported as an effective bioremediation approach to improve pesticide degradation

There were many reports that presented successful bioaugmentation of pesticides and hydrocarbon in contaminated soil and water which lack of the microbial activities as shown in Table 2-4. The possibility to improve atrazine degradation in soils by bioaugmentation was studied by Rousseaux et al. (2003). The results indicated that an inoculation of the atrazine-mineralizing strain, *Chelatobacter heintzii* Cit1, to the soils that did not have the atrazine mineralization resulted in a 3-folds increase of atrazine mineralization capacity. Peter (2000) demonstrated that oxygenation, coupled with bioaugmentation with enrichments of atrazine-mineralizing property bacteria obtained from the contaminated site, *Pseudomonas* sp. strain ADP, decreased the half-life of atrazine mineralization in unamended, anaerobic aquifer material from 730 d to 20 d. However, the oxygenation and bioaugmentation of aquifer material with these strains did not enhance the

Table 2-4 Examples of bioaugmentation researches

Matrix	Microorganism (s)	Pollutant	Reference
Soil	*Pseudomonas* sp. strain ADP	Atrazine	[Shapir et al., 1997; Lima et al., 2009]
	Klebseilla sp. CA17	Dichloroaniline	[Tongarun et al., 2008]
	Burkholderia cepacia PCL3	Carbofuran	[Plangklang & Reungsang, 2008]
	Streptomyces sp. M7	Lindane	[Benimeli et al., 2008]
	Sphingobium indicum B90A	hexachlorocyclohexane	[Raina et al., 2008]
	Mixed consortia from agricultural soil	Endosulfan	[Kumar et al., 2007]
	Agrobacterium radiobacter J14a	Atrazine	[Struthers et al., 1998]
	Mixed consortia from agricultural soil	Atrazine; alachlor	[Chirnside et al., 2007]
	Burkholderia sp. FDS-	Fenitrothion	[Hong et al., 2007]
	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 & HaBlom., 1999]
	Ralstonia 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; *R. eutrophus* H850	Polychlorinated biphenyl	[Singer et al., 2000]
	Arthrobacter RP17	Phenanthrene	[Schwartz et al., 2000]
	R. basilensis RK1	2,6-Dichlorophenol	[Steinle et al., 2000]
	Comamonas testosteroni I2	3-Chloroaniline	[Boon et al., 2003]
	Desulfitobacterium frappieri PCP-1	Pentachlorophenol	[Guiot et al., 2002; Lanthier et al., 2002]
Activated sludge	Methanogenic consortia	BTEX	[Da Silva & Alvarez, 2004]
	Pseudomonas 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; *P. putida* F1DCC	Chlorobenzenes	[Wenderoth et al., 2003]
Aquifer/ groundwater	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]

mineralization of fenamiphos within the time constraints of the experiments. The inoculation of strain B-14 (10^6 cells g^{-1}) to soil with a low indigenous population of chlorpyrifos- degrading bacteria mixed with 35 mg of chlorpyrifos kg^{-1} soil resulted in a higher degradation rate than what was observed in non-inoculated soils (Singh et al. 2004). The introduction of *Sphingobium chlorophenolicum* into soil with plants showed approximately four times faster degradation of pentachlorophenol (PCP) when compared to the non-inoculated soil (Dams et al., 2007). The degradation of endosulfan (100 mg l^{-1}) in soil slurry was most effectively achieved with 85% removal within 16 days when the endosulfan degrader, *Pseudomonas aeruginosa*, was added. Whereas, the endosulfan degradation in noninoculated control medium within same incubation period was about 16% (Arshad, 2008). The effects of inoculum size, microbial distribution, and soil nutrient amendments on the degradation of carbofuran in soil by bacteria strain C28 were studied by Duquenne et al. (1996). Results indicated that an increase in the inoculum size and the equal distribution of C28 applied to soil increased the effectiveness of carbofuran degradation. The study of Whyte et al. (1999) found that the bioaugmentation of the contaminated soils with consortia containing the greatest percentages of hydrocarbon degradative bacteria resulted in the shortest C16 mineralization of hydrocabon acclimation period. Ruberto et al. (2003) reported that bioaugmentation with the B-2-2 strain increased the hydrocarbon bioremediation efficiency (75% of the hydrocarbon was removed). They suggested that autochthonous bacterial flora from Antarctic soils is able to degrade an important fraction of the gas-oil and that bioaugmentation represents a valuable alternative tool to improve bioremediation. Garon et al. (2004) indicated the enhanced biodegradation of fluorene in slurry soil by *A. cylindrospora*, fluorene degrading bacteria, inoculation. The bioaugmentation of the Long Beach soil showed the greatest degradation in the light and heavy total petroleum hydrocarbons compared to the attenuation and biostimulation techniques.

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. 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 researches indicated that biostimulation by adding nutrients from agricultural residues is an effective tool to remediate various types of contaminant. A removal of petroleum hydrocarbon in contaminated soil was increased 1.18 times compared to non-stimulated soil (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 accelerated when zinc was added into the contaminated soil at a concentration of 140 mg/kg (Wong et al., 2005). Selenate in agricultural drainage water was removed when rice straw was added (Zhang and Frankenberger, 2002). Additions of farm manure, straw and nitrogen fertilizer stimulated microbial activity and accelerated atrazine degradation (Hance, 1973). The combination of biostimulation and bioaugmentation increase the biodegradation of *cis*-DCE and *trans*-DCE at the range of 14% and 18% degradation rate, respectively, (Olaniran et al., 2005). The additional of rice straw accelerated the hydrolysis of carbofuran to carbofuran-phenol in anaerobic flooded soil. Atagana (2003) reported that additional nutrients in the form of indigenous microbial biosupplement and sewage sludge were effective in creosote removal by increasing the total heterotrophic and creosote degrading microorganisms and increasing the reductions rate of creosote to 88.7 and 86.1%, respectively.

The combination of bioaugmentation and biostimulation might be an effective strategy to improve bioremediation. Both indigenous and augmented microorganisms could benefit from biostimulation by the addition of energy sources or electron acceptors. The study of Portzman et al. (1999) indicated that the degradation of atrazine in sequencing batch reactor augmented with *Agrobacterium radiobacter* J14a could be enhanced by supplementation with citrate as C-sources. The used *Pseudomonas sp.* ADP in combination with citrate plus succinate markedly increased *Pseudomonas* sp. ADP cell-survival and atrazine mineralization (Silva et al., 2004).

2.2.3 Bioreactors

Bioaugmentation technique can be applied both *in situ* by directly addition of the degraders to the contaminated areas and *ex situ* through biodegradation in bioreactor. *In situ* treatment although possesses the advantages of simplicity and cost-effective, it requires long time to complete the degradation and can be restrained by some limit conditions such as low permeability and heterogeneous of the contaminated matrices (Prasanna et al., 2008). Therefore, the bioreactor technology which can be specially designed in variety of configurations for maximizing the microbial activity is a present promising technology for bioremediation of the contaminant (Venkata-Mohan et al., 2004).

For bio-treatment systems of contaminated water, Sequencing Batch Reactor (SBR) are an attractive alternative to continuous system, mainly because of their simplicity and flexibility of operation, better solid retention and cost effective in comparison to continuous process (Ratusznei et al., 2000). The operation of SBR mainly consists of four steps, i.e. feeding, reaction, settling and liquid withdrawal (Zaiat et al., 2001), in which exposure time between microorganisms and contaminant, frequency of exposure and level of the respective concentration can be set independently of any inflow conditions. The contaminant degradation efficiency as well as the quality of the effluent from SBR can be easily control by adjusting Hydraulic Retention Time (HRT) or time scale of each operation steps and accelerating mass transfer and microbial activities (Zaiat et al., 2001).

SBR had been developed by many researchers for treating of various types of pesticide such as atrazine (Protzman et al., 1999), 2,4-dichlorophenoxyacetic acid (2,4-D) (Mangat and Elefsiniotis, 1999) and 2,4-dichlorophenol (2,4-DCP) (Wang et al., 2007), in which their results evidenced the enhancement of pesticides biodegradation in SBRs system.

Soil-slurry phase reactors comprising of 10-40% of soil in liquid (water) are relatively new application for soil and sediment bioremediation. It is a cost-effective alternative to less effective treatment methods such as solid phase remediation (Ramakrishna et al., 2006; Bento et al., 2005; Rahman et al., 2003). Soil slurry phase reactor is an alternative technology for decontamination of soil and sediment by minimizing the limiting effect of mass transferring on biodegradation

(Robles-Gonzalezl et al., 2008; Venkata Mohan et al., 2004). The soil-slurry treatment system can utilize both indigenous microorganisms in soil and inoculated specific degrader to degrade the contaminant which can partition itself to soil and liquid phases. Therefore, the degradation process can be taken place in both soil, water, and/or soil-water phases resulting in an enhancement of contaminant degradation (Venkata Mohan et al., 2008). Published data indicated a successful bioremediation of the hazardous substance using soil-slurry phase reactor especially pesticides such as pendimethalin (Ramakrishna et al., 2008), lindane (Quintero et al., 2006), and hexachlorocyclohexane isomers (Quintero et al., 2005; 2006). In addition, they also reported that the remediation efficiency of the contaminated soil could be effectively improved by using bioslurry reactor with the augmentation of specific microorganisms capable of degrading pesticide of interest. The researches on successive remediation of pesticides using bioslurry phase reactor and SBR were presented in Table 2-5.

2.3 Immobilization

Addition of specific degrader in to the pesticide contaminated environmental matrices was reported to improve the remediation efficiency (Rousseaux et al., 2003; Yu et al., 2003; Thompson et al., 2005). However, some limitations of applying 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 extend period of works. The supporting materials used for immobilization can act as protective agents against the effects of pH, temperature, or even substrate and product inhibition hence enhancement the cell survival (Bekatorou et al., 2004; Braud et al., 2007).

3.3.1 Immobilization techniques

Techniques for cell immobilization can be divided into 4 major categories based on the physical mechanisms (Figure 2-4) (Bekatorou et al., 2004).

Table 2-5 Recent researches on bioremediation of pesticides using soil slurry phase reactor and SBR

Contaminant	Contaminated matrix	Mode of operation	References
Bio slurry phase reactor			
Chlorpyrifos	Soil	• Sequencing batch mode with anoxic–aerobic–anoxic conditions	[Venkata Mohan et al., 2004]
Chlorpyrifos	Soil	• Sequencing batch mode with bioaugmentation under anoxic–aerobic–anoxic conditions	[Venkata Mohan et al., 2007]
Lindane	Soil	• Fed batch with bioaugmentation and/or biostimulation under anaerobic condition	[Quintero et al., 2006]
Lindane	Soil	• Batch with bioaugmentation and biostimulation under aerobic condition	[Quintero et al., 2007]
2,4-D	Clay and organic matters	• Batch with bioaugmentation and biostimulation under aerobic condition	[Robles-Gonzalez et al., 2006]
Pendimethalin	Soil	• Sequencing batch with biostimulation under aerobic condition	[Venkata Mohan et al., 2007]
pendimethalin	Soil	• Batch with bioaugmentation under aerobic condition	[Ramakrishna et al. 2007]
SBR			
Isoproturon and 2,4-D	Synthetic wastewater	• With bioaugmentation and/or biostimulation under aerobic or anaerobic condition	[Celis et al., 2008]
2,4-D	Synthetic wastewater	• With bioaugmentation and/or biostimulation under anaerobic condition	[Chin et al., 2005]
2,4-CP and 2,4-DCP	Synthetic wastewater	• With bioaugmentation and/or biostimulation under aerobic-anaerobic conditions	[Majumder and Gupta, 2009]
2,4-DCP	Synthetic wastewater	• With bioaugmentation and/or biostimulation under aerobic condition	[Wang et al., 2007]

3.3.1.1 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.

3.3.1.2 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.

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

Self-aggregation is 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.

3.3.1.4 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.

3.3.2 Bioaugmentation with cell immobilization

The support materials used for immobilization could be either synthetic polymers or natural materials like agricultural residues (Table 2-6 and 2-7). 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).

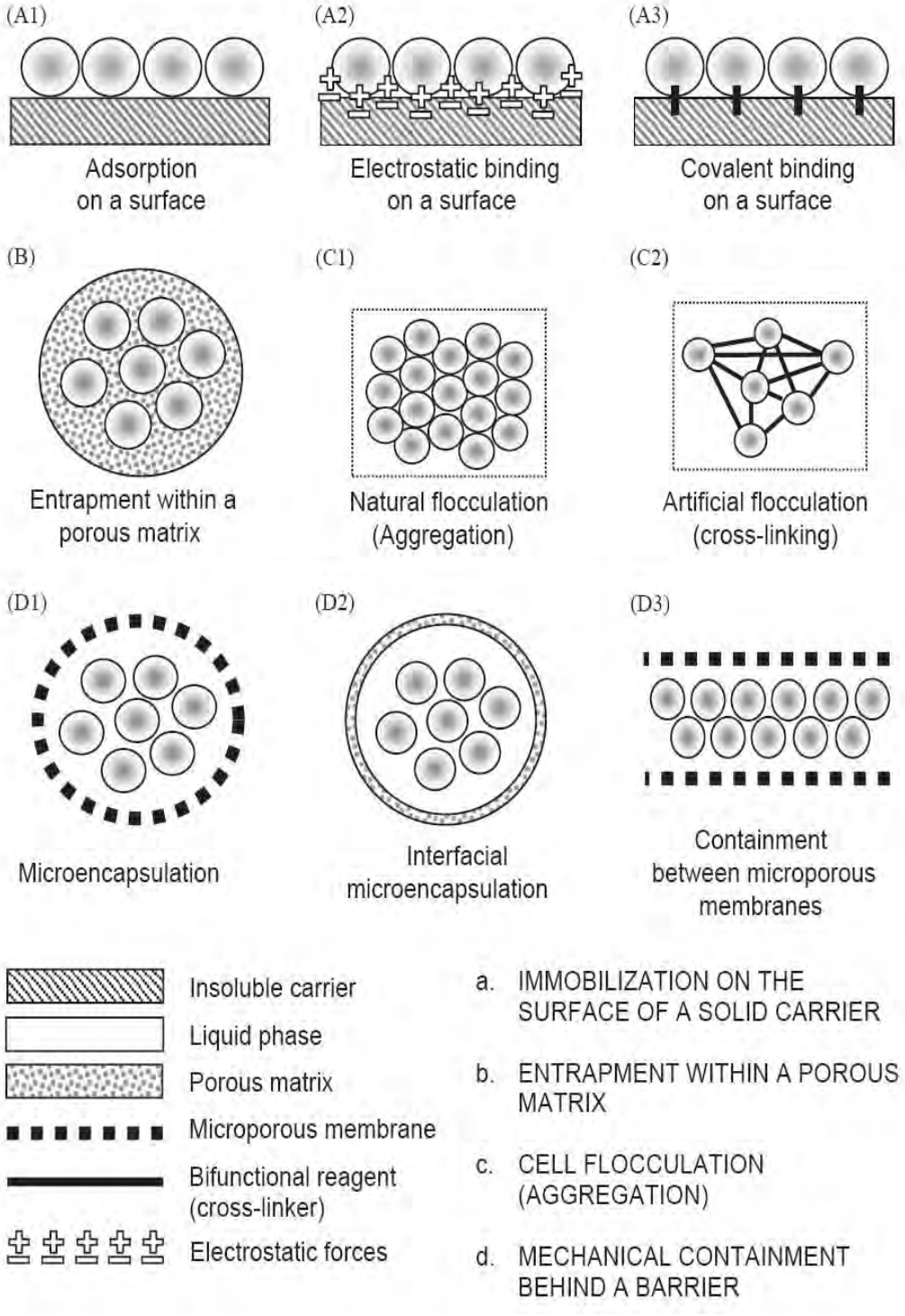

Figure 2-4 Basic methods of cell immobilization (Bekatorou et al., 2004)

Table 2-6 Examples of immobilized cell used in bioremediation of toxic substances

Contaminant	Microorganisms	Carriers	References
Acrylamide	*Pseudomonas* sp	alginate	[Nawaz et al., 1992]
3-Chloroaniline	*Xanthomonas* sp	alginate	[Ferschl et al., 1991]
3-Chlorobenzoate	*Pseudomonas acidovorans*	alginate	[Sahasrabudhe et al., 1988]
dehalogenation	CA28	alginate	[Beunink and Rehm. 1990]
4-Chloro-2-nitrophenol	*Pseudomonas* sp B13	granulated Lecaton-	[Overmeyer and Rehm.
		particles	1995]
Chlorophenols	Mixed culture	alginate	[Lee et al., 1994]
Chlorophenol	*Pseudomonas putida* US2	polyurethane	[Valo et al., 1990]
Chlorophenols	Mixed culture	celite R-633 microcarriers	[Shieh et al., 1990]
Chlorinated phenols	*Rhodococcus* spp	glass, cellulose, chitin	[Portier and Fujisaki 1986]
4-Chlorophenol	Activated sludge	alginate	[Westmeier and Rehm.1985,
4-Chiorophenol	Several strains	granulated Lecaton	Westmeier and Rehm.1987]
		particles	
p-Cresol	*Alcaligenes* sp A 7-2	granular clay	[Balfanz and Rehm.1991]
p-Cresol	*Alcaligenes* sp A 7-2	alginate	[O'Reilly et al., 1988]
Cyanuric acid	*Pseudomonas* sp	alginate	[O'Reilly and Crawford.
DDT	*Pesudomonas* sp	polyurethane	1989]
Dechlorination of spent	*Pseudomonas* sp NRRL B-	granular clay	[Ernst and Rehm. 1995]
sulphite	12228		
bleach effluents	Mixed culture	alginate	[Beunink and Rehm. 1988]
Dichloroacetic acid	*Streptomycetes* spp	polyurethane	[Zhon et al., 1993]
Glyphosate	*Xanthobacter autotrophicus*	alginate	[Heinze and Rehm. 1993]
Hydrocarbons	Mixed culture	diatomaceous earth pellets	[Hallas et al., 1992]
Inorganic cyanides	*Candida parapsilosis*	granular clay	[Omar et al., 1990]
p-Nitrophenol	*Pseudomonas putida*	agar	[Chapatwala et al., 1993]
PAHs	Mixed culture	alginate	[Heitkamp et al., 1990]
Pentachlorophenol	*(Pseudomonas* spp)		
	Mixed culture	carrageenan	[Wiesel et al., 1993]
Pentachlorophenol	*Flavobacterium* sp	diatomaceous earth	[O'Reilly et al., 1988]
Pentachlorophenol	*Flavobacterium* sp	biocarrie	[O'Reilly and Crawford .
Pentachlorophenol	*Arthrobacter* sp ATCC 33790	granular clay	1989]
Pentachlorophenol	*Phanerochaete chrysosporium*	slag of lava	[Lin and Wang. 1991]
Pentachlorophenol	*Arthrobacter* sp ATCC 33790	alginate	[Siahpush et al., 1991]
Pentachlorophenol	*Flavobacterium* sp	polyurethane	[Hu et al., 1994]

Table 2-7 Example of agricultural materials used as supporting materials for cell immobilization.

Reactions	Microorganisms	Carriers	References
Carbendazim and 2,4-Dichlorophenoxyacetatic Acid degradation	Microbial consortium from paddy soil sample	Loofa sponge	[Pattanasupong et al., 2004]
Hexavalent chromium degradation	*Rhizopus cohnii*	Sawdust	[Li et al., 2008]
Itaconic acid production	*Aspergillus terreus*	Papaya wood	[Iqbal et al., 2004]
Xylitol production	*Candida guilliermondii*	Sugarcane bagasse	[Santos et al., 2005]
Ethanol production	*Saccharomyces cerevisiae* strain AXAZ-1	Apple pieces	[Kourkoutas et al., 2006]
Polycyclic aromatic hydrocarbons (PAHs) degradation	*Phanerochaete chrysosporium*	Sugarcane bagasse	[Mohammadi and Nasernejad, 2009]
Cr(VI) degradation	*Acinetobacter haemolyticus*	Wood husk	[Zakaria et al., 2007]
Removal of nickel(II)	*Chlorella sorokiniana*	Loofa sponge	[Akhtar et al., 2004]
Clavulanic acid production	*Streptomyces clavuligerus*	Loofa sponge	[Saudagar et al., 2008]
Wine production	*Saccharomyces cerevisiae* 101	Watermelon rind pieces	[Reddy et al., 2008]
Alcohol production	*Saccharomyces cerevisiae*	Grape skins	[Mallouchos 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; 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. However, they were considered to be expensive for use in large-scale outdoor system in the fields. In addition the disposal of synthetic polymer is of the concern due to its non-biodegradable characteristic, unlike the natural materials such as agricultural residues which are biodegradable. Therefore, collateral researches on cell immobilization using natural supporting material have been developed. Agricultural

matrices are alternative support materials for cell immobilization because it is environmental friendly, locally available and cheaper than synthetic polymer.

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). Chitin and chitosan flakes obtained from shrimps wastes were used to immobilize *Rhodococcus corynebacterioides* QBTo (hydrocarbon-degrading bacterial strain) to remove crude oil from polluted seawater. The percentage of hydrocarbon removal obtained in the microcosm inoculated with immobilized cells was higher than microcosm inoculated with free cell up to two times. The results also indicated that chitin and chitosan flakes improved the survival and the activity of immobilized cells (Gentili et al., 2006). *Acinetobacter* 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 immobilized cells of genetically engineered *Escherichia 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). 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 in synthetic medium. After 4 d of incubation, carbendazim was degraded 95% and 80% by immobilized consortium, respectively, which were significantly higher than that of the free-living consortium, 12%. For 2,4-D, the consortia immobilized on the loofa sponge and the coconut fiber completely degraded within 1 d, while it took 2 d for complete degradation in the free-living (Pattanasupong et al., 2004). THTO4. At the initial toluene concentration of 30 mg l^{-1}, the maximum specific rate of TCE degradation declined from 2.28 to 1.45 $days^{-1}$ when the salinity increased from 0 to 3.5% (Lee et al., 2006). Chen et al. (2006) reported that a C/N ratio of 12:1 (OPEOn (octylphenol polyethoxylates): $(NH_4)_2SO_4$) and C/N ratio of 22:1 (OPEOn:KNO_3) were optimal for specific growth rate of *Pseudomonas nitroreducens* TX1 and OPEOn degradation rate, respectively.

Moreover, the kinetic analysis showed that the growth of *P. nitroreducens* TX1 was inhibited when the OPEOn concentration was higher than 18,000 mg l^{-1}. This helpful information was used to develop the technique for using of *P. nitroreducens* TX1 as a tool in bioremediation of OPEOn contaminated site.

2.4 Kinetic characterization

During bioremediation process, the extreme environmental variations such as pesticide concentrations and metabolic toxicities may decrease or cause adverse effects to the degradation capability of the augmented degraders both free and immobilized cell forms. In order to effectively use the isolated degraders in bioaugmentation of pesticide, the kinetic information on cell growth and pesticide degradation should be determined to understand the microbial response to the environmental variations before introducing the degrader into the environment (Bano and Musarrat, 2004; Yan et al., 2007).

In order to determine the actual effect of substrate concentration on growth of isolates degraders, the evaluation of growth kinetic parameters has been conducted. The data of specific growth rate could be evaluated by fitting to the substrate inhibition models. The models available in the literature were developed for the best explanation of the degradation kinetic base upon the microorganisms and substrate of interest such as Haldane, Luong, Edwards (double exponential model) and Aiba Models (Table 2-8).

There are some articles published the comparison between different kinetic models to express growth and degradation kinetics of microorganisms with substrate inhibition. Carrera et al. (2004) tested the several kinetics models for nitrification by ammonium and nitrite in suspended and immobilized activated sludge in biofilm system. Aiba model is the best models to describe nitrification inhibition by ammonium in both suspended and immobilized systems, whereas the Haldane model is the best model for describing nitratation inhibition by nitrite in both systems (Carrera et al., 2004). Tsuneda et al. (2002) reported that the Holdane model is the best kinetic model to describe BOD removal in fluidized-bed reactor. The Luong model was firstly propose for describing the substrate inhibition to the growth kinetic behavior of yeast during butanol production (Luong 1987). This model was also

proposed for describing the growth kinetic on volatile organic compound such as fluorobenzene (Carvalho et al. 2005) and benzene (Li et al. 2006).

Table 2-8 Examples of substrate inhibition models for growth and degradation kinetic evaluation

Model	Equation for growth kinetic(1)	Equation for degradation kinetic	Reference
Haldane	$\mu = \frac{\mu_{\max} \times S}{K_s + S + \left(\frac{S^2}{K_i}\right)}$	$k = \frac{k_{\max} \times S}{K_s + S + \left(\frac{S^2}{K_i}\right)}$	Haldane (1930)
Luong	$\mu = \frac{\mu_{\max} \times S}{K_s + S}\left(1 - \frac{S}{S_m}\right)^n$	$k = \frac{k_{\max} \times S}{K_s + S}\left(1 - \frac{S}{S_m}\right)^n$	Luong (1987)
Edwards	$\mu = \mu_{\max}\left[\exp\left(-\frac{S}{K_i}\right) - \exp\left(-\frac{S}{K_s}\right)\right]$	$k = k_{\max}\left[\exp\left(-\frac{S}{K_i}\right) - \exp\left(-\frac{S}{K_s}\right)\right]$	Edwards (1970)
Aiba	$\mu = \frac{\mu_{\max} \times S}{K_s + S}\exp\left(-\frac{S}{K_i}\right)$	$k = \frac{k_{\max} \times S}{K_s + S}\exp\left(-\frac{S}{K_i}\right)$	Aiba et al. (1968)

(1) S is initial carbofuran concentration in BSM (mg/l), $\mu_{\max}$ is maximum specific growth rate of PCL3 (/d), $k_{\max}$ is maximum carbofuran degradation rate (/d), K_s is half-saturation constant (mg/l), K_i is substrate inhibition coefficient (mg/l), S_m is substrate concentration above which net growth ceases and n is Long coefficient

CHAPTER III

ISOLATION AND CHARACTERIZTION OF CARBOFURAN DEGRADING BACTERIUM: BIODEGRADATION KINETIC IN FREE AND IMMOBILIZED CELL FORMS

3.1 Introduction

Carbofuran (2,3-dihydro-2,2 dimethylbenzofuran-7-yl methylcarbamate) is a broad-spectrum insecticide widely used in agriculture to control insects and nematodes on contact or after ingestion. Carbofuran is of environmental concern because it is soluble in water and highly mobile in soil resulting in a high potential for groundwater contamination and can cause acute toxicity to mammals through cholinesterase inhibition (EPA, 2006). In 2007, Thailand imported carbofuran in form of Furadan3G up to approximately 5,000 tons for agriculture purpose (FMC cooperation, 2008). Continuous use of carbofuran in rice field may subsequently cause contamination risk to soil and groundwater, thus a removal of carbofuran is necessary.

The important route for pesticide removal from contaminated site is through microbial degradation. Microorganisms capable of degrading specific pesticide could be found in contaminated natural matrices. They could be isolated and used for degradation of target pesticides (Zhang et al., 2006; Hong et al., 2007). These degraders are capable of using the pesticide as their energy sources, i.e., C- or N- or C and N-sources. A number of carbofuran degraders were isolated and studied including *Achromobacter* sp. WM111 (Tomasek and Karns, 1989), *Aminobacter ciceronei* ER2 (Topp et al., 1993), *Sphingomonas* sp. CFO6 (Feng et al., 1997), *Sphingomonas* sp. CD, *Sphingomonas* sp. TA (Ogram et al., 2000), *Pseudomonas aeruginosa* NJ-101 (Bano and Musarrat, 2004), *Sphingomonas* sp. SB5 (Ryeol et al., 2006), *Sphingomonas agrestis* CDS-1 (Liu et al., 2006), *Novosphingobium* sp. FND-3 (Yan et al., 2007), and *Paracoccus* sp. YM3 (Peng et al., 2008). The carbofuran degradation patterns and efficiency were different depending on the type of microorganisms and the ranges of carbofuran concentration used. Though there are

evidences on the microbial adaptation to metabolize pesticide as their energy source as described earlier, the high-strength of pesticide concentration may cause the inhibitory effects to microbial cell growth leading to a decrease of degradation capability of the degraders (Sahinkaya and Dilek, 2005; 2006, She et al., 2005; Lodha et al., 2007). Thus, in order to effectively use the isolated degraders in bioaugmentation of pesticide, the biodegradation kinetic should be determined for optimal design and operation of bioremediation systems. However, to the best of our knowledge, there is no report on microbial kinetic responsible to different carbofuran concentrations of the isolated carbofuran degraders.

Addition of specific degrader into the pesticide contaminated environmental matrices through the bioaugmentation technique was reported to improve the remediation efficiency (Rousseaux et al., 2003; Yu et al., 2003). However, some limitations of applying 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 the immobilization technique which offers many advantages over free cells including regeneration and reuse of the immobilized cells for extend period of works. The supporting materials used for immobilization can act as protective agents against the effects of pH, temperature, or even substrate and product inhibitions hence increase the cell survival (Bekatorou et al., 2004; Braud et al., 2007). However, the mass transfer of target contaminants to the degraders might be obstructed by support materials which can reduce the microbial degradation efficiency. Thus the determination of degradation kinetic for immobilized cell in comparison to free cells to a better understanding of biodegradation phenomena is needed.

In this research, the effective carbofuran degrader was isolated from carbofuran phytormediated rhizosphere soil and characterized. In addition, an attempt had been made to investigate the kinetic of carbofuran degradation by free and immobilized cell forms of the isolated degrader in order to examine its potential for using as inoculum in bioaugmentation of carbofuran residues in the environment.

3.2. Materials and Methods

3.2.1 Chemicals

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

3.2.2 Culture medium

Culture medium used in this study was C-limited Basal Salt Medium (BSM) (Mo et al., 1997) containing (in g l^{-1}): 5.57, $NaHPO_4$; 2.44, KH_2PO_4; 2.00, NH_4Cl; 0.20, $MgCl_2.6H_2O$; 0.0004, $MnCl_2.4H_2O$; 0.001, $FeCl_2.6H_2O$; and 0.001, $CaCl_2$. In order to isolate the carbofuran degraders which possessed the ability to use carbofuran as its sole C-source or sole N-source or a sole C and N-sources, three types of BSM were used. In the first media, C-limited BSM, 5 m l^{-1} of carbofuran was used as a sole C-source. In the second media, N-limited BSM, 5 mg g^{-1} of carbofuran was used as a sole N-source instead of NH_4Cl and 10 g l^{-1} of glucose was used as C-source. In the last media, C and N-limited BSM, 5 mg l^{-1} of carbofuran was used as sole C and N sources. pH of the media was adjusted to 7 before autoclaved at 121 °C for 15 min. Carbofuran solution in methanol was added after the media was sterilized. For the BSM agar, 1.5% of bactoagarose was added to BSM before sterilization.

3.2.3 Enrichment and isolation of carbofuran degraders

The attached soil around the root zone, rhizosphere soil, of rice (*Oryza sativa* L.), corn (*Zea mays* L.) and Chinese kale *(Brassica alboglabra* C.) were collected from the previous study on phytoremediation of carbofuran residues in rice field soil by Teerakul et al. (2004). In order to enrich carbofuran-degrading bacteria, 5 g rhizosphere soil sample from each plant was added to 100 ml C-, N- and C and N-limited BSM media containing 5 mg l^{-1} of carbofuran. Flask was incubated at 30 °C and shaken at 150 rpm on a rotary shaker. Every 7 days, the enriched culture showing the dissipation of carbofuran was transferred to a fresh media containing 5 mg l^{-1} of carbofuran. Four subcultures were conducted to obtain a soil-free enrichment. The

carbofuran degradation ability of carbofuran degraders in soil-free enrichment media was enhanced by subsequently inoculating 10% of the soil-free enriched culture into fresh BSM containing 20, 40 and 100 mg l^{-1} of carbofuran and incubating for 7 days, respectively. After the enhancement at 100 mg l^{-1} of carbofuran, all enrichment cultures presenting the dissipation of carbofuran were streaked on each BSM agar coated with 5 mg l^{-1} of carbofuran to obtain single colonies. Ten isolated carbofuran degraders with different single colonies were obtained. A PCR amplification followed the method as previously described by Parekh et al. (1996) indicated that all isolates degraders contain methylcarbamate degrading (*mcd*) gene coding hydrolase enzyme responsible for degrading carbofuran. Carbofuran degradation efficiency of each isolated colony was determined in liquid BSM containing 5 mg l^{-1} of carbofuran. The concentration of carbofuran in culture media was analyzed by using HPLC. The isolate named PCL3 was selected for further experiment due to its highest capability to degrade carbofuran. Cell morphology, size and arrangement of the isolate PCL3 were determined by Scanning Electron Microscopy following the method previously conducted by Rachman et al. (1998).

3.2.4 16S rRNA gene sequencing and phylogenetic analysis of the isolated carbofuran degraders

A single colony of the isolate PCL3 was whole-cell direct lysed at 70 ^{o}C for 20 min in sterile double distilled water for using as PCR template. The 16S rRNA gene of each isolate was then amplified by Px2 Themal Cycler (Thermo Electron Cooperation, USA). Conditions of PCR were initial denaturation at 94 °C for 3 min and 35 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 1 min and extension at 72°C for 1 min followed by a final extension at 72°C for 10 minutes. A nearly full-length 16s rRNA gene was amplified with the forward primer PA (5’ GTA GAG CTT ACA CTA TAT CGC AAA CTC CTA 3’) and universal reverse primer PH (5’ AAG GAG GTG ATC CAG CCG CA 3’) and Taq PCR core kit (QIAGEN, USA) as described in a manufacture’s manual. PCR products were sent to Macrogen Inc. (Soul, Korea) for gene sequencing. The 16S rRNA gene sequences with 1,430 bp were deposited at GenBank under accession number of EF990634. Alignment of different 16s rRNA gene sequences from GenBank database was performed using CLUTALX version 1.81 with neighbor-joining method. Graphic for phylogenetic trees were built by using the TREEVIEW program version 1.6.6.

3.2.5 Immobilization of the isolate PCL3

3.2.5.1 Supporting materials

Natural supporting materials i.e., corncob and sugarcane bagasse were used to immobilize the isolate PCL3 because they have a high matrix porosity and a pore size that could enhance the cell adsorption capability of immobilization. Sugarcane bagasse and corncob were cut into 0.7x0.7x0.7 cm and then 300 g of each supporting material was boiled in 3 l of 1% NaOH for 3 h to remove lignin and fibers inside the materials which might react with the cells (Bardi and Koutinas, 1994). The alkaline-boiled sugarcane bagasse and corncob were then washed three times with 3 l of distilled water, soaked in distilled water overnight and then sterilized by a tyndallization (15 min at 121°C three times spaced by at least 12-h intervals) and kept at 4 ^{o}C prior the usage.

3.2.5.2 Cell immobilization

Adsorption was used as the immobilization method in this study. This method was typically performed when the porous media were used as the support materials with the advantage of easy to operate (Bickerstaff, 1997). The immobilization technique was conducted by adding 75 g of each sterile corncob and bagasse into the sterile 250 ml NB containing 5 mg l^{-1} of carbofuran before inoculating with the isolate PCL3 (10^{6} CFU ml^{-1}). Flask was then incubated at 150 rpm, room temperature, for 48 hours. After incubation, supporting 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 washed with 0.85% NaCl by aseptic technique. This process was repeated two times. Immobilized cells were kept at 4^{o}C prior the usage. The internal cell density on the sugarcane bagasse and corncob was approximately 10^{7} CFU g^{-1} dry materials.

3.2.6 Biodegradation of carbofuran in BSM in batch cultivation

Degradation of carbofuran by free cells in comparison to the immobilized cells on corncob and sugarcane bagasse was investigated in batch system to examine the biodegradation kinetic patterns. BSM added with various carbofuran concentrations of 0-200 mg l^{-1} as a sole C-source of substrate was used as culture media. For free cell experiments, 200 ml of carbofuran supplemented with C-limited

BSM at each concentration was added into 500-ml Erlenmeyer flask before inoculating with free cells of PCL3 at approximately $5.8x10^6$ CFU ml^{-1}. Flask was then incubated at room temperature (30±3 ^oC) and shaken at 150 rpm. Culture media was sampled at days 0, 2, 4, 6, 8, 10, 14, 18, 24, and 30 to determine the number of PCL3 in the culture media by the spread plate technique. Carbofuran and its metabolites i.e., 3-ketocarbofuran and carbofuran phenol were extracted from culture media by liquid-liquid partitioning method and further analyzed by HPLC.

Biodegradation of carbofuran by the immobilized PCL3 on corncob and sugarcane bagasse was conducted in a 250-ml shake flask in batch experiments. A 100 ml of C-limited BSM containing carbofuran at each concentration were added into 250 ml flask before inoculating with 10 g (approximately $4.6x10^6$ CFU ml^{-1}) of the immobilized cell on each support material. Flasks were incubated and analyzed for carbofuran and metabolites concentrations in culture medium and number of PCL3 in support materials in a similar manner to free cell experiment. All of the experiments were performed in triplicates.

3.2.7 Analytical methods

3.2.7.1 Enumeration of carbofuran degraders

Number of the isolate PCL3 in culture media was determined by spread plate technique. The serial-diluted aliquots of culture media samples, 100 µl, was spreaded onto BSM agar coated with 5 mg l^{-1} of carbofuran and incubated at 30 °C until colonies appeared.

To determine the number of PCL3 on the supporting materials, 10 g of wet immobilized cells were sampled from culture media and washed with sterile 0.85% NaCl solution three times. The washed immobilized cells were blended to small particles using blender and then added to 50 ml sterile 0.85% NaCl solution and shaken at 250 rpm for 5 min in order to dissolve cells out from the support materials. Number of PCL3 in the liquid part was determined by spread plate technique as described above. Number of PCL3 leaked from support material was determined by counting the number of PCL3 suspended in culture media by spread plate technique.

The number of PCL3 in free cell experiment was directly converted to the biomass concentration by linear relationship, while in the

immobilization experiment, the number of PCL3 on support material and leaked cell were added together before being conducted.

3.2.7.2 Extraction and analysis of carbofuran and its metabolites

Extraction of carbofuran from culture media using a liquid-liquid partitioning method was conducted by adding 2-ml of methanol to 2-ml of culture media and then sonicated twice for 10 min each time, 50/60 voltage cycle. After sonication, carbofuran and its metabolites were extracted from the media in separation funnel with dichloromethane. This extraction was done 3 times. For the first, second and third extractions, 4, 2 and 2 ml of dichloromethane, respectively, were added to the sonicated media and hand shaken for 30 sec. The organic fraction of the samples from each extraction was collected and 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 before analyzing by HPLC (Planklang and Reungsang, 2008).

The half-lives of carbofuran in the soil were calculated by fitting to a modified first-order kinetic model using SAS program (SAS Institute, Inc., 1985; Plangklang and Reungsang, 2008). Data was analyzed by SPSS program Version 10.0 (SPSS Inc., Chicago, IL). The significance of treatments was set at *p*-value less than or equal to 0.05 by the one way ANOVA test.

3.2.8 Modeling of the biodegradation process of carbofuran

Base on the material balance, the specific carbofuran degradation rate can be expressed as follows:

$$-\frac{dS}{dt} = q_s X \qquad (1)$$

Two of the biodegradation kinetic models were used to describe the variations of relation between the biodegradation pattern and the initial carbofuran concentration. Monod model (equation (2)) was applied when carbofuran was considered as non-inhibitory compound and its inhibitory effect was neglected.

$$q_s = \frac{q_{\max} \times S}{K_s + S} = -\frac{dS}{Xdt} \qquad (2)$$

When the specific carbofuran degradation rate was observed to decrease with an increase in carbofuran concentration, the inhibitory effect of carbofuran was taken into the consideration. The substrate inhibition model used in this study was adapted from Luong model (Luong, 1987) and expressed as equation (3).

$$q_s = \frac{q_{\max} \times S}{K_s + S}\left(1 - \frac{S - S_i}{S_m - S_i}\right) = -\frac{dS}{Xdt} \qquad (3)$$

Where q_s is specific carbofuran degradation rate (mg mg^{-1} d^{-1}); X is the biomass concentration (mg l^{-1}); S is substrate concentration (mg l^{-1}); t is time (d); q_{max} is maximum specific carbofuran degradation rate (mg mg^{-1} d^{-1}); K_s is half-saturation constant (mg l^{-1}); S_m is a critical inhibitor concentration of substrate above which degradation stop; S_i is substrate inhibition coefficient (mg l^{-1}) in which the inhibitory effect of carbofuran is considered to occur in a linear manner at the initial concentration of S_i.

The parameters of the model were estimated using SIGMAPLOT®9.0 software by nonlinear regression analysis. The constraints of $1/2\,q_m \le q_{\max} \le 3q_m$ and K_s , S_m , $S_i > 0$ were given to all data set while estimating the parameters. Here q_m is the maximum specific growth rate obtained from the observed data.

3.3 Results and Discussions

3.3.1 Isolation and characterization of the isolate PCL3

Colony morphology on BSM agar coated with carbofuran of the isolate PCL3 after 4 days of incubation was white, circular, entire, convex, smooth, and opalescent with the size ranging from 0.5-1 mm in diameter. Gram stained and microscopic examination revealed that the isolate PCL3 was a gram-negative rod bacterium and motile when it was grown in BSM containing 5 mg l^{-1} of carbofuran at 30 ^{o}C. SEM analysis indicated that the isolate PCL3 was approximately 3.5-4.0 µm in length and 1.0-1.3 µm in diameter arranged singly or in short chains (2-3 cells) without observing of flagella (Figure 3-1).

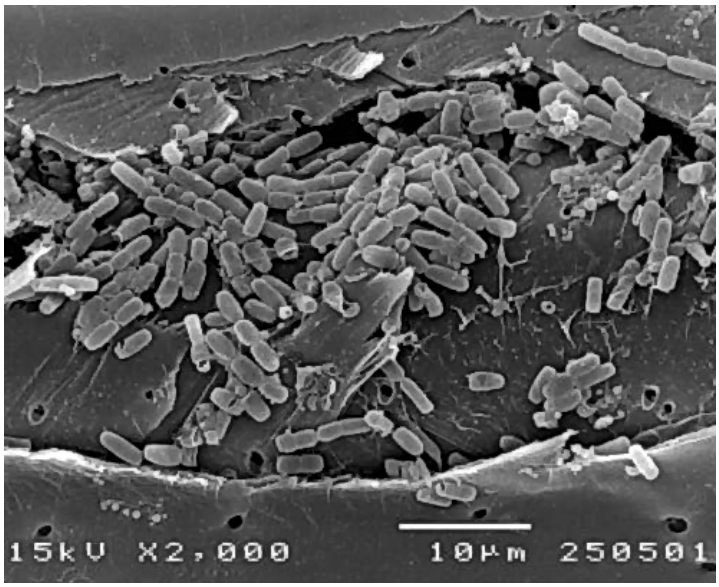

Figure 3-1. Scanning Electron Microscope (SEM) of isolate PCL3 with the magnification of 2,000X

3.3.2 16s rRNA sequencing and phylogenetic analysis

The partial sequences of 16s rRNA gene (1430 bp) of the isolate PCL3 were determined. A sequence set was compared to those available sequences in GenBank database. PCL3 showed the high similarity to the beta group of Proteobacteria, genus *Burkholderia sp.*, with the highest degree of similarity of 96% to *Burkholderia cepacia* ATCC 35254 (GenBank accession number of AY741346). Phylogenetic tree of the isolate PCL3 base on representatives of related species of the genus *Burkholderia* and known reference strains of carbofuran degraders was depicted in Figure 3-2. Carbofuran degraders isolated from previous study were mostly belonged to a group of *Proteobacteria* which exhibited fairly close relative to isolate PCL3 (Figure 3-2). *Burkholderia* species are the wide spread bacteria in nature containing more than 30 species which could be obtained from various sources, such as soil, water and sediment (Wiersinga et al., 2007). *B. pyrocinia* was reported as the carbofuran degrading bacterium which was obtained from soil contaminated with carbamate pesticides (Desaint et al., 2000). Some strains of *Burkholdria* sp. possess the ability to biodegrade others pesticides such as fenitrothion (Hayatsu et al., 2000; Tago et al., 2006; Zang et al., 2006), phathalate (Chang and Zylstra, 1999) and quinclorac (Lu et al., 2003).

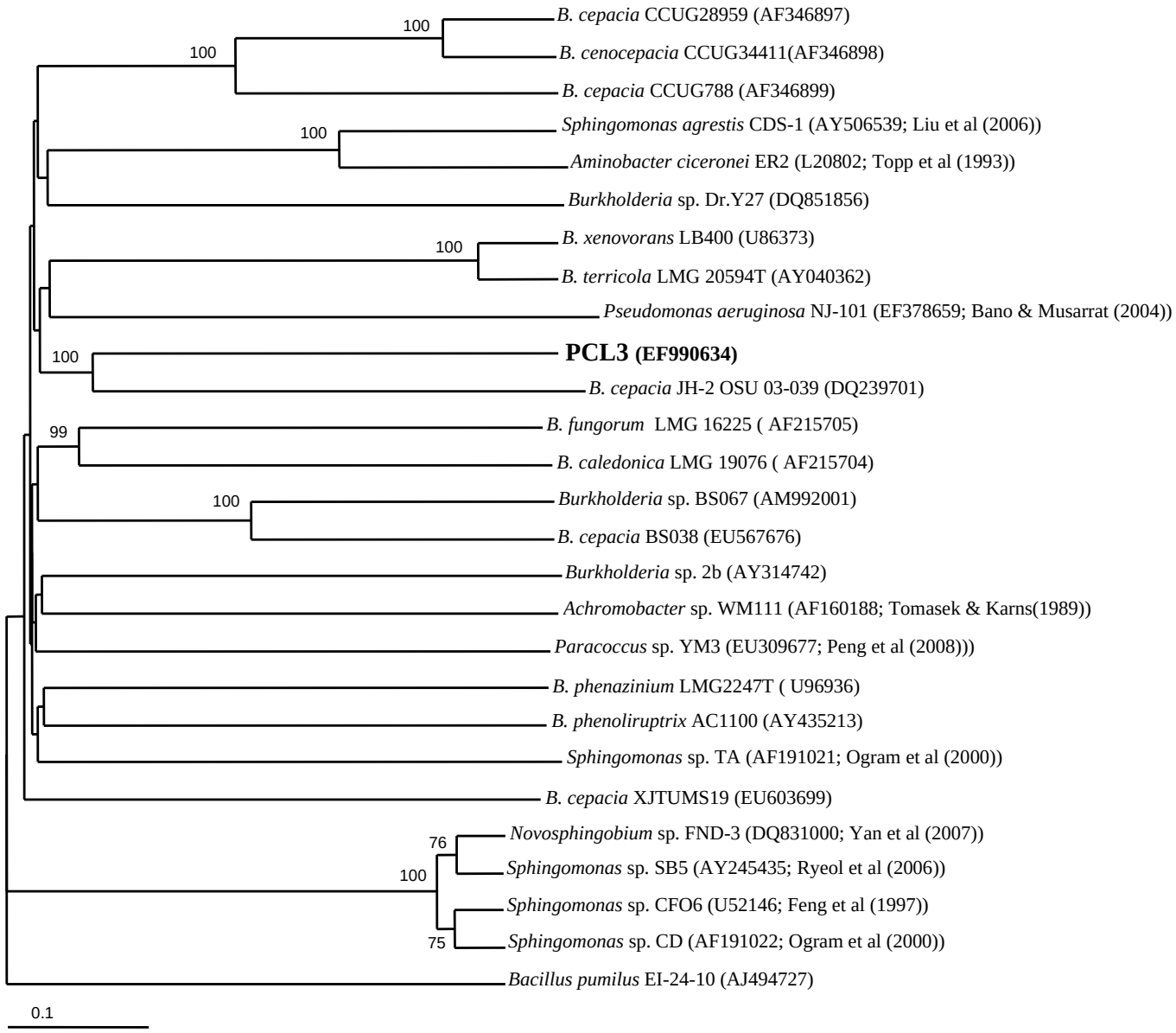

Figure 3-2. Neighbor-joining tree showing the phylogenetic position of the isolate PCL3 as compared to related species of the genus *Burkholderia* and reference strains of carbofuran degraders based on 16s rRNA gene sequences. *Bacillus pumilus* was selected as an out-group in this analysis. The GenBank accession number for each microorganism was shown in parentheses after species name. Reference for each carbofuran degrader strain was indicated in the parentheses after accession number. Bootstrap values expressed as percentage of 1000 replications of $\geq$ 70% were shown at the branch. The bar represented 0.1 substitutions per site.

3.3.3 Effect of carbofuran concentration on growth patterns of free and immobilized PCL3

The microbial cell growth of the isolate PCL3 in free cell form at various initial carbofuran concentrations of 5-200 mg l^{-1} was investigated and depicted in Figure 3-3. Cell growth curves have typically exponential, stationary and dead phase with the increase in lag phase when the initial concentration of carbofuran was increased. It was observed that carbofuran concentrations of 5 mg l^{-1} did not show any inhibitory effect on the isolate PCL3 as indicated by almost no lag phase during its growth. A relatively short lag period of 4 d was observed during the growth of the isolate PCL3 at initial carbofuran concentration of 50 mg l^{-1}, and the longer lag period of 6 and 8 d were shown in the growth patterns of PCL3 at the initial carbofuran concentrations of 100 and 150 d, respectively (Figure 3-3). At a high carbofuran concentration of 200 mg l^{-1}, cell concentration slightly increased from day 8 to 15 d then gradually decreased. This indicated that the growth of the isolate PCL3 was almost completely inhibited at this initial carbofuran concentration which might due to the fact that the acclimatization of isolated degraders in this study was carried out at the concentration of carbofuran up to 100 mg l^{-1}. Therefore, the isolated degrader might not be able to grow well at a high carbofuran concentrations greater 100 mg l^{-1}.

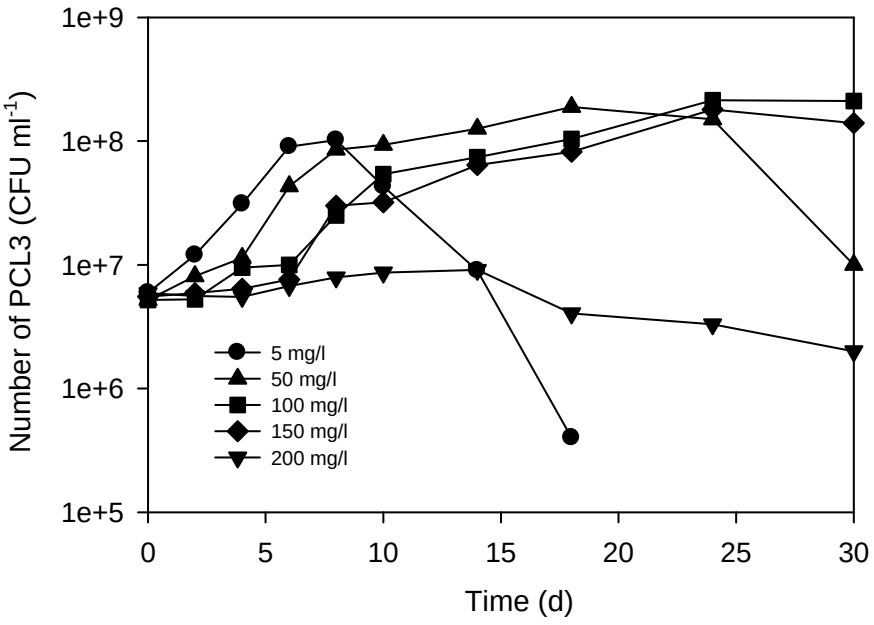

Figure 3-3. Growth of the isolate PCL3 in BSM containing different initial carbofuran concentrations.

Growth and survival of the immobilized PCL3 were determined by the numbers of carbofuran degraders in the support materials i.e., corncob and sugarcane bagasse. Number of cells leaked from support materials was observed in culture media during incubation. The numbers of the isolate PCL3 in supports and in BSM in the experiment with the initial carbofuran concentration of 50 mg l^{-1} were depicted Figure 3-4. The similar result was observed in all other experiments (data not shown). Results indicated that cell numbers on the immobilized matrices were stable in the range of 10^7-10^8 CFU g^{-1} dry support throughout 30 d of incubation without any inhibitory effect (Figure 3-4). These findings implied that the survival and stability of the isolate PCL3 could be improved through the immobilization on corncob and sugarcane bagasse. Corncob and bagasse might act as protective agents against limiting condition such as substrate and product inhibitions during incubation or shear forces (Bekatorou et al., 2004).

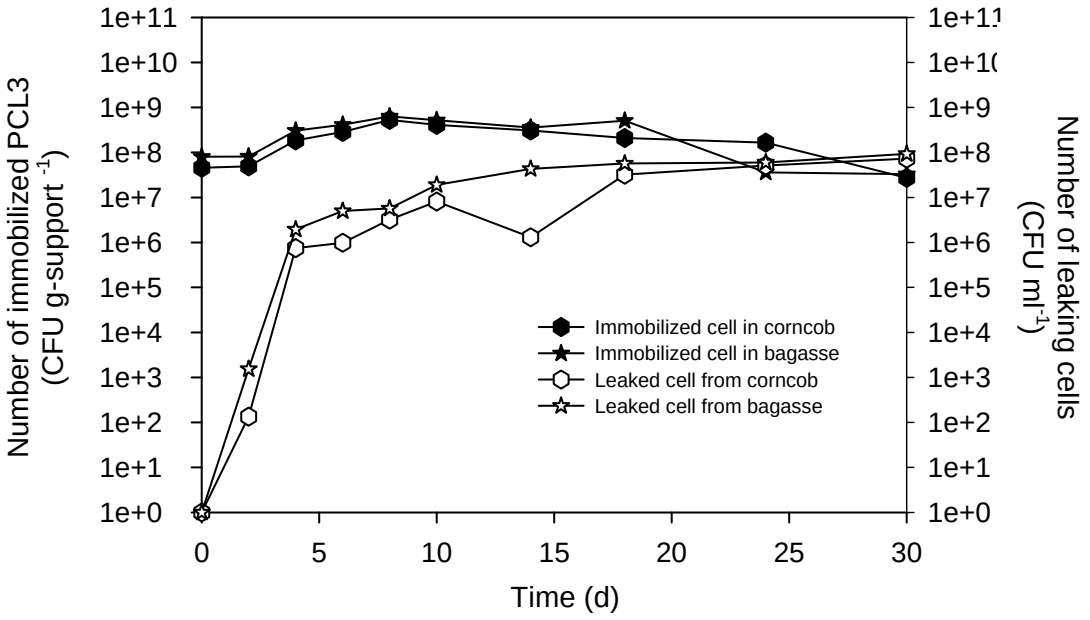

Figure 3-4. Growth and number of leaked cells of the isolate PCL3 from supports at the initial carbofuran concentration of 50 mg l^{-1}.

Cell number of PCL3 in culture media was negligible at d 0. Number of PCL3 in the liquid culture media increased to greater than 10^6 CFU ml^{-1} after 10 d of incubation, whilst the number of PCL3 in the support materials was not reduced (Figure 3-4). These results indicated that the cells were leaking out from the porous media of corncob and bagasse due to a limited space and they continued to grow in

the medium causing the increase in cell concentration. Cell leaking might be resulted from the fact that there are no barriers between the cells and the media which led to a possibility of cell detachment and relocation with potential establishment of cell-equilibrium inside the supports and culture media (Bekatorou et al., 2004; Kumar and Das, 2001).

3.3.4 Effect of carbofuran concentration on carbofuran degradation patterns of free and immobilized PCL3

The degradation profiles of carbofuran in BSM with various concentrations of carbofuran inoculated with free cells of PCL3 was depicted in Figure 3-5. Results revealed that carbofuran could be degraded immediately at the initial concentration of 5 mg l^{-1} after starting the experiment. The lag period could be observed at the initial carbofuran concentration of greater than 5 mg l^{-1}. The degradation patterns of carbofuran in BSM were correlated to the growth patterns of the isolate PCL3 (Figures 3-3). At the concentration of carbofuran between 5-50 mg l^{-1}, the growth of the isolate PCL3 reached the stationary phase when carbofuran concentration in culture media was almost completely consumed. This implied that carbofuran was incorporated to cells of the isolate PCL3 for its growth.

At the initial carbofuran concentration of 50 mg l^{-1}, the stationary phases for growth of PCL3 and carbofuran degradation were observed at the same period of incubation (Figure 3-3, 3-5). However, carbofuran could not be completely degraded when stationary phase was observed (Figures 3-3. 3-5). At the initial carbofuran concentration in BSM of 200 mg l^{-1}, the microbial growth was not observed, but a slightly decrease of carbofuran concentration (approximately 26%) was observed (Figure 3-3b). The result indicated that at a high initial carbofuran concentration, PCL3 tended to use carbofuran for maintaining the cell. In addition, there might be abiotic processes responsible for carbofuran degradation. This might be resulted from the hydrolysis and volatilization processes during shaking incubation. Though the abiotic degradation processes are not as important route as microbial degradation to carbofuran dissipation but they were contributing dissipation processes which could be found in the abiotic control as repotted in previous studies (Evert, 2002; Lalah et al., 1996).

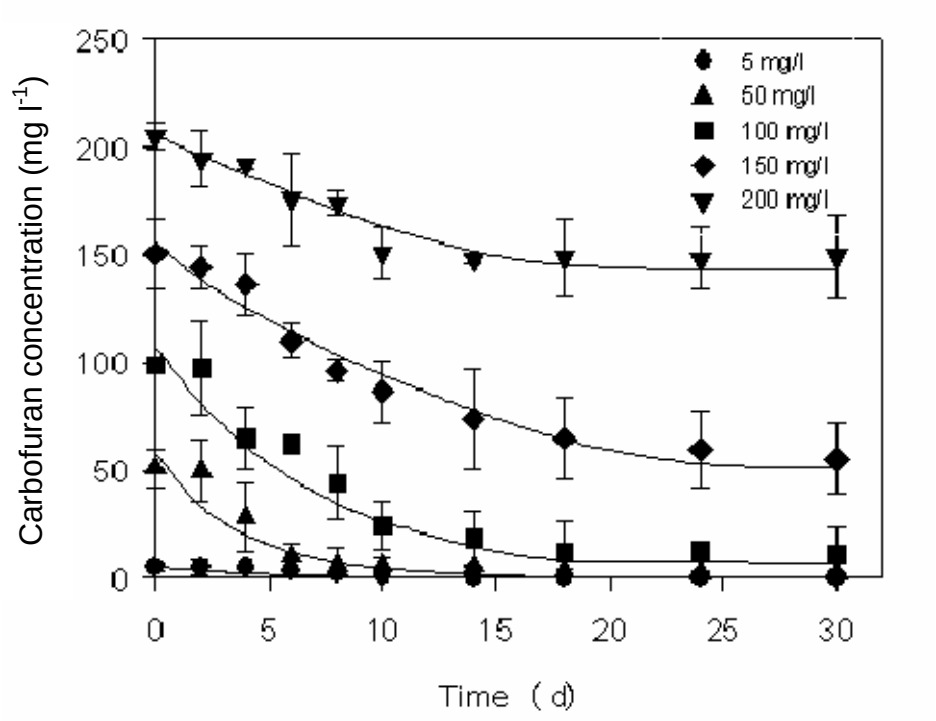

Figure 3-5. Dissipation of carbofuran in BSM inoculated with free cells of the isolate PCL3 at various carbofuran concentrations. Lines indicated the carbofuran degradation fitting to the modified first order kinetic model.

The degradation profiles of carbofuran in BSM inoculated with the immobilized PCL3 on corncob and sugarcane bagasse were depicted in Figure 3-6. Results indicated that carbofuran up to 200 mg l^{-1} could be degraded by the immobilized PCL3 on both supports without an obvious inhibitory effect. This might be due to the fact that the immobilized PCL3 could survive and grow on both support materials throughout the experiment which could responsible for effective carbofuran degradation in BSM.

Carbofuran phenol and 3-keo carbofuran were the metabolites detected during carbofuran degradation in this experiment (data not shown). The accumulation of carbofuran phenol was not observed at the end of incubation (d 30) for the experiments with the initial carbofuran concentration of 5-50 mg l^{-1}. However, at the initial carbofuran concentration of 100-150 mg l^{-1}, approximately 12.3 mg l^{-1} of carbofuran phenol could be detected at the end of incubation. This implied that PCL3 have a capability to degrade carbofuran phenol at a low concentration and was inhibited when the concentration of carbofuran phenol was increased. The detection of carbofuran phenol in the culture media might be due to the isolate PCL3 has *mcd*

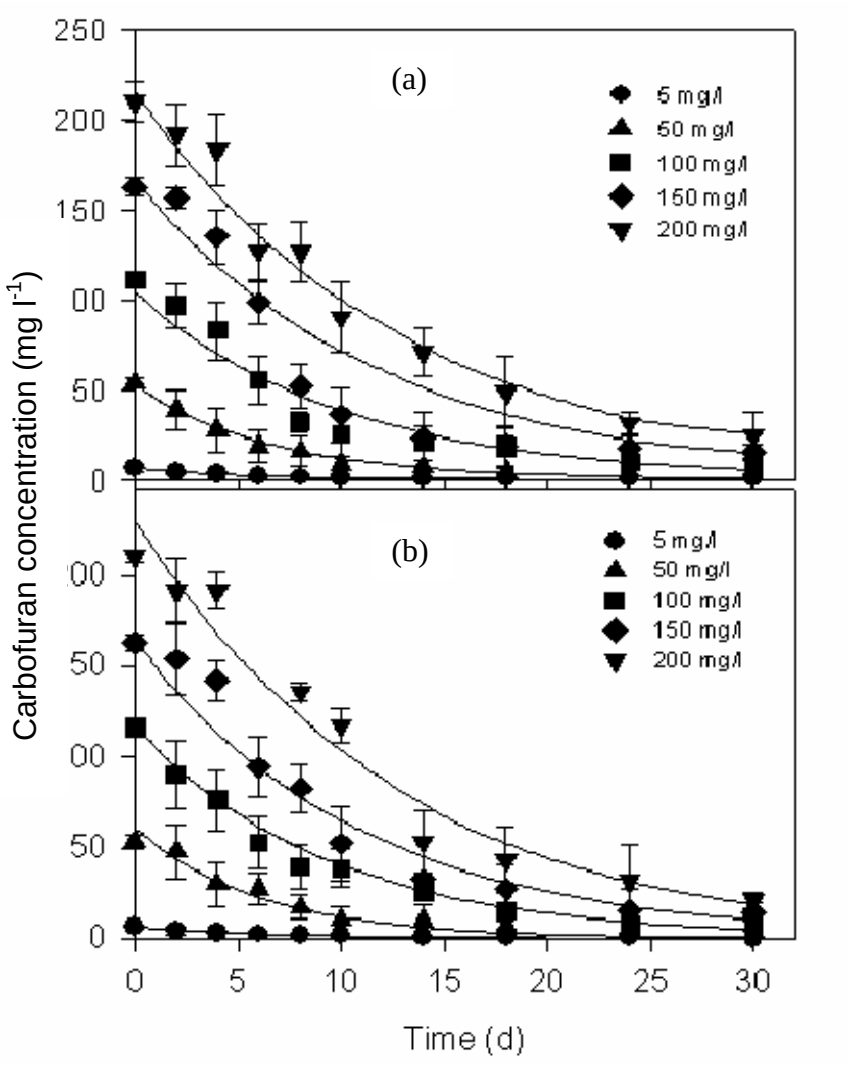

Figure 3-6. Dissipation of carbofuran in BSM inoculated with the immobilized PCL3 on corncob (a) and sugarcane bagasse (b) at various carbofuran concentrations. Lines indicated the carbofuran degradation fitting to the modified first order kinetic model.

gene (Plangklang and Reungsang, 2009) coding hydrolase enzyme responsible for degrading carbofuran. Catalyzing of carbofuran degradation at N-metyl group by hydrolase enzyme yielding carbofuran phenol was also reported in previous researches (Eisler, 1985; Head et al., 1992; Tomasek and Karns, 1989).

Accumulation of 3-keto carbofuran, approximately 2.1 mg l^{-1} could be found in all experiments after 4 d till the end of incubation. For the experiment with the carbofuran concentration of 200 mg l^{-1}, only 3-keto carbofuran was detected without the observing of carbofuran phenol. This indicated that 3-keto carbofuran might not be a result from microbial degradation.

3.3.5 Half-lives of carbofuran in BSM

The effect of immobilization on carbofuran degradation efficiency by free cells in comparison to immobilized cells was evaluated. The degradation rate coefficient of carbofuran was calculated by fitting the data to a modified first-order kinetic model in order to examine half-lives of carbofuran in BSM. The regressions coefficients, r^2, ranged between 0.92-0.98 indicating a good fit of the data to the model (Table 3-1, Figures 3-4, 3-5). Results indicated that carbofuran could be effectively degraded by free and immobilized cells at the initial carbofuran of 5-100 mg l^{-1} with the short haft-lives ranged between 2.74-6.93 d. The half-lives of carbofuran obtained from the immobilized experiment is not significantly different from that of the free cell experiment ($p>0.05$). These results implied that immobilization did not limit the accession of carbofuran in the culture media to PCL3 cell. At a concentration of carbofuran greater than 100 mg l^{-1}, longer carbofuran half-lives of 14-30 d were observed when free cells of PCL3 was used. However the carbofuran half-lives in BSM with immobilized cells slightly increased to 7-9 d (Table 3-2). These results indicated that carbofuran degradation efficiency of the isolate PCL3 at high carbofuran concentration could be enhanced by immobilization technique which was the result from the improvement of cell survival.

3.3.6 Carbofuran degradation kinetics

The relation between specific carbofuran degradation rate and initial concentration of carbofuran was depicted in Figure 3-7. The decreased specific carbofuran degradation rate with the increase in initial carbofuran concentration in free cell experiment (Figure 3-7) implied that carbofuran acts as an inhibitor on PCL3. Therefore the substrate inhibition model was used to explain the biodegradation kinetic of carbofuran by PCL3 in free cell form. The Monod model was used to explain the biodegradation kinetic of carbofuran by PCL3 in the immobilized cell form. The results indicated that there was no inhibitory effect of carbofuran at the concentration up to 200 mg l^{-1} (Fig 3-7) on the immobilized PCL3 which confirmed that the immobilized technique could protect the PCL3 cell from substrate inhibition hence enhancing carbofuran degradation efficiency. The Kinetic parameters of PCL3 in free and immobilized cell forms estimated from nonlinear regression with biodegradation kinetic models was tabulated in Table 3-2. The regressions

Table 3-1 Half-lives ($t_{1/2}$) of carbofuran in PCL3 inoculated BSM with various initial carbofuran concentrations

Initial carbofuran concentration (mg l^{-1})	Free cells of PCL3		Immobilized PCL3 on corncob		Immobilized PCL3 on sugarcane bagasse	
	$t_{1/2}$ (d)	$r^{2\,(2)}$	$t_{1/2}$ (d)	r^2	$t_{1/2}$ (d)	r^2
5[(1)]	3.32^{aA}±1.322	0.90	3.52^{aA}±1.101	0.96	4.63^{aA}±1.72	0.95
50	2.74^{aA}±1.255	0.92	3.28^{aA}±1.420	0.95	4.23^{aA}±1.35	0.96
100	4.92^{aA}±2.876	0.93	6.93^{aAB}±2.311	0.96	6.36^{aA}±2.11	0.98
150	14.44^{aB}±1.28	0.96	8.15^{bB}±2.892	0.94	7.45^{bA}±2.02	0.96
200	30.13^{aC}±7.33	0.98	9.00^{bB}±2.919	0.98	8.09^{bA}±2.73	0.93

[(1)] Comparison between treatment in row and column are significantly different (Duncan, $p \leq 0.05$) if mark different small and capital letters, respectively

[(2)] Coefficients of determination for non-linear regressions

coefficients, r^2, ranged between 0.986-0.991 indicating a good fit of the data to the model. The estimated kinetic parameters for free cells of PCL3 from the proposed substrate inhibition model were q_{max} = 0.451 d^{-1} and K_s = 171.96 mg l^{-1}. The inhibitory effect of carbofuran was considered to occur in a linear manner at the initial concentration of (S_i) of 114.86 mg l^{-1}. The maximum substrate concentration above which carbofuran degradation was completely inhibited (S_m) was 248.76 mg l^{-1}. The greater q_{max} of 0.124 and 0.198 d^{-1} and the lower K_s of 160.83 and 113.95 mg l^{-1} for the immobilized PCL3 on corncob and sugarcane bagasse, respectively was obtained (Table 3-2). From the kinetic behavior of PCL3, it was confirmed that the immobilization technique could protect the PCL3 cell from substrate inhibition hence enhancing carbofuran degradation efficiency.

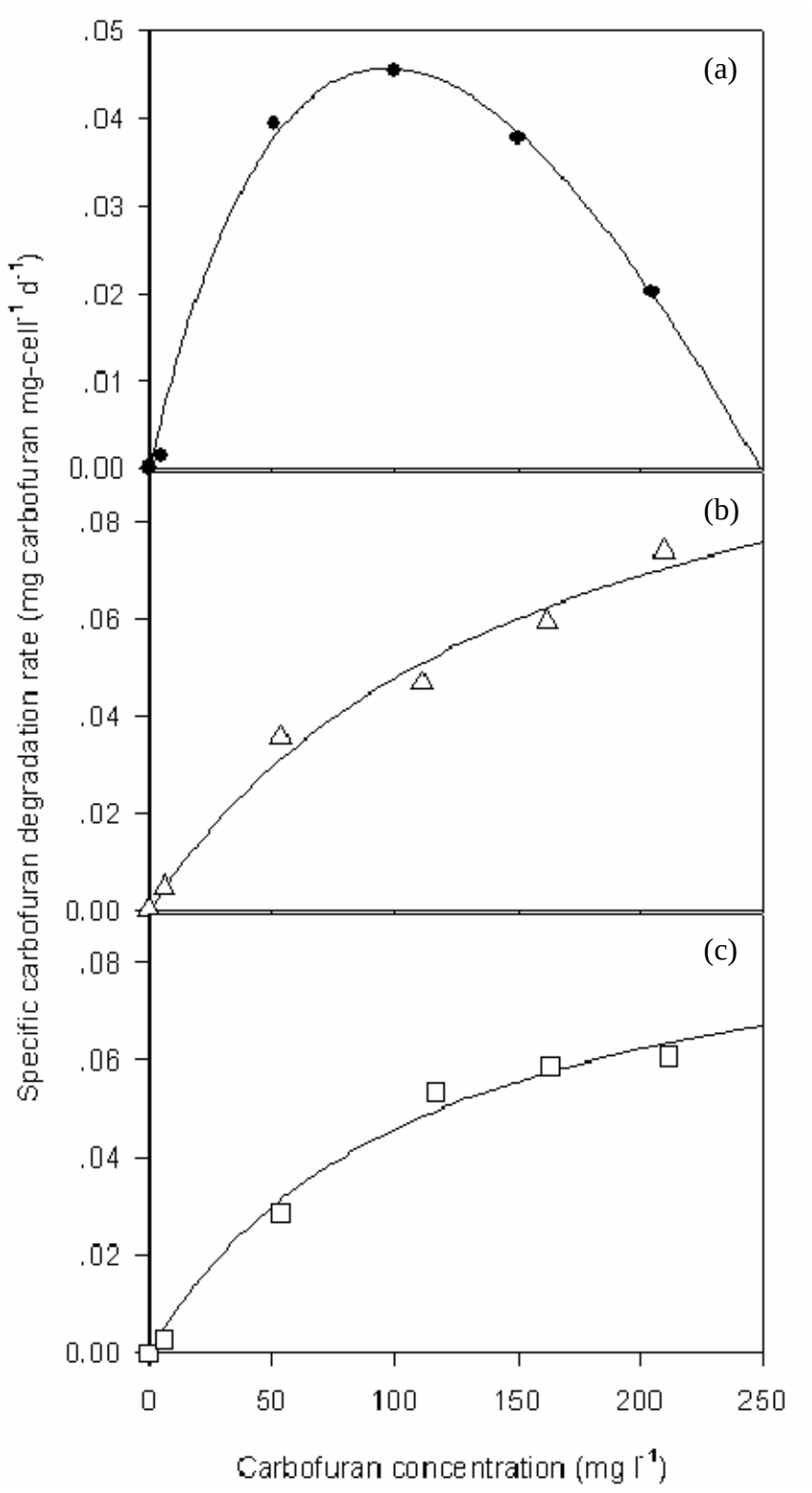

Figure 3-7. The specific degradation rate of carbofuran by PCL3 in free cell form (a) and immobilized cell form on corncob (b) and sugarcane bagasse (c) on initial carbofuran concentration.

Table 3-2 Kinetic parameters of PCL3 in free and immobilized cell forms estimated from nonlinear regression with biodegradation kinetic models

Kinetic parameters	Free cells of PCL3	Immobilized PCL3 on corncob	Immobilized PCL3 on sugarcane bagasse
	Substrate inhibition	Monod	Monod
q_{max} (d^{-1})	0.087	0.124	0.098
K_s (mg l^{-1})	171.96	160.83	113.95
S_i (mg l^{-1})	114.86		
S_m (mg l^{-1})	248.76		
r^2	0.988	0.986	0.991

3.4 Conclusions

A carbofuran degrader was identified base on 16s rDNA sequences as *Burkholderia* sp. PCL3 (GenBank accession number of EF990634). Both free and immobilized cell forms of the isolate PCL3 could effectively degrade carbofuran at the initial carbofuran concentration of 5-100 mg l^{-1} with no significantly different in the half-lives. The isolate PCL3 in free cell forms was inhibited by carbofuran concentration greater than 100 mg l^{-1} thus the substrate inhibition model was used to explain its biodegradation kinetic. Immobilization technique was able to improve the inhibitory effect of carbofuran on the isolate PCL3 at high concentration which indicated by the survival of the immobilized PCL3 at the concentration up to 200 mg l^{-1}.

CHAPTER IV

BIOAUGMENTATION OF CARBOFURAN RESIDUES IN SOIL USING *Burkholderia cepacia* PCL3 ADSORBED ON AGRICULTURAL RESIDUES

4.1 Introduction

Carbofuran (2,3-dihydro-2,2 dimethylbenzofuran-7-yl methyl carbamate) is a broad-spectrum insecticide widely used in agriculture to control insects and nematodes on contact or after ingestion. It is of environmental concern because of its relatively high solubility of 351 mg l^{-1} at 25^{o}C so it is mobile in soil resulting in a high potential for groundwater contamination. Carbofuran can cause acute toxicity to mammals through cholinesterase inhibition (LD_{50} = 2 mg g^{-1}) (EPA, 2006).

In our previous study, thirteen different bacterial strains were isolated from carbofuran phytoremediated rhizosphere soils by an enrichment technique (Plangklang, 2004). Their abilities to degrade carbofuran were investigated in Basal Salt Medium (BSM) containing 5 mg l^{-1} of carbofuran. The shortest half-life of carbofuran, 3 d, was found in C-limited BSM cultured with an isolate named PCL3, identified by 16S rRNA as *Burkholderia cepacia* (GenBank accession number of EF990634), indicating that carbofuran was used as its sole C-source. Successful biodegradation of carbofuran using the isolated degraders has been reported (Bano and Musarrat, 2004; Jiang et al., 2007; Qiu-Xiang et al., 2007; Ryeol et al., 2006; Seo et al., 2007; Slaoui et al., 2007), in which most of the carbofuran degraders used were in free cell form. However, there are some limitations in using free cells of the isolates such as low survival ability of free cells in natural conditions, low cell recovery and low capability of cell recycling (Bekatorou et al., 2004; Manohar et al., 2001). Immobilization of cells might overcome these limitations. The use of immobilized systems offers many advantages over free cells including regeneration and reuse of immobilized cells for extend period. The supporting materials may act as protective agents against the effects of pH, temperature, solvent, heavy metals or even substrate and product inhibitions, and enhancing the cell survival (Bekatorou et al., 2004). The support materials used for immobilization could be either synthetic

polymers or natural materials. Disposal of synthetic polymer is of the concern due to its non-biodegradable characteristic, unlike the agricultural residues which are natural materials and biodegradable.

Immobilized cells offer advantages over free cells, however, there is limited information on bioaugmentation of carbofuran residues using immobilized carbofuran degraders on agricultural residues. Therefore, this study was designed to examine a degradation of carbofuran residues in synthetic medium and soil by *B. cepacia* PCL3 adsorbed on corncob and sugarcane bagasse in comparison to free cells. Results from these studies would provide information on the possibility of increasing the survival of *B. cepacia* PCL3 by immobilization techniques and the application of immobilized PCL3 in bioaugmentation of carbofuran residues.

4.2 Materials and Methods

4.2.1 Chemicals and reagents

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

4.2.2 Microorganism and culture medium

The carbofuran degrader, identified by 16S rRNA as *B. cepacia* PCL3 (GenBank accession number of EF990634), was grown at 30 °C, 150 rpm in Nutrient Broth (NB) (Difco, USA) containing 5 mg l^{-1} of carbofuran for 48 h and used as inoculum for immobilization. *B. cepacia* PCL3 was previously isolated from rhizosphere soil of rice (*Oryza sativa* Linn) treated with carbofuran at a concentration of 5 mg kg^{-1}soil (Teerakun et al., 2004). This microorganism is capable of using carbofuran as a sole C-source (Plangklang, 2004).

Culture medium was a C-limited BSM (Mo et al., 1997) containing carbofuran as a sole carbon source. For BSM agar, 1.5% of bactoagar was added to BSM before sterilization. Carbofuran solution in sterile distilled water at the

concentration of 5 mg l^{-1} was coated using glass spreader on BSM agar as a sole C-source prior to use.

4.2.3 Soils

A sandy loam soil sample, 0-15 cm depth, was collected from rice fields of Ban Nonmuang, A. Muang, Khon Kaen Province. Organic carbon and nitrogen contents of the soil were 0.89% and 0.10%, respectively. Soil pH was 6.9. Soil was passed through a 2 mm sieve and kept in a plastic bag at 4 °C for two weeks before use. The concentration of carbofuran in the soil sample was 0.06 mg kg^{-1}.

4.2.4 Supporting materials

Natural supporting materials i.e., corncob and sugarcane bagasse were used to immobilize *B. cepacia* PCL3 because they have a high matrix porosity and a pore size that could enhance cell adsorption. Sugarcane bagasse and corncob were cut into 0.7x0.7x0.7 cm cubes and then 300 g of each was boiled in 3 l of 1% NaOH for 3 h to remove lignin and fibers which might react with the cells (Bardi and Koutinas, 1994). The alkaline-boiled sugarcane bagasse and corncob were then washed three times with 3 l of distilled water, soaked in distilled water overnight and then sterilized at 121^{o}C for 15 min and kept at 4 ^{o}C prior to use.

4.2.5 Cell immobilization

Cell immobilization by adsorption was conducted by adding 75 g of sterile corn cob and bagasse into the sterile 250 ml NB containing 5 mg l^{-1} of carbofuran before inoculation with the isolate PCL3 (10^{6} cfu ml^{-1}). The medium was then incubated at 150 rpm, at room temperature, for 48 hours. After incubation, supporting materials were transferred to a fresh NB containing 5 mg l^{-1} of carbofuran and incubated, as previously described, before collection by filtration through Buchner filter funnel. The support materials were washed twice with 0.85% NaCl using aseptic technique. Immobilized cells were kept at 4^{o}C prior use. The internal cell density on the sugarcane bagasse and corncob was approximately 10^{7} cfu g^{-1} dry material. The microstructure of support materials and immobilized cells were investigated by Scanning Electron Microscope (SEM) (Rachman et al., 1998).

4.2.6 Microbial degradation of carbofuran by free and immobilized cells of *B. cepacia* PCL3 in BSM

Degradation of carbofuran in BSM by *B. cepacia* PCL3 in free cells form was conducted in a 500-ml shake flask in batch experiments. C-limited BSM, 200 ml, containing 5 mg l^{-1} of carbofuran as a sole carbon source, was added into the flasks before inoculation with approximately 10^6 cfu ml^{-1} of *B. cepacia* PCL3. Flasks were then incubated at room temperature and shaken at 150 rpm. Flasks were sampled at days 0, 2, 4, 6, 8, 10, 14, 18, 24, and 30 to determine the number of PCL3 in the culture media and to extract carbofuran and its metabolites i.e., 3-ketocarbofuran and carbofuran phenol by liquid-liquid partitioning method. Carbofuran and its metabolites were analyzed by HPLC.

Degradation of carbofuran by the immobilized cells of *B. cepacia* PCL3 in BSM was conducted in 250-ml shake flask in batch experiments. C-limited BSM, 100 ml, containing 5 mg l^{-1} of carbofuran was added to each flask before inoculation with immobilized *B. cepacia* PCL3. This was conducted by weighing 10 g of the immobilized PCL3 with an approximate cell concentration of 10^6 cfu ml^{-1} on a sterile Petri dish and transferring to the flask using sterile forceps. Flasks were then incubated for 30 d and analyzed for carbofuran and metabolite concentrations for the number of PCL3 in support materials and in the culture medium as previously described.

After 30 d of incubation, immobilized cells on corncob and sugarcane bagasse were harvested by filtration through Buchner filter funnel and washed three times using a sterile BSM. Then 10 g of these immobilized cells were re-inoculated into a fresh BSM media containing 5 mg l^{-1} of carbofuran. Concentrations of carbofuran and its metabolites and cell leakage and survival were monitored for another 30 d. This step was repeated twice to investigate the reusability of immobilized cells.

The number of *B. cepacia* PCL3 in culture media, accounted as cells leaked from the support materials, was determined by plate count technique which modified from Zilli et al. (2004). To observe the cell growth and cell survival on the supporting materials, 10 g of wet immobilized cells were taken from culture media and washed with sterile 0.85% NaCl solution three times. The washed immobilized cells were blended to 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 the support materials. The number of *B. cepacia* PCL3 in the liquid phase was determined by plate count on the carbofuran-coated BSM agar.

4.2.7 Bioaugmentation of carbofuran residues in soil with free and immobilized cells of *B. cepacia* PCL3

Eight sets of bioaugmentation experiments (Table 4-1) were conducted in soil microcosms in 425 cm^3 glass jars capped with plastic lids. Carbofuran solution, at an initial concentration of 5 mg kg^{-1} dry soil was spiked into 20 g dry weight of soil in each glass jar and well mixed by hand stirring. Either free cells or immobilized cells of *B. cepacia* PCL3 on corncob and sugarcane bagasse, was added into the soil at the initial cell concentration of 10^6 cfu g^{-1} dry soil. The initial moisture content of the soil samples was adjusted to 15-18% before incubation at an average room temperature of 29±2^{O}C in the dark. Soil samples were sacrificed at days 0, 5, 10, 15, 25, 35 and 60 and further extracted with an Accelerated Solvent Extractor, ASE 100 (Dionex, USA). Concentrations of carbofuran and its metabolites were determined by HPLC. At each sampling date, total numbers of carbofuran degraders including bacteria and fungi in the soil were counted by spread plate technique on carbofuran-coated BSM agar. The half-lives of carbofuran in soils were calculated by fitting time-course concentration data to a modified first-order kinetic model. Three sets of control were included, i.e., soil without inoculation (treatment A) and autoclaved soil with

Table 4-1 Soil bioaugmentation experiment

Treatment	Experimental set up
A	Soil
B	Soil + free cells of PCL3
C	Soil + immobilized PCL3 on corncob
D	Soil + immobilized PCL3 on sugarcane bagasse
E	Autoclaved soil + free cells of PCL3
F	Autoclaved soil + immobilized PCL3 on corncob
G	Autoclaved soil + immobilized PCL3 on sugarcane bagasse
H	Autoclaved soil + autoclaved corncob
I	Autoclaved soil + autoclaved sugarcane bagasse

inoculation of *B. cepacia* PCL3 with both free (treatment E) and immobilized cell forms (treatment F, G). The abiotic degradation control was sterilized soil mixed with sterilized immobilized matrices (treatment H, I). The experiment was set up as previously described.

4.2.8 Sorption of carbofuran to support materials

Adsorption isotherms were determined by conducting batch equilibrium experiments. De-lignified corncob and sugarcane bagasse were air-dried overnight and milled into small pieces using blender and passed through 2 mm sieve. Then a total of 0.25 g of air-dried and de-lignified corncob or sugarcane bagasse was put into a 250 ml conical flask and mixed with 50 ml of 0.01 M $CaCl_2$ solution containing carbofuran at carbofuran concentrations of 0.05, 0.1, 1.0, 5.0, 10.0, and 20.0 mg l^{-1}. Flasks were shaken at a constant speed of 100 rpm for 48 h at an average room temperature of 29±2^{O}C. After 48 h, the solution was passed through a Whatmann filter paper No. 1 and the filtrate was extracted by liquid-liquid partitioning method and quantified for carbofuran concentration by HPLC. The data were fitted to the Freundlich equation (Sposito, 1980) to describe the kinetic of carbofuran sorption to corncob and sugarcane bagasse.

4.2.9 Extraction and analysis of carbofuran and its metabolites

Extraction of carbofuran from culture media using a liquid-liquid partitioning method was conducted by adding 2-ml of methanol to 2-ml of culture media and then sonicated twice for 10 min each time, 50/60 voltage cycle. After sonication, carbofuran and its metabolites were extracted from the media in separation funnel with dichloromethane. This extraction was done three times. For the first, second and third extractions, 4, 2 and 2 ml of dichloromethane, respectively, was added to the sonicated media and hand shaken for 30 sec. The organic fraction of the samples from each extraction was collected and 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 before analyzing by HPLC.

Carbofuran and its metabolites were extracted from 13-g soil samples by an Accelerated Solvent Extractor ASE 100 (Dionex, USA) prior to analysis by HPLC following the conditions described by Planklang and Reungsang (2008).

The half-lives of carbofuran in the soil were calculated by fitting to a modified first-order kinetic model using SAS program (SAS Institute, Inc., 1985; Plangklang and Reungsang, 2008). Data were analyzed by SPSS program Version 10.0 (SPSS Inc., Chicago, IL). The significance of treatments was set at *p*-value less than or equal to 0.05 by the one way ANOVA test.

4.2.10 PCR amplification

The oligonucleotide primer pair of mcdF1 and mcdR1 (Parekh et al., 1996) was used for specific amplification of a 590 bp fragment from the methyl carbamate degrading (*mcd*) gene region of the isolate PCL3. The *mcd* gene codes for hydrolase enzyme responsible for carbofuran degradation. Colony PCR condition was conducted followed the method as previously described by Parekh et al. (1996).

4.3 Results and Discussion

4.3.1 Scanning Electron Microscope (SEM)

The microstructure of support materials and immobilized cells were depicted in Figure 4-1. The photograph showed that corncob (Figure 4-1A) has more porosity than sugarcane bagasse (Figure 4-1B). It is evident from the photograph that *B. cepacia* PCL3 attached to the support materials inside their porous space which indicated that *B. cepacia* PCL3 was immobilized on corncob and sugarcane bagasse by adsorption mechanism. Cells were rod shape with approximately 5 µm in length and 1.2 µm in diameter when they were immobilized on corncob (Figure 4-1C). When cells were immobilized on sugarcane bagasse, the size of cells was approximately 3.5 µm in length and 1.3 µm in diameter (Figure 4-1D). Difference in cell shape could be the results of nutrient acquisitions and different attachment mechanisms to different surfaces (Young, 2007).

4.3.2 Degradation of carbofuran in BSM

This experiment investigated the ability of immobilized *B. cepacia* PCL3 to degrade carbofuran in BSM in comparison to free cells. Degradation of carbofuran in BSM was described by a modified first-order kinetic model (Figure 4-2). The corresponding kinetic data fitted to a modified first-order kinetic model was tabulated in Table 4-2. The regressions coefficients, r^2, ranged between 0.80 and 0.99

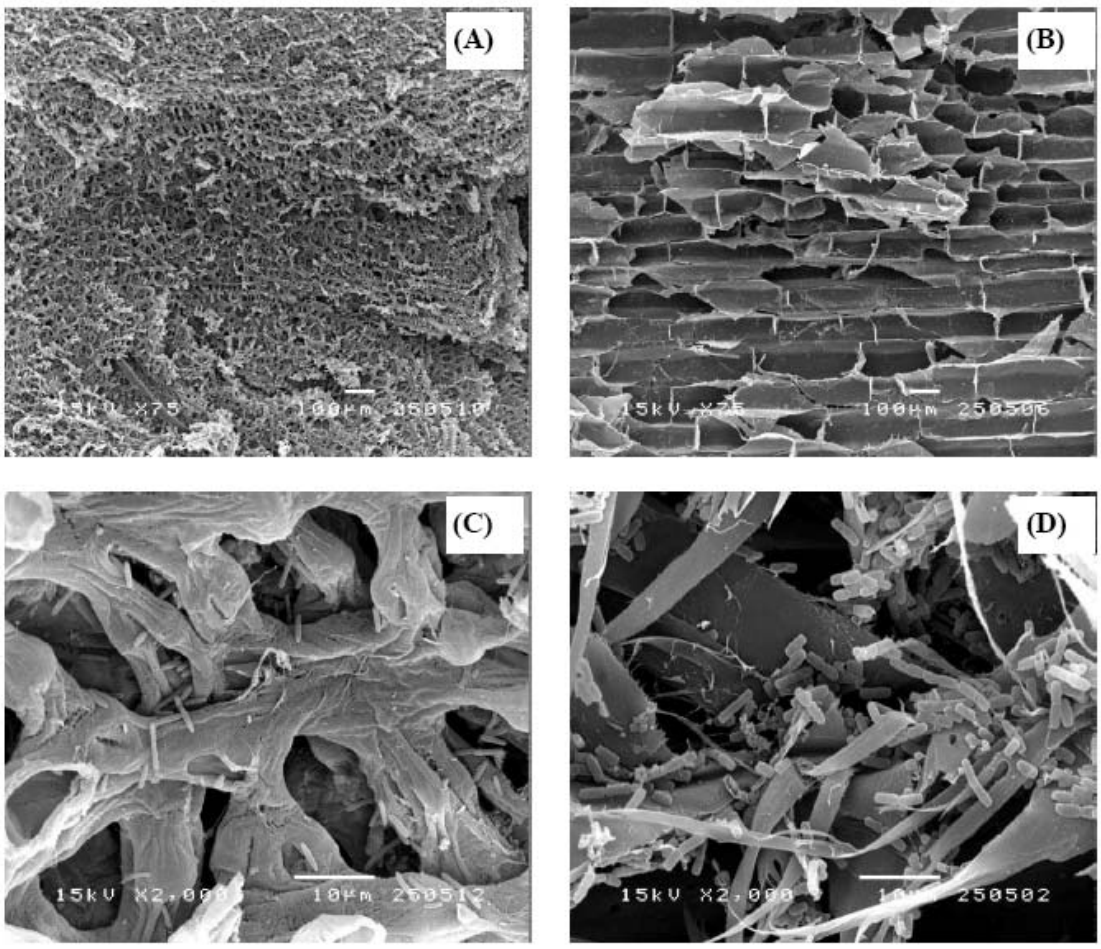

Figure 4-1. Scanning electron micrographs studies of support materials i.e., (a) corncob, and (b) sugacne bagasse and immobilized *B. cepacia* PCL3 on (c) corncob and (d) sugarcane bagasse.

indicating a good fit of the data to the first-order kinetic model (Table 4-2). Carbofuran was rapidly degraded by *B. cepacia* PCL3 in both free and immobilized cells forms on corncob and on sugarcane bagasse with $t_{1/2}$ of 3, 3 and 4 d, respectively (Table 4-2), which suggested that immobilization did not decrease carbofuran degrading activity of *B. cepacia* PCL3 in BSM. The high matrix porosity and pore size of these two materials might enhance the absorption capacity and substrate transfer to the immobilized cells (Gu et al., 1994; Jimoh, 2004). Support materials suitable for immobilization through adsorption mechanism should not be only good packing materials for bacterial growth, but they should be effective for physical adsorption of the contaminant to support a transfer of the contaminant to the cells (Ma et al., 2006).

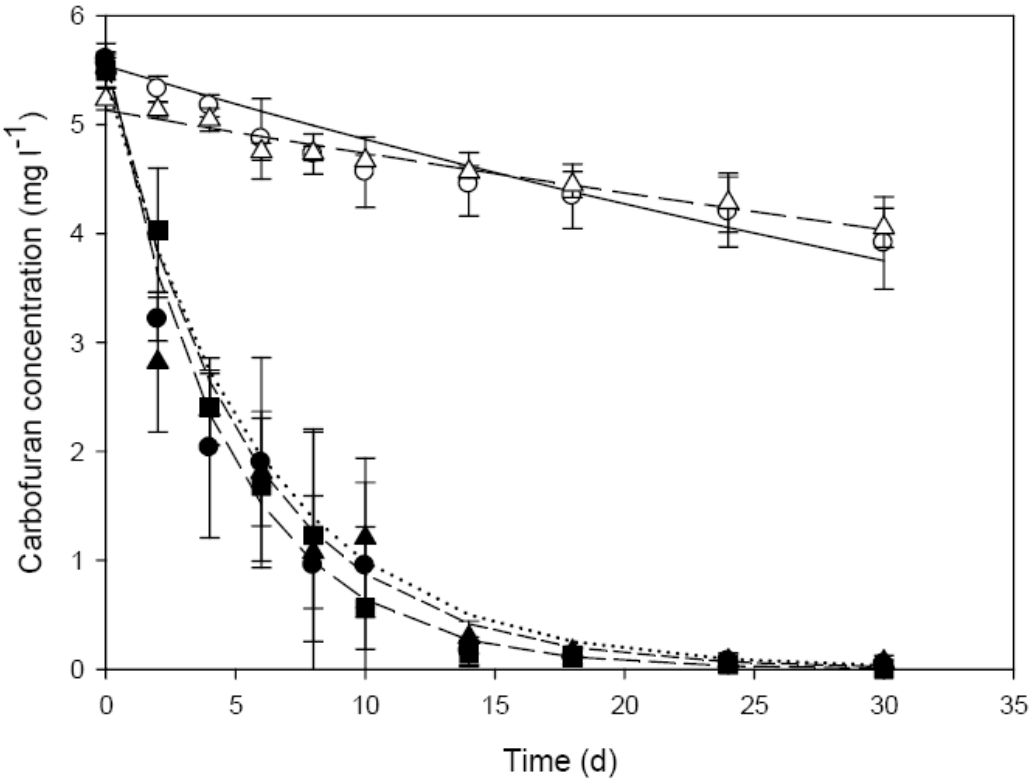

Figure 4-2. Dissipation of carbofuran in BSM inoculated with *B. cepacia* PCL3 (■ = free cell, ● = immobilized cell on corncob, ▲= immobilized cell on sugarcane bagasse) and autoclaved support materials without PCL3 (○= corncob, △ = sugarcane bagasse). Lines indicated the carbofuran degradation fitting to the first order kinetic model).

Table 4-2 Degradation rate coefficients (k_1) and half-lives ($t_{1/2}$) of carbofuran in BSM.

Treatment	k_1 (/d)	$t_{1/2}$ (d) [(1)]	r^2 [(2)]
BSM + Autoclaved corncob	0.013 [a] ±0.004	55[a] ±15.3	0.88
BSM + Autoclaved sugarcane bagasse	0.028 [a] ± 0.002	87[a] ±17.4	0.90
BSM + Free cells	0.230 [b] ± 0.056	3[b] ±0.8	0.91
BSM + Immobilized PCL3 on corncob	0.280 [b] ± 0.093	3[b] ±1.2	0.90
BSM + Immobilized PCL3 on sugarcane bagasse	0.200 [b] ± 0.051	4[b] ±1.0	0.97

(1) Comparison between treatment in column are significantly different (Duncan, $p \leq 0.05$) if mark different small letters

(2) Coefficients of determination for non-linear regressions

Growth and survival of immobilized cells were determined by the numbers of carbofuran degraders in the support materials. Cell numbers on the immobilized matrices were stable in the range of 10^7-10^8 cfu g^{-1} dry support material throughout 30 d of incubation (Figure 4-3) while free cells of *B. cepacia* PCL3 were found to increase from 10^6 to 10^8 cfu ml^{-1} at day 8 and then decreased continuously over time (Figure 4-3). These findings implied that the survival and stability of *B. cepacia* PCL3 could be improved through immobilization on corncob and sugarcane bagasse. Corncob and bagasse might act as protective agents against limiting condition such as substrate and product inhibition during incubation or shear forces (Bekatorou et al., 2004). In addition, these data suggested that the growth of immobilized *B. cepacia* PCL3 was limited by the supporting materials. Space in the immobilization matrix could limit cell density (Muyima and Cloete, 1995).

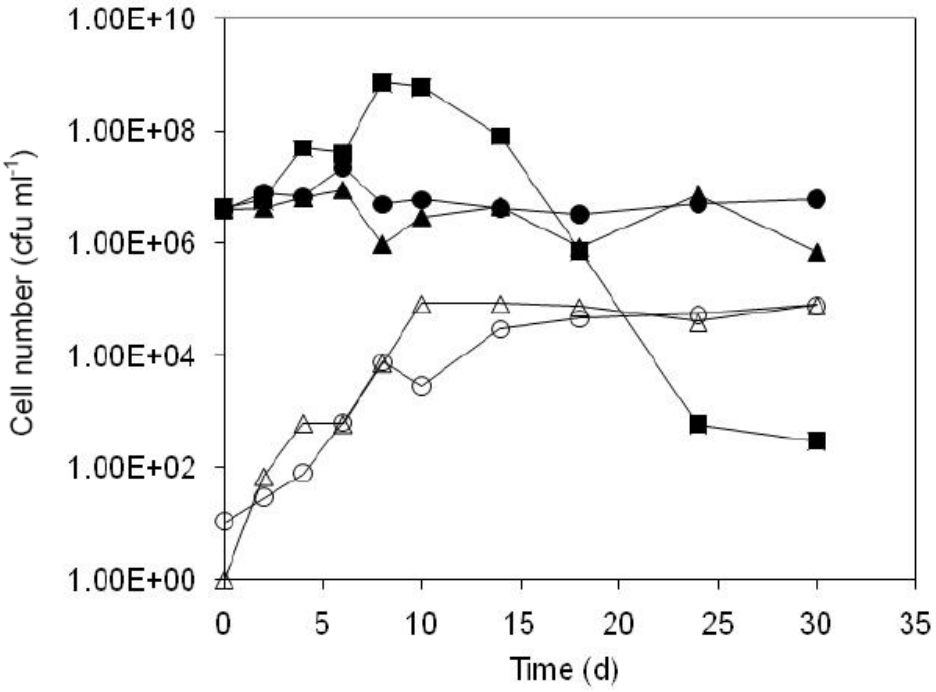

Figure 4-3. Cell numbers of *B. cepacia* PCL3 (■ = free cells, ● = immobilized cells on corncob, ▲ = immobilized cells on sugarcane bagasse) and cell leaking from ○ = corncob, △ = sugarcane bagasse.

Leakage of cells from the support materials was represented by a cell concentration observed in culture media during incubation. At day 0, number of the PCL3 cells in the liquid phase in BSM was negligible. After day 10, number of the

PCL3 in the liquid phase of the BSM was 10^5 cfu ml^{-1} (Figure 4-2) (10% of the number of cells immobilized in the support materials), whilst the number of *B. cepacia* PCL3 in the support materials was not reduced (Figure 4-3). These results indicated that the cells were leaking out from the porous media of corncob and bagasse due to a limited space and they continued to grow in the medium causing the increase in cell concentration. The profile of cell leakage was similar to that of the growth curve of *B. cepacia* PCL3 in BSM (Figure 4-3) indicating viability of the leakage cells which could increase the cell number in culture media. Cell detachment might be resulted from the fact that there are no barriers between the cells and the media which led to a possibility of cell detachment and relocation with potential establishment of cell-equilibrium inside the supports and culture media (Bekatorou et al., 2004). In the adsorption process, cells were immobilized on a supporting material by physical adsorption due to electrostatic forces or by covalent binding between the cell membrane and the supporting materials (Bekatorou et al., 2004). Previous research by Kumar and Das (2001) reported a similar finding in which the daughter cells produced from binary fission of the immobilized cells of *Enterobactor cloacae* IIT-BT 08 leaked to the culture media when there was no free space on the porous support materials.

The major metabolite from biodegradation in BSM was carbofuran phenol (Table 4-3) suggesting that *B. cepacia* PCL3 may release hydrolase enzyme for degrading carbofuran (Eisler, 1985; Head et al., 1992). Therefore, the investigation on the presence of methyl carbamate degrading gene (*mcd* gene) which encodes hydrolase enzyme in the PCL3 was conducted. The PCR product of 590 bp was obtained (Figure 4-4) thus assured that PCL3 produces hydrolase enzyme. Not only carbofuran phenol but also 3-keto carbofuran were the metabolites detected in the study. 3-keto carbofuran was the metabolite that was detected in all treatments including in the abiotic controls (Table 4-3). This metabolite is normally detected when the oxidation of carbofuran is taken placed.

Loss of carbofuran in the abiotic control might be resulted from the oxidation, the volatilization process occurred during the incubation and a continuous shaking, as well as the sorption of carbofuran to the support materials. Although the oxidation and volatilization are not as important as microbial degradation to carbofuran dissipation, they were contributing to dissipation processes which could be found in abiotic control as reported in previous studies (Lalah et al., 1996).

Table 4-3 Carbofuran metabolites found in BSM augmended with free cell of PCL3 (FC), the immobilized PCL3 on corncob (IMC-CC) and sugarcane bagasse (IMC-SCB), autoclaved corncob (AC) and autoclaved sugarcane bagasse (ASCB).

Time	Carbofuran phenol conc. (mg l^{-1})					3-Keto carbofuran conc. (mg l^{-1})				
(d)	AC	ASCB	FC	IMC-CC	IMC-SCB	AC	ASCB	FC	IMC-CC	IMC-SCB
0	nd	nd	nd	nd	nd	nd	nd	nd	0.06	nd
2	nd	nd	0.49	0.60	0.64	0.17	0.21	0.50	0.25	0.51
4	nd	nd	0.95	1.20	1.73	0.20	0.18	0.58	0.33	0.47
6	nd	nd	0.10	1.88	1.52	0.22	0.22	0.44	0.44	0.43
8	nd	nd	1.67	0.75	1.56	0.17	0.25	0.43	0.21	0.34
10	nd	nd	2.22	1.40	2.01	0.19	0.27	0.20	0.22	0.27
14	nd	nd	0.33	nd	1.37	0.19	0.18	0.45	0.19	0.12
18	nd	nd	1.20	1.29	0.61	0.19	0.10	0.37	0.10	0.32
24	nd	nd	0.51	0.56	0.70	0.10	0.28	0.17	0.17	0.12
30	nd	nd	0.13	nd	nd	0.17	0.25	0.25	0.18	0.28

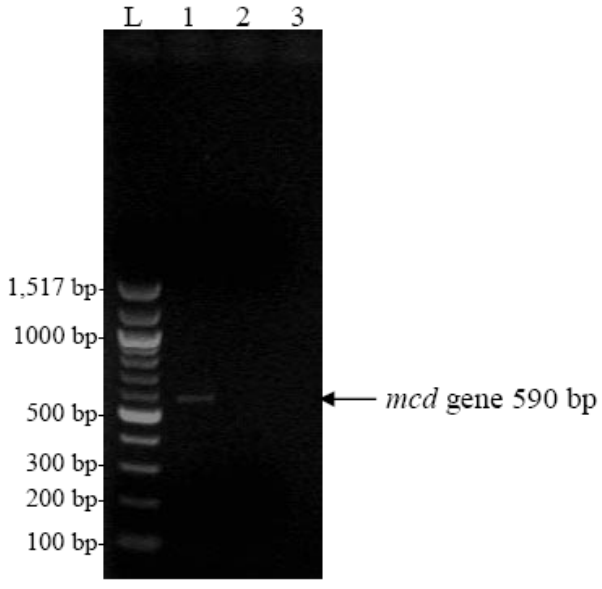

Figure 4-4. Agarose gel showing PCR products obtained from cells of *B. cepacia* PCL3 using primer specific for the *mcd* gene. L is molecular weight ladder (100 bp, New England BioLab Inc.). Lanes 1-3 are PCR products from PCL3, *E. coli* DH5α and deionized water, respectively.

Percentage of carbofuran dissipated in the abiotic controls i.e., BSM with autoclaved corncob and sugarcane bagasse were 12.7% and 26.7%, respectively, which might be caused by the sorption of carbofuran onto the surface and porous of the support materials during the incubation. Sorption coefficients (K_f) of carbofuran to corncob and sugarcane bagasse were found to be 0.03 and 0.30 mg kg^{-1}, respectively. Low K_f values may be resulted from a delignification treatment of corncob and sugarcane bagasse by NaOH before used in the sorption experiment. K_f values suggested that sugarcane bagasse could be a better sorbent than corncob and facilitate the dissipation of carbofuran.

4.3.3 Reusability of immobilized cells

This experiment examined a possibility of reusing the immobilized cells to degrade carbofuran in BSM. Degradation of carbofuran in BSM by reused immobilized PCL3 was depicted in Figure 4-5. Results demonstrated that the immobilized cells could be

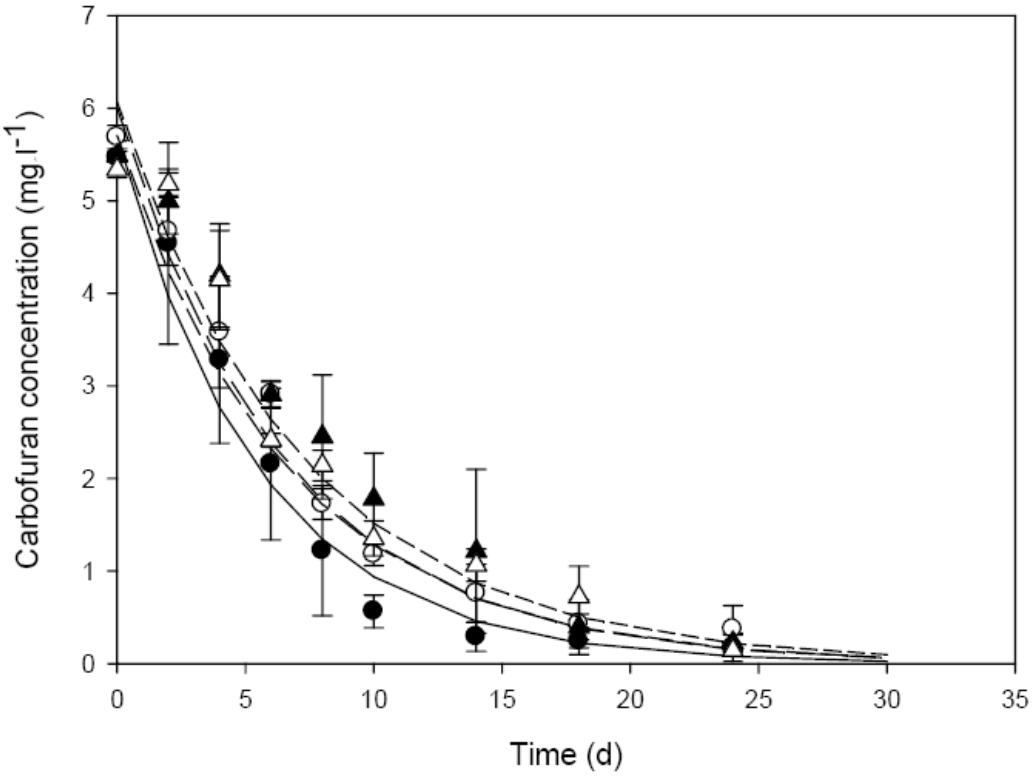

Figure 4-5. Degradation of carbofuran in BSM by reused immobilized *B. cepacia* PCL3 on corncob (● = first reuse, ○ = second reuse) and on sugarcane bagasse (▲ = first reuse, △ = second reuse). Lines indicated the carbofuran degradation fitting to the first order kinetic model.

reused twice while retaining carbofuran degradation ability ($t_{1/2}$ of 4-5 d in BSM) (Table 4-4) indicating an advantage of using immobilized cells over free cells. However, decrease in cell survival and ability of supporting materials to retain cells in their structures were observed as the number of reuse steps in BSM increased (Figure 4-6). These may due to a reduction in particle size from 0.7x0.7x0.7 cm to 0.4x0.4x0.4 cm of both support materials in BSM caused by soften structures after delignification. In consequence, an increase in cell leakage from supporting materials occurred. The decay of corncob and sugarcane bagasse implied that the immobilization of cells on these support materials could be environmental friendly causing less disposal problems than would occur for synthetic materials.

Table 4-4 Degradation rate coefficients (k_1) and half-lives ($t_{1/2}$) of carbofuran in BSM

Immobilized *B. cepacia* PCL3	k_1 (d^{-1})	$t_{1/2}$ (d) [(1)]	R^2 [(2)]
On corncob			
First reuse	0.18 [a] ± 0.064	4[a]±1.2	0.94
Second reuse	0.15 [a]±0.030	5[a]±1.1	0.95
On sugarcane bagasse			
First reuse	0.15 [a]±0.036	5[a]±1.2	0.96
Second reuse	0.14 [a]±0.003	5[a]±0.1	0.99

[(1)] Comparison between treatment in column are significantly different (Duncan, $p \leq 0.05$) if mark different small letters

[(2)] Coefficients of determination for non-linear regressions

3.3.4 Bioaugmentation of carbofuran residues in soil with immobilized *B. cepacia* PCL3

This experiment investigated an ability of the immobilized *B. cepacia* PCL3 on corncob and sugarcane bagasse to remediate carbofuran residues in soil in comparison to free cells and indigenous microorganisms. Dissipation of carbofuran in soil by the immobilized PCL3 was shown in Figure 4-7. The corresponding kinetic data fitting to a modified first-order kinetic model was tabulated in Table 4-5. Results revealed that the degradation of carbofuran in soil augmented with *B. cepacia* PCL3 in both free and immobilized cells forms on corncob and on sugarcane bagasse (treatment B, C, D; $t_{1/2}$ of 13-17 d) was more rapid than in soil without augmentation

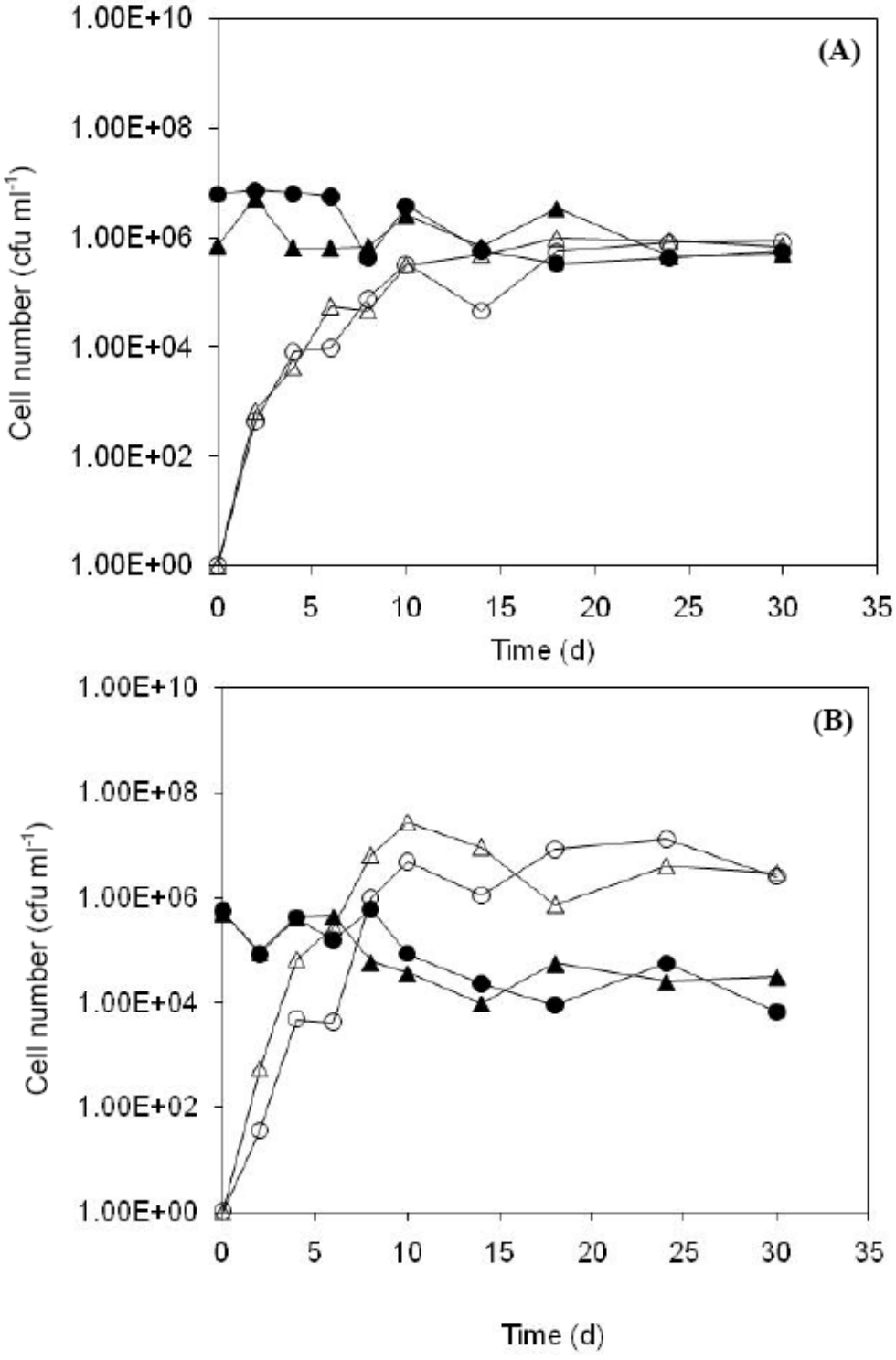

Figure 4-6. Cell numbers of immobilized *B. cepacia* PCL3;● = on corncob,▲ = on sugarcane bagasse, leaking cells; ○ = from corncob, △ = from sugarcane bagasse: (A) = first reuse and (B) = second reuse.

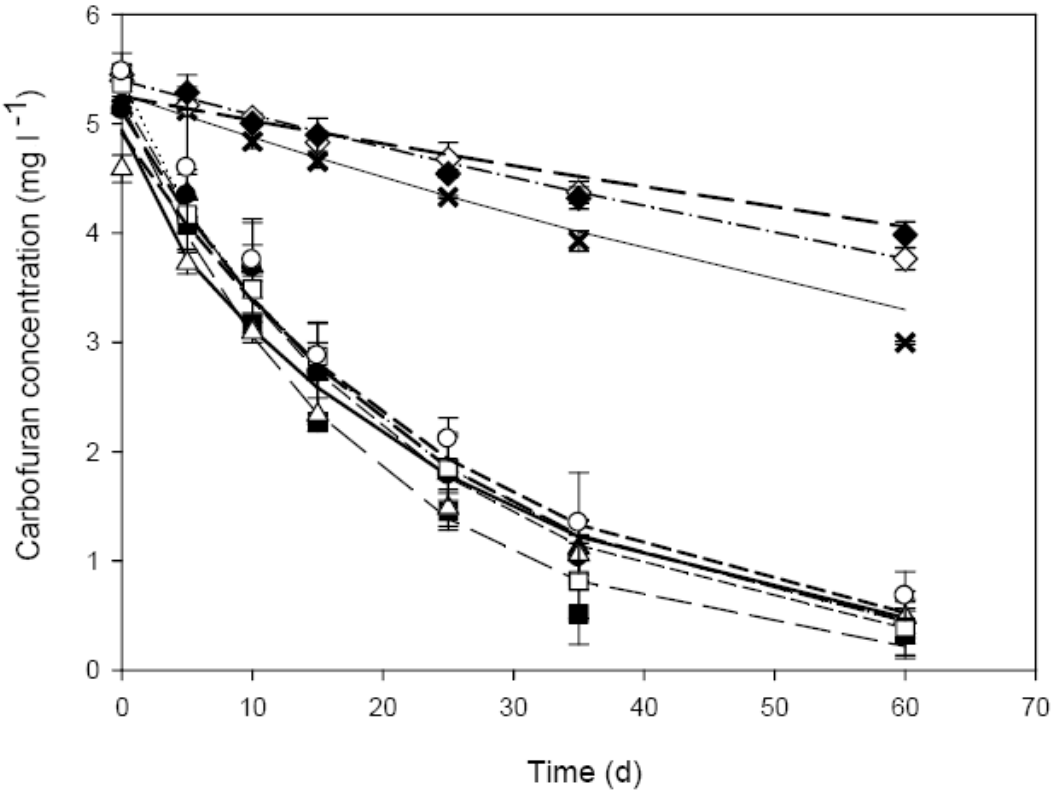

Figure 4-7. Degradation of carbofuran residues in soil (closed symbols) and in autoclaved soil (open symbols) (■, □ = free cell of PCL3; ●, ○ = immobilized PCL3 on corncob; ▲, △ = immobilized PCL3 on sugarcane bagasse; ✖ = indigenous microorganisms; ◆ = autoclaved corncob without PCL3; ◇ = autoclaved sugarcane bagasse without PCL3). Lines indicated the carbofuran degradation fitting to the first-order kinetic model.

(treatment A; $t_{1/2}$ of 74 d) (Table 4-5) which again demonstrated the ability of *B. cepacia* PCL3 to degrade carbofuran in soil as well as the potential for its use in bioaugmentation for bioremediation of carbofuran residues in soil.

The carbofuran degradation ability of PCL3 was evident in the augmented autoclaved soil (treatment E, F, G) indicated by a much shorter half-lives of carbofuran (15-19 d) than the abiotic control (treatment H, I; 114-119 d) (Table 4-5). Other evidence to support this finding is that the half- lives of carbofuran in the augmented soil (13-17 d) (treatment B, C, D) (Table 4-5) were not significantly different ($p \leq 0.05$) from degradation in augmented autoclaved soil (15-19 d) (treatment E, F, G) (Table 4-5).

Table 4-5 Degradation rate coefficients (k_1) and half-lives ($t_{1/2}$) of carbofuran in soil

Treatment	k_1 (d^{-1})	$t_{1/2}$ (d) [(1)]	r^2 [(2)]
Soil	0.0094 [a] ±0.0003	74[a] ±2.0	0.88
Soil + free cells of PCL3	0.0523 [b]±0.0163	13[b]±4.4	0.99
Soil + immobilized PCL3 on corncob	0.0435 [b]±0.0068	16[b]±2.6	0.97
Soil + immobilized PCL3 on sugarcane bagasse	0.0401 [b]±0.0097	17[b]±1.7	0.94
Autoclaved soil + free cells of PCL3	0.0462 [b]±0.0107	15[b]±3.5	0.91
Autoclaved soil + immobilized PCL3 on corncob	0.0369 [b]±0.0052	19[b]±2.5	0.99
Autoclaved soil + immobilized PCL3 on sugarcane bagasse	0.0416 [b]±0.0039	17[b]±3.5	0.93
Autoclaved soil + autoclaved corncob	0.0058 [c]±0.0003	119[c]±6.2	0.88
Autoclaved soil + autoclaved sugarcane bagasse	0.0061 [c]±0.0003	114[c]±5.1	0.89

[(1)] Comparison between treatment in column are significantly different (Duncan, $p \leq 0.05$) if mark different small letters

[(2)] Coefficients of determination for non-linear regressions

Carbofuran degradation by the immobilized cells on corncob (treatment C, F) and sugarcane bagasse (treatment D, G) ($t_{1/2}$ of 16-19 d) and free cell forms (treatment B, E) (13-15 d) of *B. cepacia* PCL3 were not significantly different ($p \leq 0.05$) (Table 4-5) suggesting that the immobilization did not worsen or improve carbofuran degradation activity of *B. cepacia* PCL3.

Carbofuran phenol was the only metabolite found in soil microcosms study with a biological activity (Table 4-6). The accumulation of carbofuran phenol at day 60 in the treatments with the PCL3 together with the indigenous microorganisms (treatments B, C and D) was lower than the treatments with the PCL3 or the indigenous microorganisms alone (treatments A, E, F and G). Results suggested that the PCL3 and the indigenous microorganisms might work closely to degrade carbofuran phenol which could be accounted as another advantage of the bioaugmentation.

Growth, survival and leakage of the immobilized cells at day 60 of incubation were investigated in order to evaluate the stability of immobilized cells when they were inoculated into soil. Results revealed that cell numbers of *B. cepacia* PCL3 on the immobilized matrices increased from 10^7 cfu g^{-1} dry support material to

Table 4-6 Carbofuran phenol detected in soil microcosms treatments

Time	Treatment								
(d)	A	B	C	D	E	F	G	H	I
0	nd	nd	nd	nd	nd	nd	nd	nd	nd
5	0.16	0.30	0.22	0.28	0.24	0.29	0.23	nd	nd
10	0.54	2.49	2.31	2.03	2.30	1.96	2.05	nd	nd
15	0.37	2.65	2.38	2.04	2.10	2.59	2.35	nd	nd
25	0.82	3.05	2.17	2.79	2.42	1.23	2.47	nd	nd
35	1.27	2.36	1.71	1.99	2.15	2.05	2.36	nd	nd
60	1.05	0.26	0.12	0.21	1.54	1.17	1.02	nd	nd

10^8 cfu g^{-1} dry support materials at day 60 of incubation (Figure 4-8). In comparison, number of inoculated free cells of *B. cepacia* PCL3 was found to decrease in soil from 10^6 cfu g^{-1} dry soil to 10^5 cfu g^{-1} dry soil at day 60 (Figure 4-8). These findings indicated that immobilization technique via absorption on corncob and sugarcane bagasse could improve the survival of *B. cepacia* PCL3 when it was inoculated to the soil.

Leakage of cells from support materials was represented by a cell number observed in soil inoculated with the immobilized cells (Figure 4-8). Results were similar to those obtained in the BSM experiment.

Since corncob and sugarcane bagasse are organic materials. They were degraded after 60 days of incubation in soil, therefore preventing us from reusing the immobilized PCL3 cells in soil treatment. Investigation into supporting materials with stronger structure after delignification in provision of a possibility to reuse the immobilized PCL3 cells to remediate carbofuran contaminated soils is being conducted.

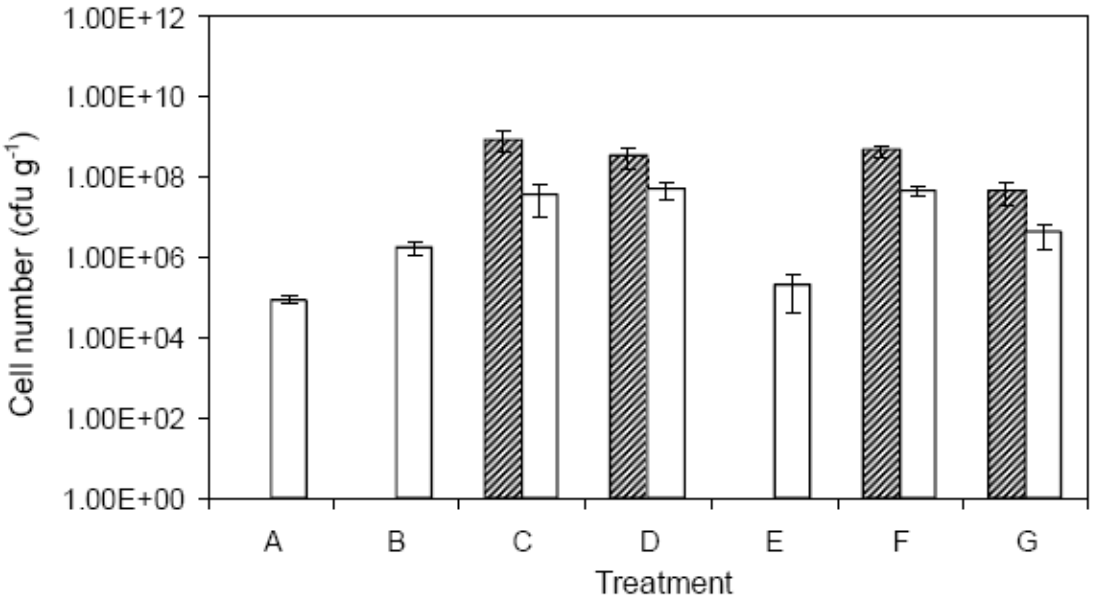

Figure 4-8. Total numbers of carbofuran degraders; ▨ = in support materials (cfu g^{-1} support), ☐ = in soil (cfu g^{-1} soil) at day 60 of incubation. Treatment A= soil; B= Soil + free cells of PCL3; C= Soil + immobilized PCL3 on corncob; D = Soil + immobilized PCL3 on sugarcane bagasse; E = Autoclaved soil + free cells of PCL3; F = Autoclaved soil + immobilized PCL3 on corncob; G = Autoclaved soil + immobilized PCL3 on sugarcane bagasse.

CHAPTER V

BIOAUGMENTATION OF CARBOFURAN BY *Burkholderia cepacia* PCL3 IN A BIOSLURRY PHASE SEQUENCING BATCH REACTOR

5.1 Introduction

Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate) is a broad-spectrum insecticide widely used in agriculture to control insects and nematodes on contact or after ingestion. Carbofuran is of environmental concern because it is soluble in water and highly mobile in soil, resulting in a high potential for groundwater contamination (EPA, 2006). In 2003, Thailand imported carbofuran in solid and liquid forms up to 826.6 and 45.5 m, respectively, for using in agriculture especially in rice fields (Department of Agriculture, 2003). Continuous use of carbofuran in the rice fields may subsequently exacerbate the risk of contamination of the soil and groundwater; thus, the removal of carbofuran is necessary.

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 (Yan et al., 2007; Bano and Musrrat, 2004). 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, the so-called bioaugmentation technique, is reported to be an effective bioremediation approach for improving pesticide degradation in contaminated soils and water that lack indigenous microbial activity (Dams et al., 2007). In addition to bioaugmentation, biostimulation (organic and/or inorganic additives that stimulate the activity of indigenous microorganisms) has also been reported to enhance the degradation rate of the contaminants of interest (Robles-Gonzalez et al., 2008).

Bioaugmentation and biostimulation can both be applied *in situ* by directly adding degraders and/or additives to the contaminated areas and *ex situ* through biodegradation in a bioreactor. Though *in situ* treatment has the advantages of simplicity and cost-effectiveness, it requires a long time period to complete the

degradation and can be restrained by some limiting conditions such as low permeability and heterogeneity of the contaminated matrices (Prasanna et al., 2008). Therefore, bioreactor technology which can be specially designed in a variety of configurations to maximize microbial activity has drawn our attention as a way to bioremediate carbofuran.

Soil-slurry phase reactors comprised of 10-40% soil in liquid (water) are a relatively new application for soil and sediment bioremediation. It is an alternative technology for decontamination of soil and sediment which minimizes the limiting effect of mass transfer on biodegradation (Robles-Gonzalez et al., 2008; Venkata-Mohan et al., 2004). Contaminant in a soil-slurry treatment system can partition itself to the soil and liquid phases and can be utilized by both indigenous microorganisms in the soil and inoculated specific degraders. Therefore, the degradation process can take place in the soil, water, and/or soil-water phases, resulting in an enhancement of contaminant degradation (Venkata-Mohan et al., 2008). Published data have indicated successful bioremediation of hazardous substances, especially pesticides such as pendimethalin (Ramakrishna et al., 2008) and hexachlorocyclohexane isomers (Quintero et al., 2005; 2006), using a soil-slurry phase reactor. In addition, it has been reported that the efficiency of remediation of contaminated soil could be effectively improved by using a bioslurry reactor augmented with specific microorganisms capable of degrading the pesticide of interest. However, to the best of our knowledge, there has been no report on carbofuran remediation in a bioaugmented soil-slurry phase reactor.

In the present study, the performance of a soil slurry phase reactor with a sequencing operation system in removing carbofuran from soil was investigated. The bioaugmentation and biostimulation treatments were applied to the soil slurry phase bioreactor to enhance the carbofuran degradation efficiency in soil. The kinetic aspects of carbofuran degradation in the soil slurry system were further studied to select the most effective strategy for remediating carbofuran contaminated soil.

5.2 Materials and Methods

5.2.1 Immobilization of *B. cepacia* PCL3

5.2.1.1 Microorganism preparation

The carbofuran degrader, identified by 16s rRNA as *B. cepacia* PCL3 (GenBank accession number of EF990634), was used in this experiment. This microorganism is capable of using carbofuran as a sole C-source (Plangklang and Reungsang, 2008). It was grown in 100 ml nutrient broth (NB) containing 5 mg l^{-1} carbofuran at 30°C and 150 rpm for 36 h and was used as seed inoculum for immobilization.

5.2.1.2 Supporting material preparation

Corncob was used as a support material to immobilize *B. cepacia* PCL3. This material has high matrix porosity and a pore size that could enhance the cell adsorption capability during immobilization. Corncob was cut into 0.7x0.7x0.7 cm pieces using a knife and then 300 g of cut corncob was boiled in 3 l of 1% NaOH for 3 h to remove lignin and fibers inside the materials which might react with the cells (Bradi 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.

5.2.1.3 Cell immobilization

Adsorption was used as the immobilization method in this study. This method is typically performed when porous media are used as support materials with the advantage of ease of operation. The immobilization technique was conducted by adding 75 g of sterile corncob to 250 ml of sterile NB containing 5 mg l^{-1} carbofuran before inoculating with PCL3 (10^{6} cfu ml^{-1}). The flask was then incubated at 150 rpm, at room temperature, for 48 h. After incubation, the support material was transferred to fresh NB containing 5 mg l^{-1} of carbofuran and incubated, as previously described, before harvesting by filtration through a Buchner filter funnel and washing with 0.85% NaCl by an aseptic technique. This process was repeated two times. Immobilized cells were kept at 4^{o}C until use in further experiments. The internal cell density on the corncob after immobilization was approximately 10^{7} cfu g^{-1} dry material.

5.2.2 Soil

A sandy loam soil sample, 0-15 cm in depth, was collected from the rice fields of Ban Nonmuang, A. Muang, Khon Kaen Province. Organic carbon and nitrogen content of the soil were 0.89% and 0.10%, respectively, and the soil pH was 6.9. The soil was passed through a 2 mm sieve and stored in a plastic bag at 4°C. Background carbofuran in the soil sample detected by HPLC was 0.06 mg kg^{-1}.

5.2.3 Soil slurry preparation

Air-dried soil was spiked with carbofuran at a concentration of 20 mg/kg dry soil, well mixed by hand stirring, and kept at 4^{o}C for 24 h to induce the homogenous sorption of carbofuran over the soil particles. For soil slurry preparation, the impregnated soil was added to distilled water at a ratio of 1 g soil:20 ml distilled water before feeding to the reactor.

5.2.4 Soil slurry phase reactor configuration

A 2-L blue bottle was used as a reactor in this study with a working volume of 1.5 l and a suitable inlet and outlet arrangement as shown in Figure 5-1. Oxygen was supplied from an air compressor through an air diffuser at the flow rate of 600 ml min^{-1} at the bottom of the bottle. The contents of the reactor were continuously mixed by a magnetic stirrer.

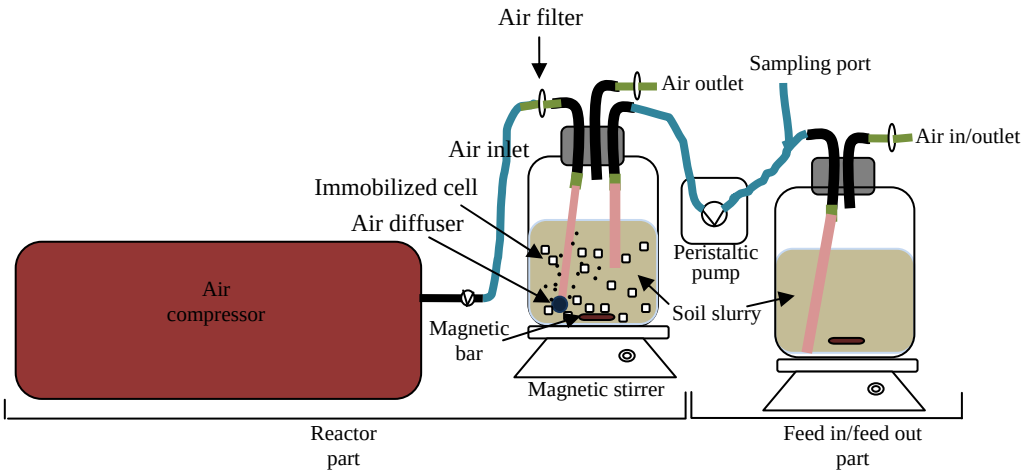

Figure 5-1. Schematic diagram of soil slurry phase reactor (not subject to scale)

5.2.5 Soil slurry phase reactor operation

In this experiment, eight soil slurry phase reactors (A-H) (Table 5-1) were operated with an initial carbofuran concentration of 20 mg kg^{-1} dry soil. The soil slurry was prepared as described above and fed to the reactor at a flow rate of 25 ml min^{-1} using a peristaltic pump. For the bioaugmented reactors (A, C, and H), 100 g wet corncob immobilized with *B. cepacia* PCL3 (10^7 cfu g $corncob^{-1}$) were added to the reactor before feeding in the soil slurry. For the biostimulated reactors (G and H), molasses (1,500 mg COD l^{-1}) was mixed with the soil slurry before feeding to the reactor. Each reactor was aerobically operated in sequencing batch mode with a total cycle period of 84 h (HRT). The cycle period consisted of 1 h of fill phase, 82 h of react phase, and 1 h of decant phase. Aerobic conditions in the reactor were maintained by pumping air through an oxygen diffuser at an air flow rate of 600 ml min^{-1}. During the react phase, the reactor was continuously stirred using a magnetic

Table 5-1 Soil slurry phase reactor treatment

Reactor	Experimental set up	Purpose
A	Soil slurry + immobilized PCL3	- To investigate carbofuran degradation in soil slurry in the presence of both immobilized PCL3 and indigenous microorganisms
B	Soil slurry	- To investigate carbofuran degradation in soil slurry in the presence of only indigenous microorganisms
C	Autoclaved soil slurry + immobilized PCL3	- To investigate carbofuran degradation in soil slurry in the presence of only immobilized PCL3
D	Soil slurry + autoclaved corncob	- To investigate the effect of corncob as a C-source for indigenous microorganisms and the biostimulation effect on carbofuran removal
E	Autoclaved soil slurry + autoclaved corncob	- Abiotic control to determine the effect of corncob on dissipation of carbofuran in soil slurry
F	Autoclaved soil slurry	- Abiotic control
G	Soil slurry + molasses (1500 mg COD l^{-1})	- To investigate the biostimulation effect of molasses on carbofuran degradation ability of indigenous microorganisms in soil slurry
H	Soil slurry + immobilized PCL3 + molasses (1500 mg COD l^{-1})	- To investigate the effects of bioaugmentation and biostimulation techniques on carbofuran degradation

stirrer to homogenize the contents and maintain air diffusion in the reactor. The soil slurry was sampled every 6 h via the sampling port using a peristaltic pump. The concentrations of carbofuran and its metabolites, i.e. carbofuran phenol and 3-keto carbofuran, in both the soil and liquid phases were determined by extraction using a liquid-liquid partitioning method followed by HPLC. The pH values of the soil slurry were measured using a digital pH meter (Sartorius, Germany). The number of carbofuran degraders in the soil slurry was enumerated by a drop-plate technique.

5.2.6 Analysis method

5.2.6.1 Extraction of carbofuran in soil slurry

In order to extract carbofuran and its metabolites from the soil and liquid phases, the soil slurry was centrifuged at 6 000 rpm and 25^{o}C for 15 min to separate the liquid from the soil. The liquid phase was filtered through cellulose acetate paper number 1 (Whatman, England) prior to the liquid-liquid partitioning extraction procedure (Plangklang, 2004). Briefly, 2 ml of methanol were added to 2 ml of liquid sample and then the mixture was sonicated twice for 10 min on a 50/60 voltage cycle. After sonication, carbofuran and its metabolites were extracted in a separation funnel with dichloromethane. This extraction was performed 3 times with 4, 2, and 2 ml of dichloromethane. The organic fractions from the extractions were collected, pooled, and evaporated in the fume hood, then re-dissolved in 4 ml of 60% methanol and passed through a 0.45 μm nylon membrane syringe filter before analysis by HPLC.

The soil phase was air dried at room temperature (30±2^{o}C) and weighed in an HDPE tube, and 60% methanol was added at a ratio of 1:200 (w/v) soil:methanol. The tube was horizontally shaken at 200 rpm for 30 min and further centrifuged at 6 000 rpm and 10^{o}C for 10 min to separate the supernatant. This step was repeated twice and the supernatants were pooled and filtered through cellulose acetate paper number 1 prior to the liquid-liquid partitioning extraction procedure described above. The final volume of the extract was adjusted to 0.4 ml and then filtered through a nylon membrane filter disc before analysis by HPLC. Percent recovery of this procedure was 94.4%.

5.2.6.2 HPLC analysis of carbofuran and its metabolites concentrations

The extracts were analyzed using a Shimadzu 10-A HPLC equipped with a 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 (60:40); flow rate, 1 ml min^{-1}; ambient temperature. External standard linear calibration curves for carbofuran, carbofuran phenol, and 3-keto carbofuran were used to quantify their concentrations in the aqueous phase. The observed concentrations were characterized by peak areas.

5.2.6.3 Soil slurry phase reactor performance

The performance of the soil slurry phase bioreactor for remediation of carbofuran was evaluated by determining the carbofuran degradation efficiency (*E*) as given in equation (1):

$$E(\%) = \left[\frac{C_0 - C_t}{C_0} \right] \times 100 \quad (1)$$

where C_o is the initial carbofuran concentration (g) in the soil phase plus the liquid phase and C_t is the carbofuran concentration (g) in the soil slurry, the soil phase plus the liquid phase, at the end of reactor operation (84 h).

5.2.6.4 Kinetic analysis of carbofuran degradation in soil phase

The experimental data obtained from soil slurry phase reactor operation were studied by fitting to zero-, first-, and second-order kinetic equations as described in equations (2), (3), and (4), respectively, in order to understand the kinetic aspect of carbofuran degradation:

$$C = C_0 - k_0 t \quad (2)$$

$$C_t = C_0 e^{-k_1 t} \quad (3)$$

$$1/C_t = 1/C_0 + k_2 t \quad (4)$$

where C is the mean concentration (mg) of carbofuran as a function of time (t) in hours, k_0 (mg h^{-1}), k_1 (h^{-1}), and k_2 (mg^{-1} d^{-1}) are the zero-, first-, and second-order degradation rate constants (d^{-1}), respectively.

The half-lives of carbofuran in the soil phase were calculated from linear regression analysis using the following equations:

$$t_{1/2},0^0 = \frac{C}{2k_0} \quad (5)$$

$$t_{1/2},1^0 = \frac{0.693}{k_1} \quad (6)$$

$$t_{1/2},2^0 = \frac{1}{k_2 C} \quad (7)$$

5.2.6.5 Enumeration of carbofuran degraders in soil slurry by the drop plate technique

The number of carbofuran degraders in the soil slurry was determined by the drop-plate technique. The serial-diluted aliquots of the soil slurry samples, 20 µL, were plated onto basal salt medium (BSM) agar coated with 5 mg l^{-1} carbofuran and incubated at 30°C until colonies appeared. BSM agar (Mo et al., 1997), pH 7, contains (in g l^{-1}): 5.57, $NaHPO_4$; 2.44, KH_2PO_4; 2.00, NH_4Cl; 0.20, $MgCl_2.6H_2O$; 0.0004, $MnCl_2.4H_2O$; 0.001, $FeCl_3.6H_2O$; 0.001, $CaCl_2$, and 1.5% agar was added to the medium before autoclaving at 121°C for 15 min. Carbofuran solution in sterile distilled water, as the sole C-source, was coated on the BSM agar at a concentration of 5 mg l^{-1} using a glass spreader prior to use.

5.3 Results and Discussion

5.3.1 Soil slurry phase reactor performance

5.3.1.1 Non-augmented and abiotic reactors

The degradation profiles and degradation efficiency of carbofuran in the soil slurry for the non-augmented reactors (B and D) and abiotic control reactors (E and F) as a function of operation time are depicted in Figures 5-2 and 5-3, respectively. Reactor B, which had only indigenous microorganisms in the soil, exhibited marked carbofuran degradation (Figure 5-2) with a relatively high efficiency of 67.69% (Figure 5-3). This indicated that there might be some carbofuran degraders presented as native microflora in the soil. The rice field where the soil samples were collected had a history of carbofuran application with a background

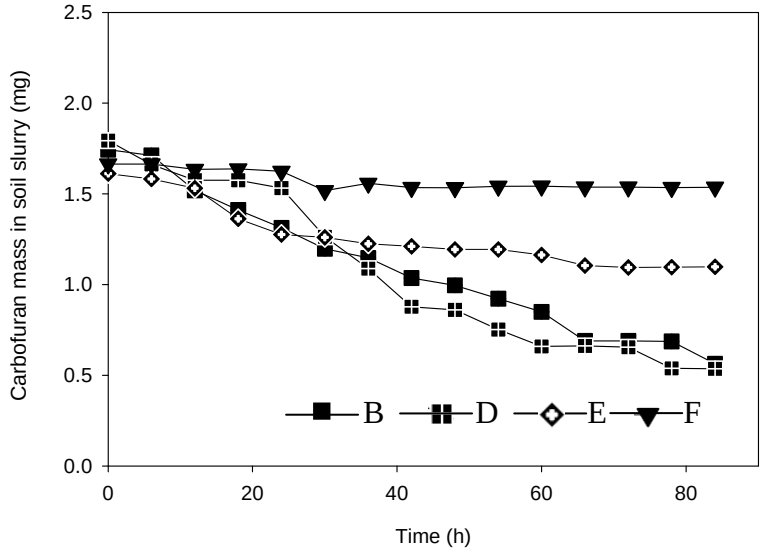

Figure 5-2. Degradation profiles of carbofuran in soil slurry phase reactors without bioremediation treatment (B: Soil slurry; D: Soil slurry + autoclaved corncob; E: Autoclaved soil slurry + autoclaved corncob; F: Autoclaved soil slurry)

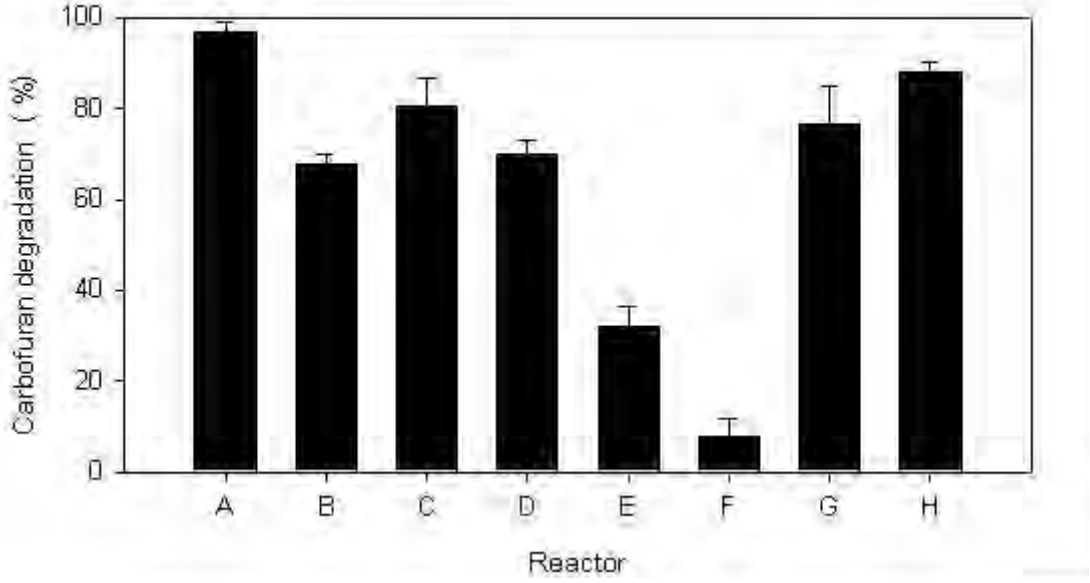

Figure 5-3. Performance of carbofuran in soil slurry phase reactors (A: Soil slurry + immobilized PCL3; B: Soil slurry; C: Autoclaved soil slurry + immobilized PCL3; D: Soil slurry + autoclaved corncob; E: Autoclaved soil slurry + autoclaved corncob; F: Autoclaved soil slurry; G: Soil slurry + molasses; H: Soil slurry + immobilized PCL3 + molasses)

concentration of 0.06 mg kg^{-1} carbofuran; therefore, microorganisms in the soils would have been able to adapt to use carbofuran as an energy source (Plangklang and Reungsang, 2008).

The addition of autoclaved corncob to the soil slurry (reactor D) increased the carbofuran degradation efficiency to 70.12% (Figure 5-3). In addition, the abiotic experiments (i.e. autoclaved soil slurry with corncob and no additional PCL3 treatment) demonstrated an adsorbance of 31.86% of carbofuran (Figure 5-3), which implied that corncob could act as a sorbent for the removal of carbofuran. Sorption coefficient (K_f) of carbofuran to corncob was found to be 0.03 mg kg^{-1} (Plangklang and Reungsang, 2008). Abiotic control (reactor F, autoclaved soil slurry) showed an insignificant carbofuran degradation profile (Figure 5-2). However, a relatively low carbofuran degradation efficiency of 7.70% was found (Figure 5-3). This might have resulted from oxidation and volatilization processes due to the continuous aeration during reactor operation. Although the oxidation and volatilization are not as important as microbial degradation to carbofuran dissipation, they were contributing to dissipation processes which could be found in abiotic control as reported in previous studies (Evert, 2002; Lalah et al., 1996).

5.3.1.2 Effect of bioremediation treatments

The effects of bioaugmentation, biostimulation, and bioaugmentation together with biostimulation treatments on carbofuran degradation in the soil slurry phase reactor were investigated in this experiment. The carbofuran degradation profiles in soil slurry phase reactors with bioremediation treatments as a function of operation time are depicted in Figure 5-4. A significant improvement in carbofuran degradation in the soil slurry could be found when these bioremediation techniques were compared to non-augmented experiments. The highest carbofuran degradation of 96.97% could be achieved with the bioaugmentation treatment (addition of immobilized PCL3, reactor A), followed by degradation of 88.23% by bioaugmentation together with biostimulation treatments (addition of PCL3 and molasses, reactor H) and degradation of 76.70% by biostimulation treatment (addition of molasses; reactor G) (Figure 5-3). Reduction in carbofuran degradation efficiency after the addition of molasses to the augmented reactor could be due to the preferential use of sugars in molasses than carbofuran since sugars have less complex structures and are easier to be metabolized by microorganisms (Liu et al., 2008). In

addition, there might be some adverse effects from substances contained in molasses, such as metal ions (Liu et al., 2008) or by-products of molasses metabolism, on the carbofuran degraders in the reactor.

The effectiveness of bioaugmentation treatment was evident in reactor C containing autoclaved soil slurry and immobilized PCL3 with a carbofuran degradation of 80.65% (Figure 5-3). These results confirmed that bioaugmentation was an effective technology to improve carbofuran degradation in contaminated matrices.

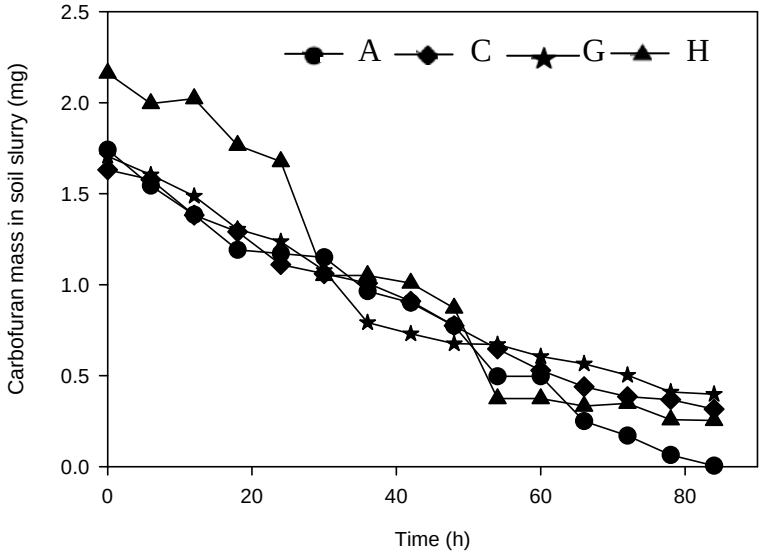

Figure 5-4. Degradation profiles of carbofuran in soil slurry phase reactors with bioremediation treatments (A: Soil slurry + immobilized PCL3; C: Autoclaved soil slurry + immobilized PCL3; G: Soil slurry + molasses; H: Soil slurry + immobilized PCL3 + molasses)

Our previous research (Plangklang and Reungsang, 2008) investigated carbofuran degradation in soil and rhizosphere soil microcosms in which soils were collected from the same rice field as presented in this study. The experiments were conducted using soil microcosms with an initial carbofuran concentration of 5 mg kg^{-1} dry soil. The bioaugmentation technique, free cell of PCL3 inoculation, was applied to both kinds of soil in order to improve the carbofuran degradation efficiency. When compared to carbofuran degradation in soil microcosms, the soil slurry phase bioreactor showed a better performance in carbofuran degradation. The shortest time to achieve 90% carbofuran degradation in the bioaugmented soil microcosms was 50

d (Plangklang and Reungsang, 2008), which was 14.3 times longer than using the soil slurry phase treatment (3.5 d) (Figure 5-4), even though the initial carbofuran concentration in the soil microcosms was 4 times lower than in the soil slurry phase reactor. These findings indicate that the bioslurry phase reactor could be applied for the purpose of significantly enhancing carbofuran degradation efficiency in the soil. The aeration together with mechanical mixing might responsible for this trend by improving the mass transfer rate and contact among microorganisms, carbofuran, and nutrients, and hence increasing the rates of carbofuran biodegradation. This finding was similar to the results from previous studies (Venkata-Mohan et al., 2006; 2004; Quintero et al., 2005; 2006).

Carbofuran phenol and 3-keto carbofuran were observed to be the metabolites in both soil and liquid phases in the reactors with biological activity (reactors A, B, C, D, G and H) (data not shown). In the soil phase, carbofuran phenol and 3-keto carbofuran could be observed in reactors A, B, C, D and H from 6 to 42 h while, in reactors D and G, these metabolites could be found until the end of reactor operation. Higher concentrations of carbofuran metabolites were found in the liquid phase than in the soil phase, and the accumulation of carbofuran phenol and 3-keto carbofuran in the liquid phase could be found in all bioreactors until the end of reactor operation except for reactors A and H. A decrease in carbofuran metabolites during reactor operation implied that the microorganisms in the soil slurry phase reactors might be able to metabolize carbofuran metabolites as their energy sources, as was reported in a study by Yan et al. (2007).

5.3.2 Substrate partitioning between soil and liquid phases

Carbofuran partitioning in the soil and liquid phases during soil slurry phase reactor operation was investigated (Figures 5-5 and 5-6). Substrate partitioning in the abiotic control reactor (reactor F) (Figure 5-5) indicated the actual mechanism of carbofuran partitioning in the two phases of the slurry system was insignificant. After start-up of the reactor, carbofuran rapidly desorbed from the soil to the aqueous phase (Figure 5-5). The partitioning of carbofuran approached steady state at 30 h of reactor operation with carbofuran concentrations in the soil and liquid phases of approximately 0.75 and 0.79 mg, respectively (Figure 5-5). Reactor E, autoclaved soil with autoclaved corncob added, showed the same trend of carbofuran partitioning as reactor F (Figure 5-5). The relatively high desorption of carbofuran from the soil into

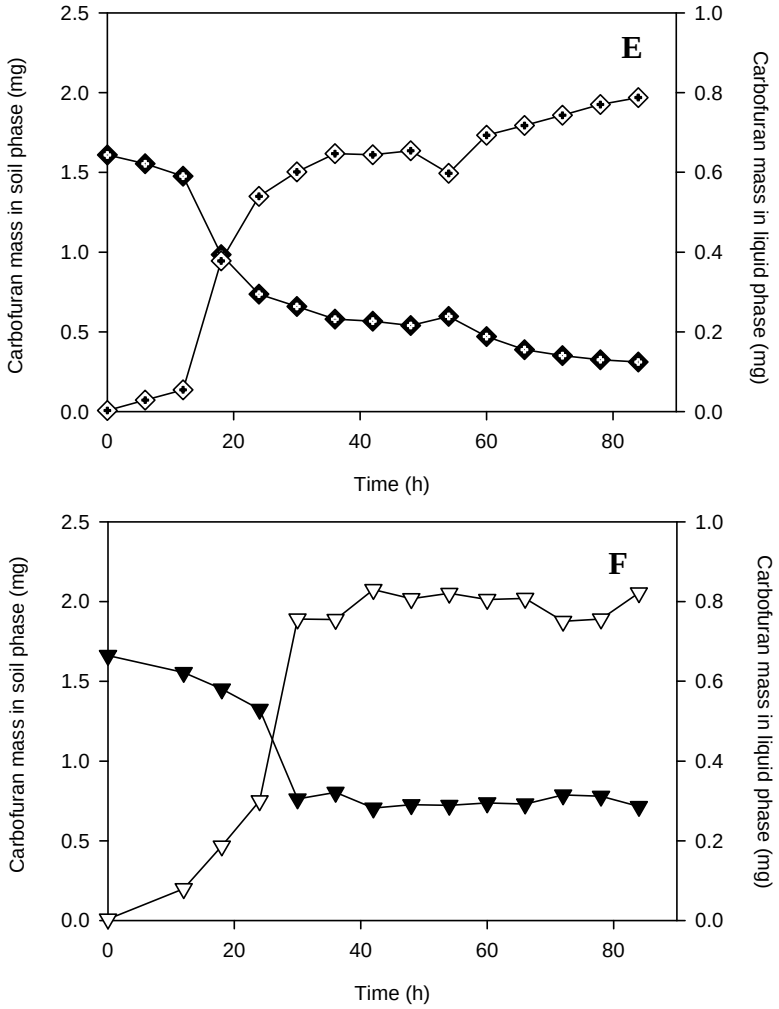

Figure 5-5. Carbofuran partitioning between soil (close symbols) and liquid (open symbols) phases in abiotic control soil slurry phase reactors (E: Autoclaved soil slurry + autoclaved corncob; F: Autoclaved soil slurry)

the liquid phase might be attributed to the hydroponic nature of carbofuran, i.e. its high water solubility of 351 mg l^{-1} at 25°C and low adsorption coefficient (Koc) of 22 (Evert, 2002). Desorption of carbofuran from the soil to the liquid phase could facilitate the mass transfer and increase contact between the microorganisms and carbofuran, which might result in an improvement in carbofuran degradation efficiency in the soil slurry phase reactor.

Carbofuran partitioning in the reactors with microbial activity (Figure 5-6), i.e. bioaugmentation (reactors A, C and H), biostimulation (reactors G and H), and indigenous microorganisms (reactors B and D) was obviously different from that in

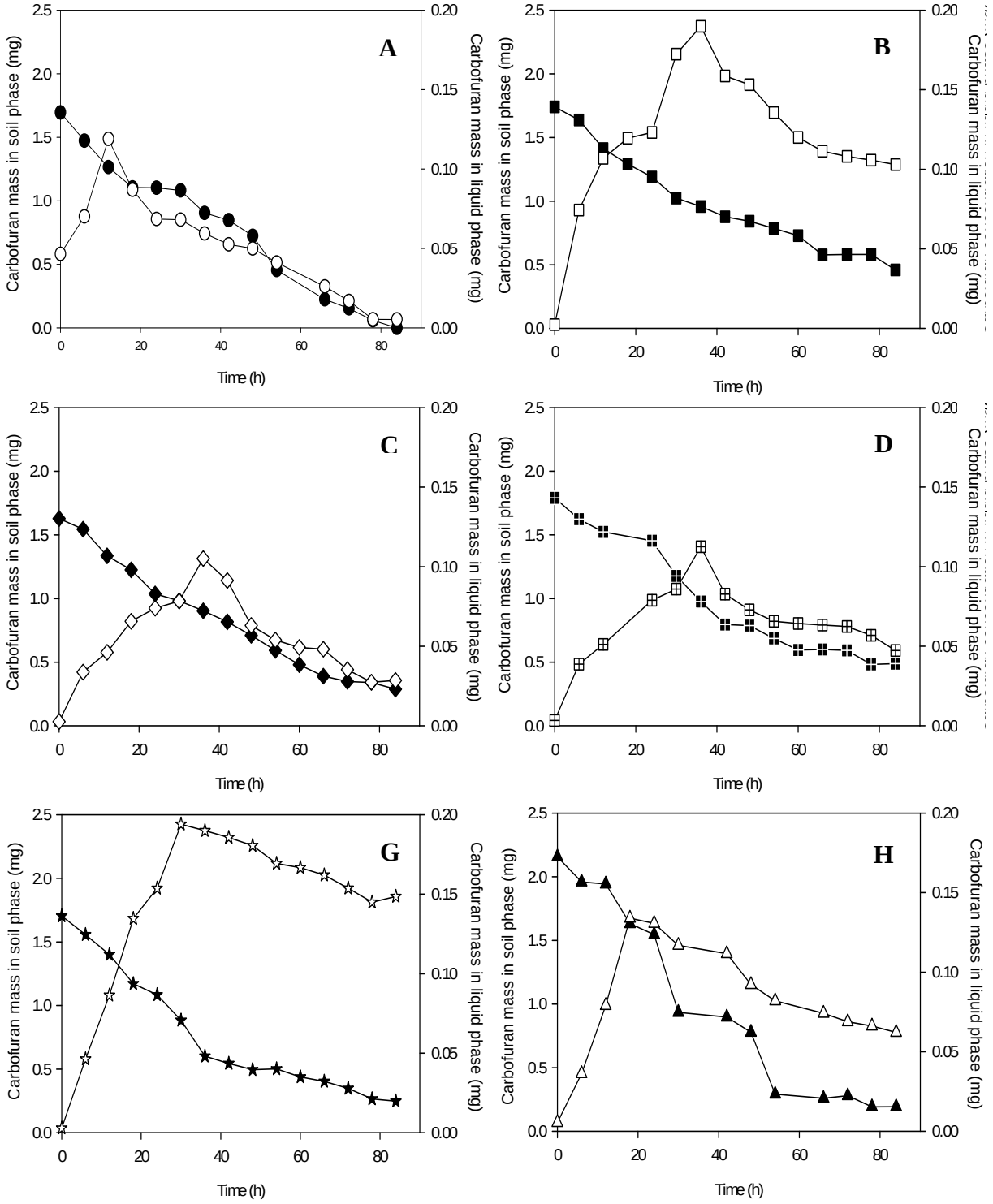

Figure 5-6. Carbofuran partitioning between soil (close symbols) and liquid (open symbols) phases in soil slurry phase reactors with biological activity (A: Soil slurry + immobilized PCL3; B: Soil slurry; reactor C: Autoclaved soil slurry + immobilized PCL3; D: Soil slurry + autoclaved corncob; G: Soil slurry + molasses; H: Soil slurry + immobilized PCL3 + molasses)

the abiotic reactors (E and F). In reactors A and H, the carbofuran concentration in the liquid phase increased until 18 h of reactor operation, reaching maximum concentrations of 0.11 and 0.13 mg, respectively, and then rapid degradation of carbofuran was observed (Figure 5-6). The continuous decrease in carbofuran concentration in the soil phase continued until the end of the experiment. This result indicated that desorbed carbofuran in the liquid phase was subjected to continuous biological degradation; therefore, desorption was enhanced due to the partitioning effect. The same partitioning patterns of carbofuran in the soil and liquid phases could be found in reactors B, D, C and G, with longer times of 30-36 h taken to approach the maximum carbofuran concentrations in the liquid phase of 0.11-0.19 mg (Figure 5-6). As the carbofuran degradations in reactors A and H were more rapid and started earlier than in the other reactors, it could be concluded that PCL3 together with indigenous microorganisms in reactors A and H were more effective in enhancing carbofuran degradation than PCL3 (reactor C) or indigenous microorganisms (reactors B, D and G) alone in the reactor.

5.3.3 pH values and CFU variations

Soil slurry pH and CFU (colony forming unit) were investigated during soil slurry phase reactor operation as important parameters indicating microbial activity. The results indicated that pH variation in the reactors with biological activity (reactors A, B, C, D, G, and H) was more obvious compared to the abiotic control reactors (E and F) (Figure 5-7). In reactors A, B, and C a slight increase in the pH of the soil slurry was observed during the early period of reactor operation and a further decrease in the soil slurry pH was found after 28 h (reactor A) and 42 h (reactors B and C) of reactor operation. An tendency of the soil slurry pH to increase was observed in reactors D, G, and H throughout reactor operation. The variation in soil slurry pH could result from the products obtained from biodegradation activity. An increase in the soil slurry pH might be due to the alkaline nature of by-products formed during the metabolism of carbofuran and organic matter by the microorganisms inhabiting the soil slurry (Prasanna et al., 2008). Subsequently, a decrease in the soil slurry pH in reactors A, B, and C might due to the formation of CO_2 from mineralization of the metabolic intermediates formed (Venkata-Mohan et al., 2006). Reactors E and F showed relatively stable soil slurry pH values during

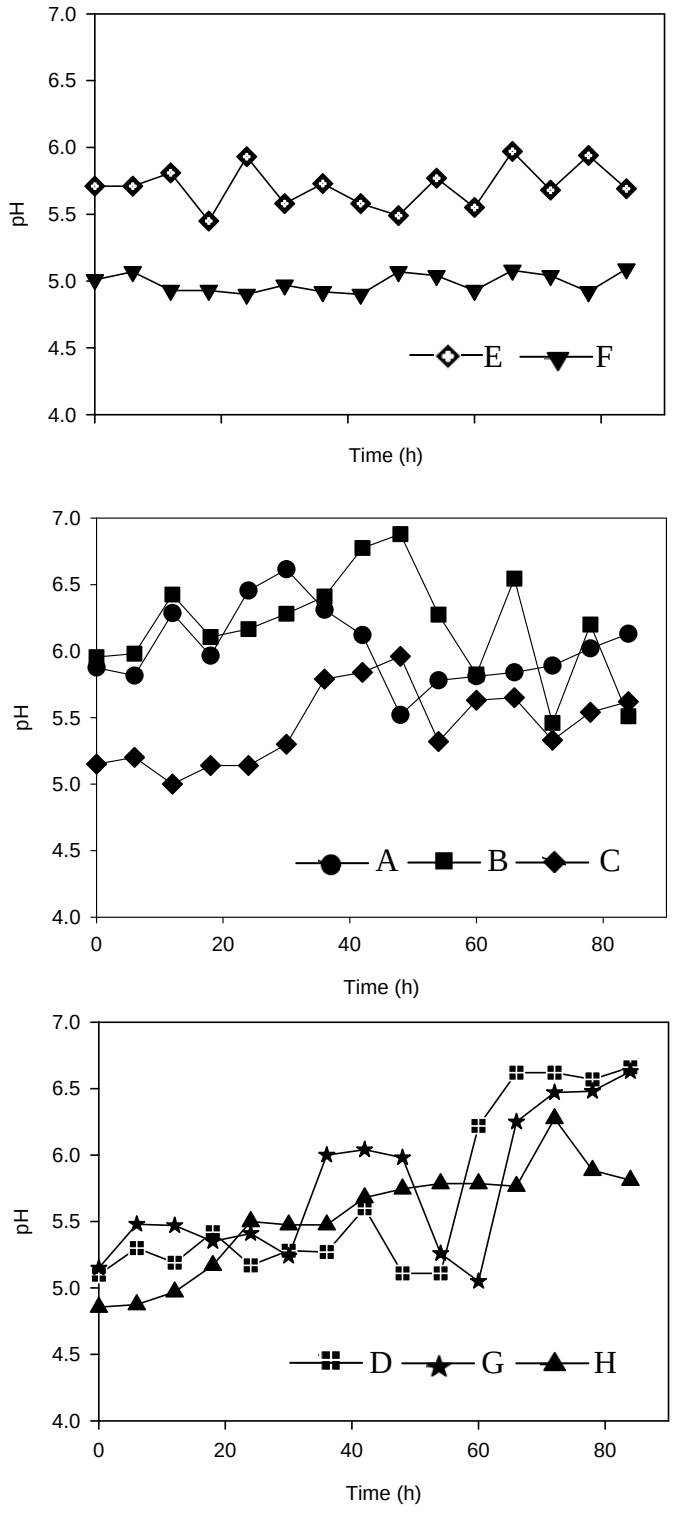

Figure 5-7. The temporal variation of soil slurry phase pH during reactor operation

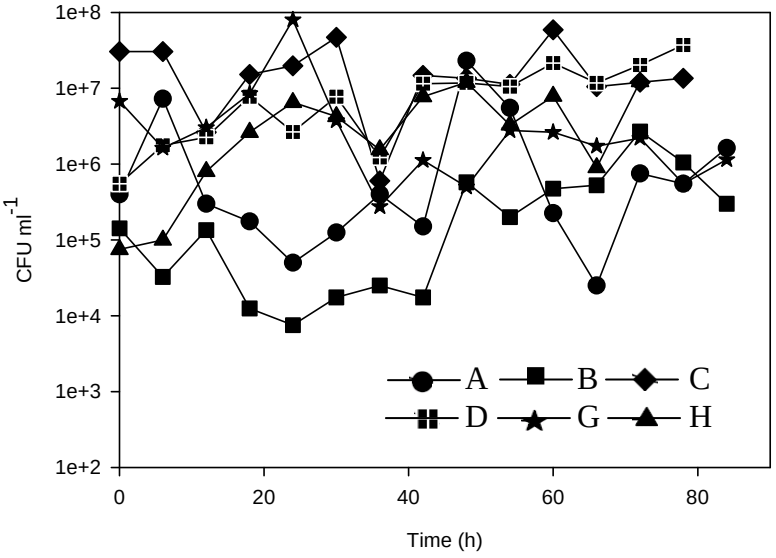

Figure 5-8. The temporal CFU variation during soil slurry phase reactor operation

operation (Figure 5-7), which might due to the absence of microbial activity (Venkata-Mohan et al., 2008).

The number of carbofuran degraders in the soil slurry phase reactors with biological activity in terms of CFU (Figure 5-8) was determined on a BSM agar plate coated with carbofuran as a sole carbon source. The results indicated the successful development of indigenous microbial numbers in the soil slurry phase reactor, which was shown by the marked increase in the numbers of carbofuran degraders (by one order of magnitude) in reactors operated with indigenous microorganisms alone (reactors B and D) (Figure 5-8). However, the number of indigenous carbofuran degraders in the stimulated reactor (G) decreased by one order of magnitude, which might due to the toxic effects of by-products from molasses components and the metabolism of molasses by the indigenous microorganisms themselves. In the augmented reactor (A), the number of carbofuran degraders in the soil slurry obviously varied over time. Though the CFU in reactor A decreased markedly by two orders of magnitude in some periods of operation (6-24 h and 42-66 h) (Figure 5-8), the greatest carbofuran degradation efficiency was obtained in this reactor. This result implied that carbofuran degradation did not depend on the efficacy of free cell of carbofuran degraders suspended in the soil slurry, but might mainly

depend on the immobilized PCL3 adsorbed on the support. In reactor H, to which was added both immobilized PCL3 and molasses, the number of carbofuran degraders increased by two orders of magnitude (Figure 5-8). This result was different from the CFU variation in reactor G, which showed the negative effect of molasses on the growth of carbofuran degraders in the soil slurry. The explanation for this phenomenon is that PCL3 in immobilized cell form could be protected from the toxic effects of the by-products from molasses components or molasses metabolism (Bekatorou et al., 2004). In contrast, molasses might well be used by PCL3 as an energy source, resulting in continuous growth of the cell and leakage to the soil slurry due to the limited space in the support material. This was attributed to an increase in the number of carbofuran degraders in the soil slurry with a decrease in carbofuran degradation efficiency, as previously described.

5.3.4 Kinetic degradation of carbofuran in soil slurry phase reactors

The kinetic aspect of carbofuran degradation in the soil slurry was examined in this study using zero-, first-, and second-order equations. The kinetic parameters of carbofuran degradation in the soil phase derived from analysis of the output of the three equations along with R^2 values are shown in Table 5-2. A reasonably good correlation was indicated by R^2 of more than 0.90. In the abiotic control reactor (F), the carbofuran degradation pattern did not follow any of the kinetic equations (R^2 = 0.64-0.66) (Table 5-2). Reactors A, C, E, and H showed a good correlation with the zero-order kinetic equation (R^2 = 0.93-0.98) (Table 5-2), which indicated that the degradation process in these reactors was independent of carbofuran concentration and the carbofuran degradation would not be inhibited by carbofuran concentration. Reactors B, D, and G showed a good correlation with the first-order kinetic equation (R^2 = 0.97-0.99) (Table 5-2), revealing that the rate of degradation was directly proportional to the concentration of carbofuran in the soil phase of these reactors.

The half-lives of carbofuran were calculated and are depicted in Table 5-2. The half-life of carbofuran in the non-augmented reactor (B) was 46.2 h, which was longer than the half-lives of carbofuran in the reactors with bioremediation treatments (reactors A, C, D, G, and H) of 30.13-40.76 h (Table 5-2), indicating an improvement in carbofuran degradation by the bioaugmentation and/or biostimulation techniques. In this study the shortest half-life of carbofuran in the soil phase of 30.13 h was

Table 5-2 Kinetic parameters for carbofuran degradation in soil phase

Reactor	Order of equation	Degradation rate constant	Degradation half-life (h)	Kinetic equation	R^2
A	Zero	0.020 g h^{-1}	39.75	$C_t = 1.59-0.020t$	0.98
	First	0.037 h^{-1}	18.73	$C_t=2.43e^{-0.037t}$	0.85
	Second	0.083 g^{-1} d^{-1}	7.11	$1/C_t=0.083t+0.59$	0.43
B	Zero	0.015 g h^{-1}	53.00	$C_t = 1.59-0.015t$	0.95
	First	0.015 h^{-1}	46.20	$C_t=1.71e^{-0.015t}$	0.99
	Second	0.017 g^{-1} d^{-1}	27.06	$1/C_t=0.017t+0.46$	0.95
C	Zero	0.016 g h^{-1}	47.81	$C_t = 1.53-0.016t$	0.97
	First	0.021 h^{-1}	33.00	$C_t=1.79e^{-0.021t}$	0.96
	Second	0.034 g^{-1} d^{-1}	5.88	$1/C_t=0.034t+0.20$	0.91
D	Zero	0.016 g h^{-1}	52.50	$C_t = 1.68-0.016t$	0.93
	First	0.017 h^{-1}	40.76	$C_t=1.84e^{-0.017t}$	0.97
	Second	0.019 g^{-1} d^{-1}	21.58	$1/C_t=0.019t+0.41$	0.96
E	Zero	0.015 g h^{-1}	46.00	$C_t = 1.38-0.015t$	0.96
	First	0.021 h^{-1}	33.00	$C_t=1.50e^{-0.021t}$	0.93
	Second	0.031 g^{-1} d^{-1}	15.16	$1/C_t=0.03t+0.47$	0.96
F	Zero	0.011 g h^{-1}	66.82	$C_t = 1.47-0.011t$	0.66
	First	0.010 h^{-1}	69.30	$C_t=1.04e^{-0.010t}$	0.65
	Second	0.010 g^{-1} d^{-1}	71.00	$1/C_t=0.010t+0.71$	0.64
G	Zero	0.017 g h^{-1}	44.12	$C_t = 1.50-0.017t$	0.91
	First	0.023 h^{-1}	30.13	$C_t=1.72e^{-0.023t}$	0.98
	Second	0.039 g^{-1} d^{-1}	5.13	$1/C_t=0.038t+0.20$	0.93
H	Zero	0.026 g h^{-1}	39.42	$C_t = 2.05-0.025t$	0.93
	First	0.032 h^{-1}	21.66	$C_t=2.67e^{-0.032t}$	0.93
	Second	0.061 g^{-1} d^{-1}	7.38	$1/C_t=0.047t+0.45$	0.80

obtained in reactor G (Table 5-2), but the greatest efficiency of carbofuran removal was obtained in reactor A (96.97%) (Figure 5-3), with a short half-life of carbofuran in the soil of 39.75 h (Table 5-2). To understand this phenomenon, we further investigated the kinetics of the degradation of carbofuran in the liquid phase after starting the degradation process in each reactor. The kinetic parameters along with R^2 for carbofuran degradation in the liquid phase of each reactor with the best correlation

Table 5-3 Kinetic parameters for carbofuran degradation in liquid phase

Reactor	Order of equation (best fitted)	Degradation rate constant	Degradation half-life	Kinetic equation	R^2
A	Zero	0.001	50.00	$C_t = 0.10-0.001t$	0.98
B	First	0.011	63.00	$C_t=0.24e^{-0.011t}$	0.93
C	First	0.027	25.67	$C_t=0.25e^{-0.027t}$	0.94
D	First	0.011	63.00	$C_t=0.12e^{-0.011t}$	0.90
G	Second	0.034	148.53	$1/C_t=0.034t+5.05$	0.97
H	First	0.013	53.31	$C_t=0.17e^{-0.013t}$	0.98

equations are presented in Table 5-3. The degradation process could not be observed in reactors E and F; therefore the degradation kinetics were not examined in these reactors. The carbofuran half-life in the liquid phase of reactor G (148.53 h) was significantly longer than that in the liquid phase of reactor A (50 h) (Table 5-3). In addition, the carbofuran degradation process in reactor A started after 18 h of reactor operation, which was earlier than in reactor G (Figure 5-6). Therefore, we could conclude that reactor A was the most effective treatment to remediate carbofuran, as indicated by the highest performance (96.97%, Figure 5-3) and shortest half-lives in the soil (Table 5-2) and the liquid phase (Table 5-3) of 39.75 h and 50 h , respectively. The carbofuran degradation pattern in the liquid phase of reactor B followed the second-order kinetics equation, indicating dependence on the concentrations of both the carbofuran and intermediates formed during the biodegradation process.

5.4 Conclusions

The soil slurry phase reactor was an effective technique for removing carbofuran from the soil. Desorption of carbofuran from the soil to the liquid phase offered favorable conditions for the biological degradation process of carbofuran. Bioaugmentation treatment could be applied together with the soil slurry phase bioreactor to enhance the carbofuran degradation efficiency in the soil. Variations of pH and CFU during the degradation process correlated with biological activity in the soil slurry phase reactors. Soil slurry augmented with immobilized PCL3 (reactor A) was the most effective treatment for bioremediating carbofuran.

CHAPTER VI

BIODEGRADATION OF CARBOFURAN IN SEQUENCING BATCH REACTOR AUGMENTED WITH THE IMMOBLIZED *Burkholderia cepacia* PCL3 ON CORNCOB

6.1 Introduction

Carbofuran (2,3-dihydro-2,2 dimethylbenzofuran-7-yl methylcarbamate) is a broad-spectrum insecticide widely used in agriculture to control insects and nematodes on contact or after ingestion. Carbofuran is of environmental concern because it is soluble in water and highly mobile in soil resulting in a high potential for groundwater contamination and can cause acute toxicity to mammals through cholinesterase inhibition (EPA, 2006). In 2007, Thailand imported carbofuran in form of Furadan3G up to approximately 5,000 tons for agriculture purpose (FMC cooperation, 2008). Contamination of carbofuran to the aqueous environment could be occurred by the results of discharges from manufacturing plants and rinsate, storage sites, accidental spills, and surface runoff through agricultural area where carbofuran was applied.

One of the effective routes for pesticide removal is microbial degradation by specific degrader and/or indigenous microorganisms. Previous research reported a discovery of microorganisms capable of degrading carbofuran (Xiang et al., 2008; Yan et al., 2007; Ryeol et al., 2006; Feng et al 1997) and other pesticides such as atrazine (Shapir et al., 1997; Lima et al., 2009), dichloroaniline (Tongarun et al., 2008) and fenitrothion (Hong et al., 2007) from contaminated natural matrices. These degraders could use the pesticide as their energy sources, i.e., C- or N- or C and N-sources. Addition of microbial cultures capable of degrading pesticide, or so-called bioaugmentation technique, is reported as an effective bioremediation approach to improve pesticide degradation in contaminated soil and water that lack of the microbial activities (Dams et al., 2007; Plangklang and Reungsang, 2008). Not only bioaugmentation but also biostimulation, the addition of organic and/or inorganic

amendments to stimulate the activity of indigenous microorganisms, has been reported to enhance a degradation rate of contaminants of interest (Robles-Gonzalezl et al., 2008).

Bioaugmentation and biostimulation can be applied both *in situ* by directly adding degraders and/or amendments to the contaminated areas and *ex situ* through biodegradation in bioreactor. Though *in situ* treatment possesses the advantages of simplicity and cost-effective, it requires long time to complete the degradation and can be restrained by some limit conditions such as low permeability and heterogeneous of the contaminated matrices (Prasanna et al., 2008). Therefore, the bioreactor technology which can be specially designed in variety of configurations to maximize the microbial activity has drawn our attention to bioremediate carbofuran.

Sequencing batch reactors (SBRs) has recently become an attractive alternative tool to remove the various kinds of pesticides from contaminated water due to its simplicity and flexibility of operation, better solid retention and cost effective in comparison to continuous process (Ratusznei et al., 2000). The operation of SBRs consists of four steps, i.e. feeding, reaction, settling and liquid withdrawal (Zaiat et al., 2001), in which exposure time between microorganisms and contaminant, frequency of exposure and level of the respective concentration can be set independently of any inflow conditions. The contaminant degradation efficiency as well as the quality of the effluent from SBRs can be easily controlled by adjusting Hydraulic Retention Time (HRT) or time scale of each operation steps and accelerating mass transfer and microbial activities (Zaiat et al., 2001). Previous studies demonstrated the enhancement of pesticides biodegradation in SBR system such as atrazine (Protzman et al., 1999), 2,4-dichlorophenoxyacetic acid (2,4-D) (Mangat and Elefsiniotis, 1999), 2,4-dichlorophenol (2,4-DCP) (Wang et al., 2007), and isoproturon (Celis et al., 2008).

The evidences on biodegradation of carbofuran in synthetic medium and soil have been published by many researchers (Tomasek and Karns, 1989; Topp et al., 1993; Feng et al., 1997; Ogram et al., 2000; Bano and Musarrat, 2004; Ryeol et al., 2006; Yan et al., 2007; Xiang et al., 2008; Plangklang and Reungsang, 2008). However, to the best of our knowledge, there is very limited information available on its biodegradation potential in the bioreactors. Therefore, the main objective of this study was to explore the performance of SBRs in bioremediation of carbofuran. The

effects of HRT, biostimulation technique, and carbofuran concentration on carbofuran degradation efficiency in SBRs were investigated. The kinetic aspects of carbofuran degradation in the SBRs were further studied to explain the degradation behavior in SBR treatments.

6.2. Materials and Methods

6.2.1 Chemicals

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

6.2.2 Feed medium

Culture medium used in this study was C-limited Basal Salt Medium (pH7) containing (in g l^{-1}): 5.57, $NaHPO_4$; 2.44, KH_2PO_2; 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, $CaCl_2$. Carbofuran stock solution in methanol, at the given concentrations was added to BSM after it was sterilized by autoclaving at 121 ^oC for 15 min before using as feed medium.

6.2.3. Immobilization of *B. cepacia* PCL3

6.2.3.1 Microorganism preparation

The carbofuran degrader, identified by 16s rRNA as *B. cepacia* PCL3 (GenBank accession number of EF990634), was used in this experiment. This microorganism is capable of using carbofuran as a sole C-source (Plangklang, 2004). It was grown in 100 ml nutrient broth (NB) containing 5 mg l^{-1} carbofuran at 30°C and 150 rpm for 36 h and was used as seed inoculum for immobilization.

6.2.3.2 Supporting material preparation

Corncob was used as a support material to immobilize *B. cepacia* PCL3. This material has high matrix porosity and a pore size that could enhance the cell adsorption capability during immobilization. Corncob was cut into 0.7x0.7x0.7 cm pieces using a knife. Three hundred g of cut corncob was boiled in 3 l

of 1% NaOH for 3 h to remove lignin and fibers inside the materials which might react with the cells (Bradi 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.

6.2.3.3 Cell immobilization

Adsorption was used as the immobilization method in this study. This method is typically performed when porous media are used as support materials with the advantage of ease of operation. The immobilization technique was conducted by adding 75 g of sterile corncob to 250 ml of sterile NB containing 5 mg l^{-1} carbofuran before inoculating with PCL3 (10^{6} CFU ml^{-1}). The flask was then incubated at 150 rpm, at room temperature, for 48 h. After incubation, the support material was transferred to fresh NB containing 5 mg l^{-1} of carbofuran and incubated, as previously described, before harvesting by filtration through a 0.45 µm filter using Buchner filter funnel and washing with 0.85% NaCl by an aseptic technique. This process was repeated two times. Immobilized cells were kept at 4^{o}C until used in further experiments. The internal cell density on the corncob after immobilization was approximately 5.3x10^{8} CFU g^{-1} dry corncob.

6.2.4 Reactor configuration

A 2-L blue bottle was used as a reactor in this study with an operating liquid volume of 1.5 l and a suitable inlet and outlet arrangements as shown in Figure 6-1. Four ports were installed in the reactor for filling, decanting and sample

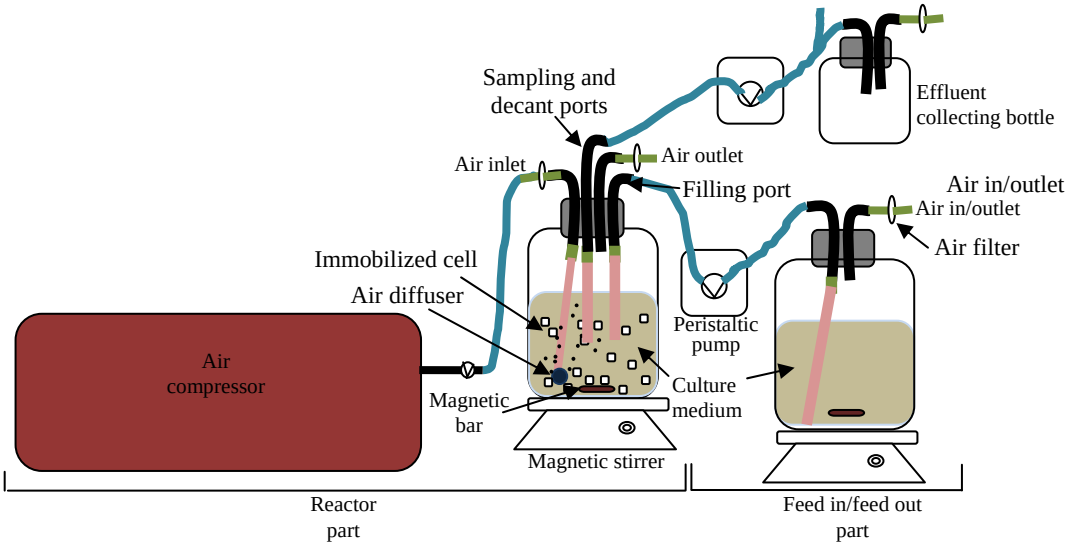

Figure 6-1. Schematic diagram of the bioreactor used (not subjec to scale)

collection, air supplying and gas venting. Air was supplied from an air compressor through an air diffuser at the bottom of the bottle. The contents of the reactor were continuously mixed by a magnetic stirrer while feeding and decanting were carried out by appropriately calibrated peristaltic pumps.

6.2.4 Reactor operation

6.2.4.1 Carbofuran degradation in batch system

Carbofuran degradation in the bioaugmented reactor was firstly studied in batch system. The sterilized reactor was added with 200 g wet corncob immobilized *B. cepacia* PCL3 (10^8 CFU g^{-1} dry corncob, 40% moisture content). A 1.5-l BSM with an initial carbofuran concentration of 20 mg l^{-1} was fed to the reactor at a flow rate of 25 ml min^{-1} by using peristaltic pump. The reactor was then operated at room temperature (28-30 ^oC) with continuous mixing by using magnetic stirrer. Dissolved Oxygen (DO) level in the medium was maintained at above 2.5 mg l^{-1} by pumping air through oxygen diffuser at the air flow rate of 600 ml min^{-1}. The reactor was operated until the carbofuran in the culture medium was completely degraded. During the reactor operation, culture medium in the reactor was sampled at every 6 h to determine the carbofuran and its metabolites concentrations. An abiotic control reactor was inoculated with autoclaved corncob which was conducted in a similar manner. Results from the batch experiment would be used as the basic information for SBR operation.

6.2.4.2 SBR experiments

Carbofuran degradation in SBRs was started as a similar manner to batch system. The reactors were aerobically operated in the sequencing batch mode with a fixed total cycle period of 48 h without a settling step in all treatments. The cycle period consisted of 1.0 h of fill, 46 h of react and 1.0 h of decant phases. The conditions for SBR operation were set base upon the results from the batch experiment. During the react phase, the reactor was continuously aerated and stirred to homogenize the contents and air diffusion. Cultured medium was sampled after finished the filling phase and at the end of each cycle period (effluent), via the sampling port by using peristaltic pump, to determine carbofuran and its metabolites

concentrations. Number of PCL3 on the supporting material was determined at the initial and at the end of reactor operation in all SBRs treatments.

6.2.4.2.1 Effect of HRT

The effect of HRT on the carbofuran degradation efficiency in SBRs was firstly investigated by varying the feed volume from 215 to 500 ml. The increasing feed volume resulted in a shorter HRT (from 14 to 6 d) in which the corresponding of feed volume to HRT was expressed as equation (1). At the end of first cycle period (48 h), 215 ml of culture medium was decanted from the reactor and the fresh BSM with 20 mg l^{-1} of carbofuran at the same volume was fed to the reactor before starting the react phase. The HRT was subsequently decreased from 14 d to 10, 8 and 6 d, respectively, after the reactor reached steady state which was indicated by a constant carbofuran concentration in the effluent (deviation of less than 5% from cycle to cycle). The optimum HRT, the shortest HRT which remain the complete degradation of carbofuran, was further used as the basic information for SBR biostimulation experiments.

$$HRT = \left[\frac{V_{reactor}}{V_{fed}} \right] \times t_c \qquad (1)$$

Where $V_{reactor}$ is the operating volume (1.5 l), V_{fed} is the feeding volume and t_c is the cycle period (48 h).

6.2.4.2.2 Biostimulation of SBRs

In order to stimulate carbofuran degradation ability of immobilized PCL3, various types of C-sources (10 g l^{-1}), i.e. molasses and cassava pulp and N-sources (0.1 g l^{-1}), i.e. brewery yeast spent powder and rice bran were used as organic amendments. These organic amendments were chosen because they are commonly agricultural wastes in Thailand. Each amended materials was sterilized by autoclaving and then added to the feed medium before feeding to the reactor. As the experimental results indicated that the optimum HRT was 8 d, thus the SBRs biostimulation experiments were operated with a total cycle period of 48 h as similar procedures described earlier. However, the short HRT of ¾ of optimum HRT was used in this experiment based on the assumption that biostimulation would be able to provide a greater efficiency of carbofuran removal than the experiment without

biostimulation. In each treatment, the reactor was operated until achieving a steady state. The optimal amendment for carbofuran degradation in SBRs was used in the further experiment.

6.2.4.2.3 Effect of carbofuran concentration

This experiment was conducted to obtain the maximum concentration of carbofuran with the remaining of high carbofuran removal efficiency of SBRs. The SBRs were operated separately at high carbofuran concentrations of 40 and 80 mg l^{-1} at the HRT of 6 d. The reactors were amended with the nitrogen source that yield the highest carbofuran degradation from the previous experiment (3.2.4.2.2) The conditions for SBR experiments were summarized in Table 6-1.

Table 6-1. SBR experiments

Condition	HRT (d)				Biostimulation of SBR*				Carbofuran conc. (mg l^{-1})		
	14	10	8	6	CP	ML	RB	SY	20	40	80
Cycle period (h)	48	48	48	48	48	48	48	48	48	48	48
Feed (h)	1	1	1	1	1	1	1	1	1	1	1
React (h)	46	46	46	46	46	46	46	46	46	46	46
Decant (h)	1	1	1	1	1	1	1	1	1	1	1
HRT (d)	14	10	8	6	6	6	6	6	6	6	6
Carbofuran conc. in feed medium (mg l^{-1})	21.02 ±1.03	20.98 ±2.11	21.32 ±0.74	20.66 ±0.74	20.59 ±2.04	20.00 ±2.55	21.96 ±1.02	21.43 ±2.36	21.96 ±1.02	39.08 ±3.44	80.42 ±4.50
Feed volume (ml)	215	300	375	500	500	500	500	500	500	500	500
Supplement	-	-	-	-	CP	ML	RB	SY	RB	RB	RB

* CP is cassava pulp (10 g l^{-1}), ML is molasses (10 g l^{-1}), RB is rice bran (0.1 g l^{-1}) and SY is spent yeast from beer fermentation process (0.1 g l^{-1}).

6.2.5 Analytical methods

6.2.8.1 Analysis of carbofuran and its metabolites concentrations

Extraction of carbofuran from culture media using a liquid-liquid partitioning method was conducted by adding 2-ml of methanol to 2-ml of culture media and then sonicated twice for 10 min each time, 50/60 voltage cycle. After sonication, carbofuran and its metabolites were extracted from the media in separation funnel with dichloromethane. This extraction was done 3 times. For the

first, second and third extractions, 4, 2 and 2 ml of dichloromethane, respectively, were added to the sonicated media and hand shaken for 30 sec. The organic fraction of the samples from each extraction was collected and 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 before analyzing by HPLC following the conditions described by Planklang and Reungsang (2008).

6.2.8.2 SBRs performance

The performance of the SBRs for remediation of carbofuran was evaluated by determining the carbofuran degradation efficiency (*E*) as given in equation (1):

$$E\ (\%) = \left[\frac{C_{in} - C_{eff}}{C_{in}} \right] \times 100 \qquad (2)$$

where C_{in} is the carbofuran concentration (mg l^{-1}) in the medium after finished filling phase of each cycle period (influent) and C_{eff} is the carbofuran concentration in culture medium (mg l^{-1}) at the end of the same cycle period (effluent).

6.2.8.3 Kinetic of carbofuran degradation

The kinetic rate constants in regard to carbofuran degradation in batch reactors and SBRs were calculated by fitting to a modified first-order kinetic model using SAS program (SAS Institute, Inc., 1985; Plangklang and Reungsang, 2008). The data used for the kinetic rate constants calculation in the batch reactors were the carbofuran concentrations over time, until complete degradation was achieved. For SBRs, the data of carbofuran degradation over time in each cycle period after the reactor reached steady state were used.

6.2.8.4 Enumeration of carbofuran degraders

The number of *B. cepacia* PCL3 in suspended form, accounted as cells leaked from the support materials, was determined by the plate count technique which modified from Zilli et al. (2004). To observe the number of PCL3 on corncob, 10 g of wet immobilized cells were taken from culture media and washed with sterile 0.85% NaCl solution three times. The washed immobilized cells were blended to 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.

6.3 Results and Discussions

6.3.1 Degradation of carbofuran in batch system

This experiment investigated the ability of immobilized *B. cepacia* PCL3 on corncob to degrade carbofuran in BSM in batch bioreactor. Carbofuran was rapidly degraded by immobilized PCL3 from 0-160 h of reactor operation and then gradually degraded (Figure 6-2). The degradation profile of carbofuran in BSM could be well described by a modified first-order kinetic model with the regressions coefficients, r^2 of 0.96. The half-life of carbofuran in the augmented degrader was 52 h. The complete degradation of carbofuran was achieved at 336 h (14 d) of reactor operation

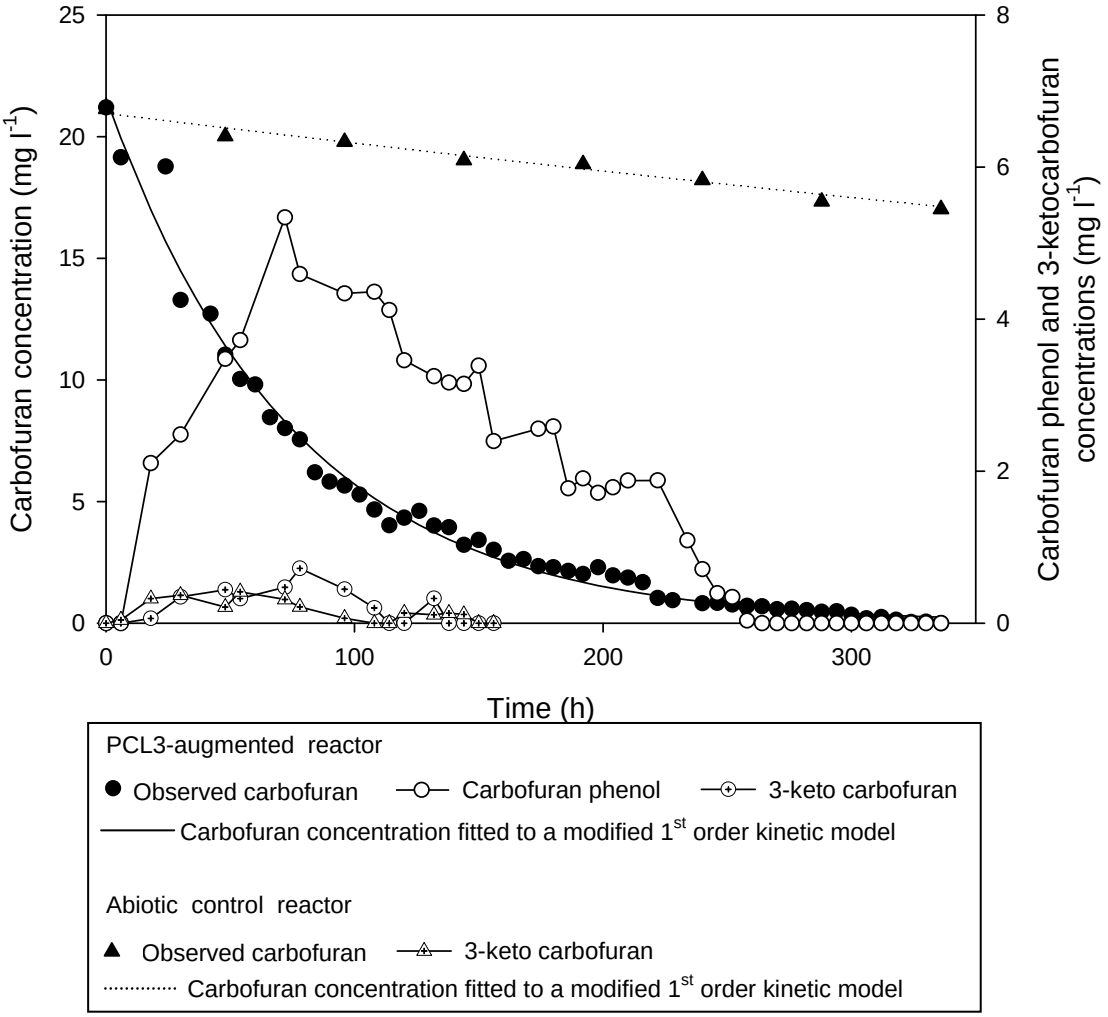

Figure 6-2. Carbofuran degradation in batch reactors

(Figure 6-2). Carbofuran phenol was the major metabolite observed during carbofuran degradation. The accumulation of carbofuran phenol could be observed until 72 h of reactor operation and then it started to decrease and could not be detected in BSM after 258 h of reactor operation (Figure 6-2). Results indicated that PCL3 could metabolize carbofuran and carbofuran phenol as its energy sources.

Carbofuran was slowly degraded in abiotic control reactor with the removal percentage of approximately 15% at 14 d of reactor operation (Figure 6-2), suggesting that degradation of carbofuran was mainly resulted from biological activity of the immobilized PCL3. Not only carbofuran phenol but also 3-keto carbofuran were the metabolites detected in this study (Figure 6-2). 3-keto carbofuran was the metabolite that was detected in both PCL3-augmented reactor and abiotic control. This metabolite is normally detected when the oxidation of carbofuran is taken placed.

Our previous research (Plangklang and Reungsang, 2009) investigated carbofuran degradation in BSM by immobilized PCL3 on corncob. The experiments were conducted in batch system in shake-flask scale with the working volume of 150 ml and initial carbofuran concentration of 5 mg l^{-1}. When compared to carbofuran degradation in the flask experiment, the degradation of carbofuran in the present augmented reactor showed a better performance in carbofuran degradation. The shortest time to achieve complete degradation of carbofuran and carbofuran phenol in the flask experiment was 30 d, which was 2.1 times longer than that in bioreactor treatment (14 d) (Figure 6-1), despite the fact that the initial carbofuran concentration in the flask was 4 times lower than in the reactor experiment (5 mg l^{-1}). These findings indicate that the carbofuran remediation efficiency was significantly enhanced by using the bioreactor technology. The aeration together with mechanical mixing might responsible for this trend by improving the mass transfer rate and contact among microorganisms and carbofuran and hence increasing the rates of carbofuran biodegradation.

At the end of reactor operation, the number of immobilized PCL3 on corncob increased approximately two times (from $4.6x10^8$ to $9.1x10^8$ CFU g^{-1} dry corncob). Number of PCL3 in suspended form increased continuously during 3 days of reactor operation, after that it was stable at approximately $8.4x10^7$ CFU ml^{-1} (data not shown). These results indicated that the cells were leaking out from the porous of

corncob due to a limited space and they continued to grow in the medium causing the increase in cell concentration. Cell detachment might be resulted from the fact that there are no barriers between the cells and the media which led to a possibility of cell relocation with potential establishment of cell-equilibrium inside the supports and culture media (Bekatorou et al., 2004).

6.3.2 Degradation of carbofuran in SBRs

6.3.2.1 Effect of HRT

Effect of HRT, by mean of feed volume variation, on the performance of SBRs in removal of carbofuran was investigated. Results indicated that carbofuran was completely degraded when the SBRs were operated at the HRT of 14 to 8 d (Figure 6-3). When the HRT was further decreased to 6 d, the carbofuran degradation efficiency decreased from 100% to approximately 73.50% (Figure 6-3). This might due to the fact that a decrease of HRT led to a higher carbofuran concentration accumulated in the culture medium (Figure 6-3), which generally needs a longer time for complete degradation. Numbers of PCL3 in the immobilized form at the starting and at the steady stead of each HRT were not markedly different (Figure 6-4). However, the numbers of PCL3 in suspended form which accumulated in culture medium decreased with the decrease of HRT (Figure 6-4). As PCL3 in both suspended and immobilized forms could responsible for carbofuran degradation in SBRs, a decrease in number of suspended PCL3 could be another reason for the decrease of carbofuran degradation efficiency at HRT of 6 d. From these results, it could be concluded that the optimal HRT for carbofuran remediation in SBR was 8 d.

Carbofuran phenol was the main metabolite observed during SBRs operation. A high accumulation of carbofuran phenol in culture medium at a shorter HRT was detected (data not shown) which can cause a toxic effect to PCL3, thus resulting in a lower carbofuran degradation at the HRT of 6 d.

6.3.2.2 Effect of biostimulation

The effect of organic amendments on carbofuran degradation efficiency of SBRs was examined at the HRT of 6 d. The carbofuran degradation efficiencies of stimulated SBRs were depicted in Figure 6-5. Results indicated that the addition of nitrogen sources, i.e. rice bran (RB) or spent yeast powder (SY) led to a

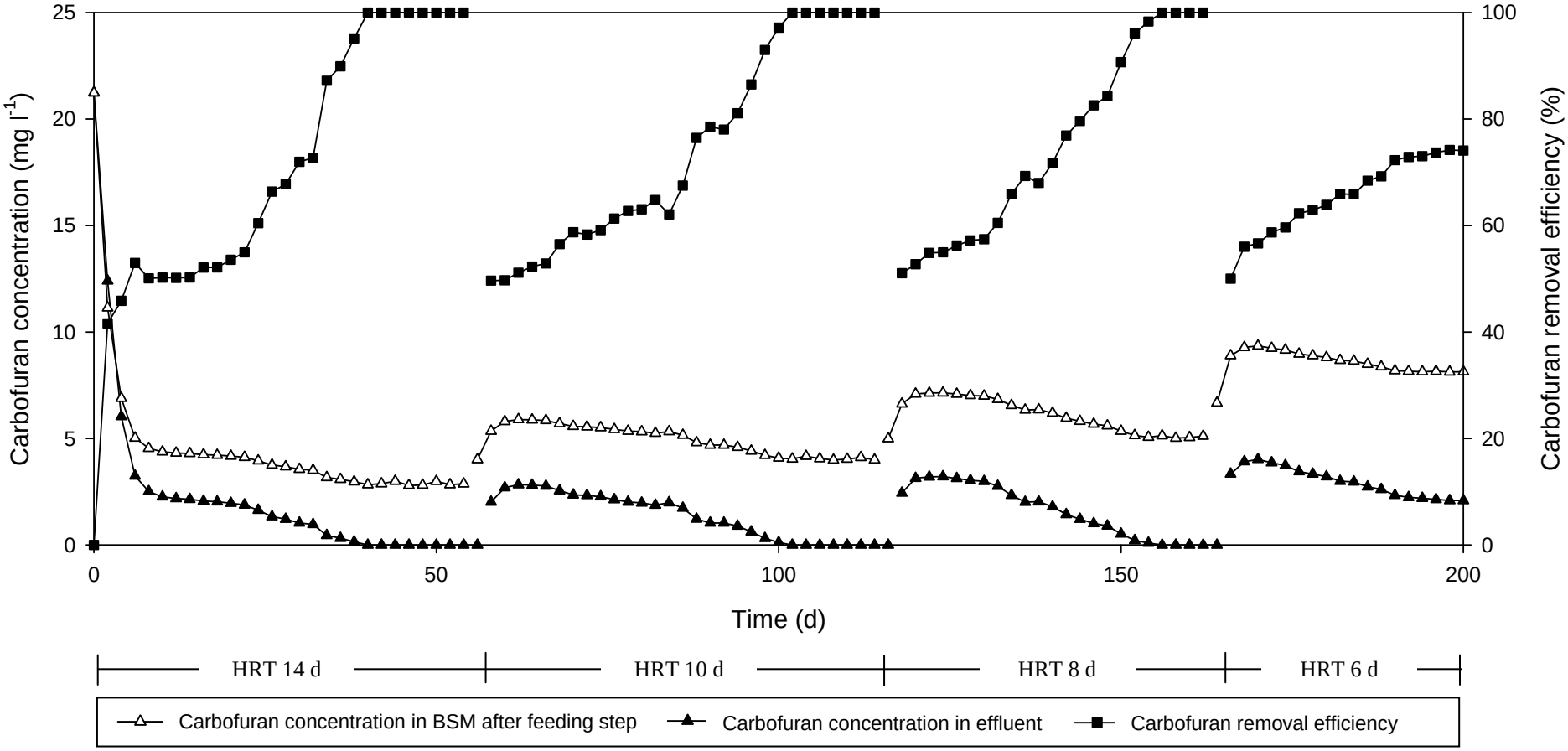

Figure 6-3. Effect of HRT on carbofuran degradation efficiency in SBR

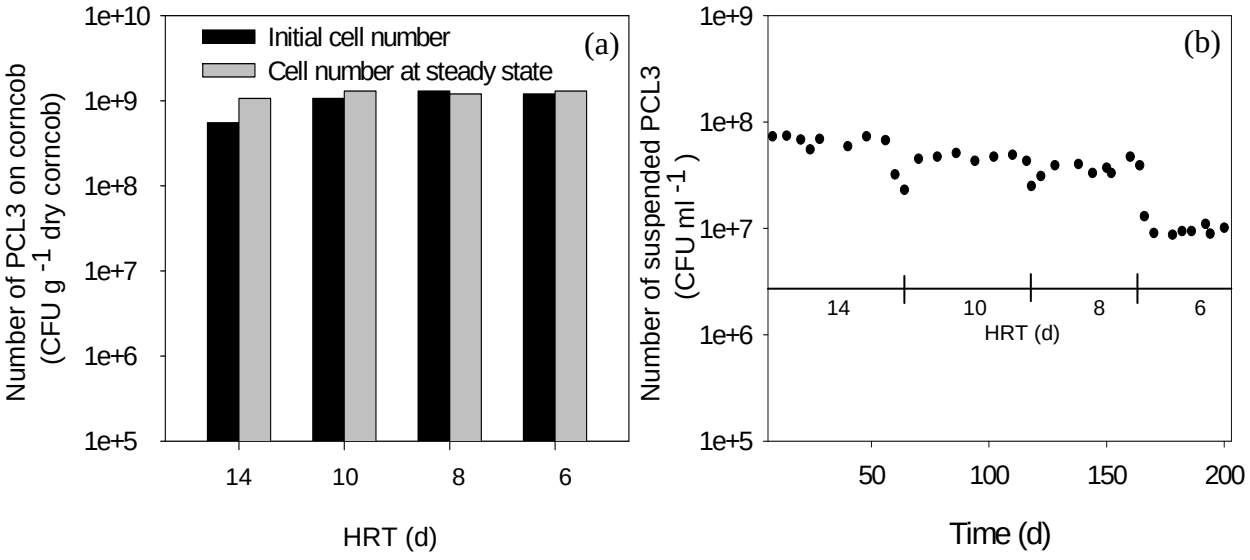

Figure 6-4. Number of PCL3 in immobilized (a) and suspended (b) forms in SBRs operated at the varying of HRT.

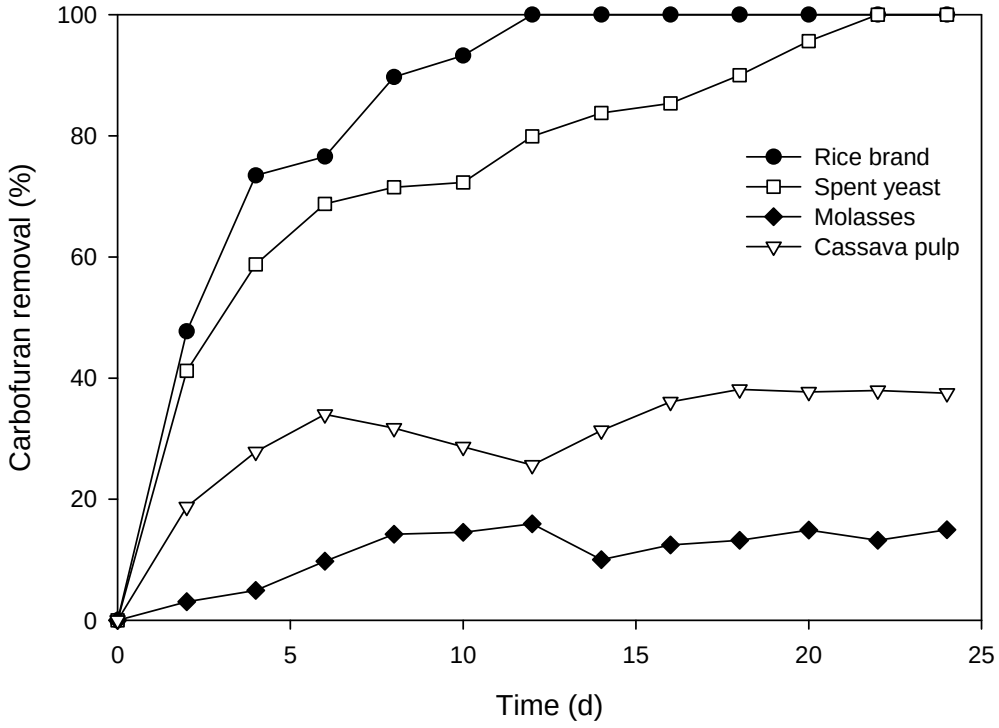

Figure 6-5. Effect of organic amendments on carbofuran degradation efficiency of SBRs

complete degradation of carbofuran in SBRs (Figure 6-5). However, the addition of supplementary carbon sources, i.e. cassava pulp (CP) or molasses (ML) resulted in a decrease in carbofuran degradation efficiency from 74% for non stimulated reactor to 37.5 and 15.0%, respectively (Figure 6-5). These results implied that by adding supplementary nitrogen sources to the culture medium, the degradation of carbofuran could be enhanced and supplementary carbon sources are not required. It might be because PCL3 utilized carbofuran as its carbon source, thus the added supplementary carbon sources could be competitive to carbofuran resulting in a decrease of carbofuran degradation efficiency.

The numbers of PCL3 in immobilized and suspended forms in the stimulated SBRs were shown in Figure 6-6. Results revealed that in the SBRs stimulated with ML, RB or SY, the number of immobilized PCL3 on corncob at the steady state increased approximately two times as compared to initial number of immobilized cell. The addition of CP to SBR resulted in an approximately 5 times decrease of number of immobilized PCL3 (Figure 6-6). This result implied that CP or degradation products from CP might contain the toxic substances for PCL3 which could reduce its ability to degrade carbofuran. In addition, CP, in general, contains 40-200 mg kg^{-1} cyanide which could cause a toxic effect to bacteria (Srinorakutara et al., 2006). These could be the reasons for the decrease in carbofuran degradation efficiency of SBR added with CP.

The number of suspended PCL3 in SBRs stimulated with RB and SY increased and stable at approximately $6.3x10^{7}$ CFU ml^{-1} (Figure 6-6), while a lower number of $4.3x10^{5}$ CFU ml^{-1} was observed in SBR added with CP. This might be the result of toxic effects of CP as described earlier. In SBR stimulated with ML, number of suspended PCL3 was approximately $9.4x10^{9}$ CFU ml^{-1} (Figure 6-6) which was markedly higher than that in the other stimulated reactors. This result was not coincide with the carbofuran degradation efficiency in ML stimulated SBR (Figure 6-5). The explanation for this result is that ML contains sugar which is more favorable substrate and has less complex structures than carbofuran, thus PCL3 could metabolite ML for its growth without the degradation of carbofuran.

As the SBRs stimulated with RB achieved the complete carbofuran within the shortest time (Figure 6-5), RB was considered as the best organic amendment and was used in the further experiment.

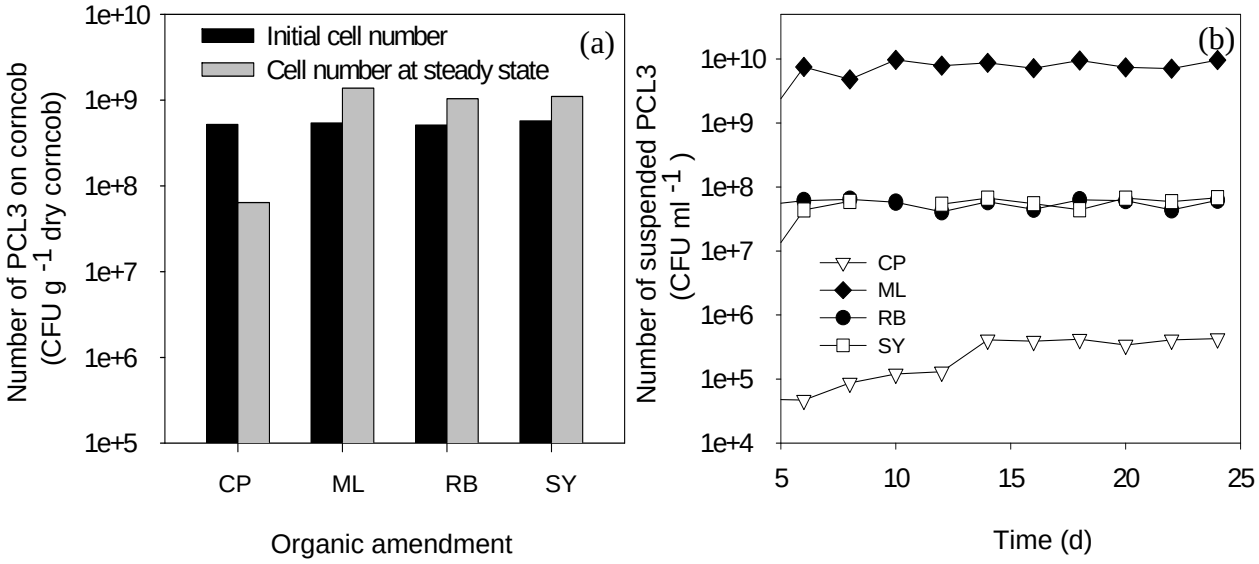

Figure 6-6. Number of PCL3 in immobilized (a) and suspended (b) forms in biostimulated SBRs; CP is cassava pulp (10 g l^{-1}), ML is molasses (10 g l^{-1}), RB is rice bran (0.1 g l^{-1}) and SY is spent yeast from beer fermentation process (0.1 g l^{-1}).

6.3.2.3 Effect of carbofuran concentration

The SBRs stimulated by RB was conducted at the HRT of 6 d with the increase in the carbofuran concentrations in feed medium from 20 to 40 and 80 mg l^{-1}. The percentage of carbofuran degradation patterns after 24 d of reactor operation are shown in Figure 6-7. At the carbofuran concentrations in feed medium of 20 mg l^{-1}, the reactor displayed a rapid adaptation behavior about 12 d before achieving the complete degradation of carbofuran. When the carbofuran concentration in feed medium was at 40 mg l^{-1}, PCL3 needed a longer time, 20 d, to achieve the complete degradation (Figure 6-7). The further increase in carbofuran concentration in the feed medium to 80 mg l^{-1} resulted in a partial degradation of carbofuran in SBR of 84% (Figure 6-7). The number of PCL3 in immobilized and suspended forms were depicted in Figure 6-8. Results indicated that number of PCL3 in immobilized form increased approximately 1.7 times at the carbofuran concentration in feed medium of 40 mg l^{-1} and number of suspended PCL3 was stable at approximately $1.6x10^{6}$ CFU ml^{-1} (Figure 6-8). At the carbofuran concentration in feed medium of 80 mg l^{-1}, the number of immobilized PCL3 was reduced from $5.4x10^{8}$ to $1.2x10^{7}$ CFU g^{-1} dry

corncob (Figure 6-8). In addition, the number of suspended PCL3 was stable at only $1.3x10^3$ CFU ml^{-1}. These results indicated that the growth of PCL3 in SBRs could be inhibited at the carbofuran concentration in feed medium of 80 mg l^{-1} which resulting in a decrease in the carbofuran degradation efficiency in SBRs.

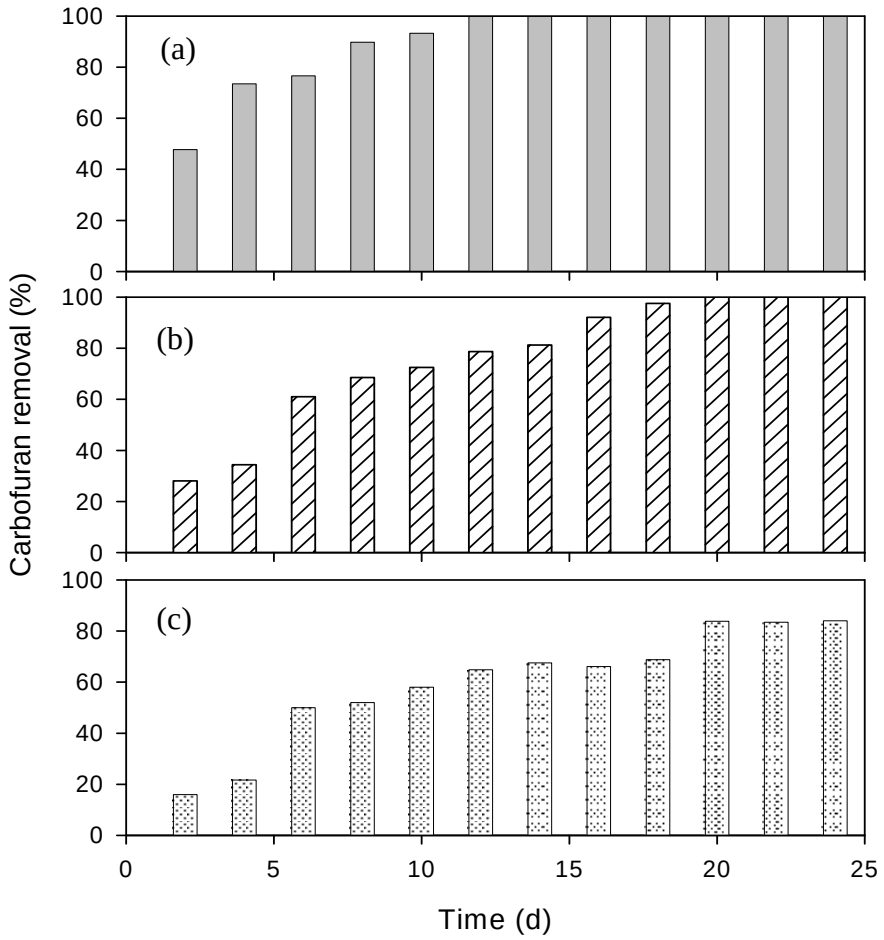

Figure 6-7. Effect of carbofuran concentrations on the performance of SBRs; (a) is 20 mg l^{-1}, (b) is 40 mg l^{-1} and (c) is 80 mg l^{-1}.

6.3.3 Kinetic of carbofuran degradation

The kinetic rate constants associated with carbofuran degradation in batch reactors and SBRs at the steady state were calculated and the result was summarized in Table 6-2. Overall biodegradation of carbofuran could be well described by the modified first-order kinetic model indicated by the regressions coefficients, r^2, ranged between 0.97-0.99 (Table 6-2). The microbial degradation of carbofuran has also been reported to follow the first order kinetic model (Plangklang

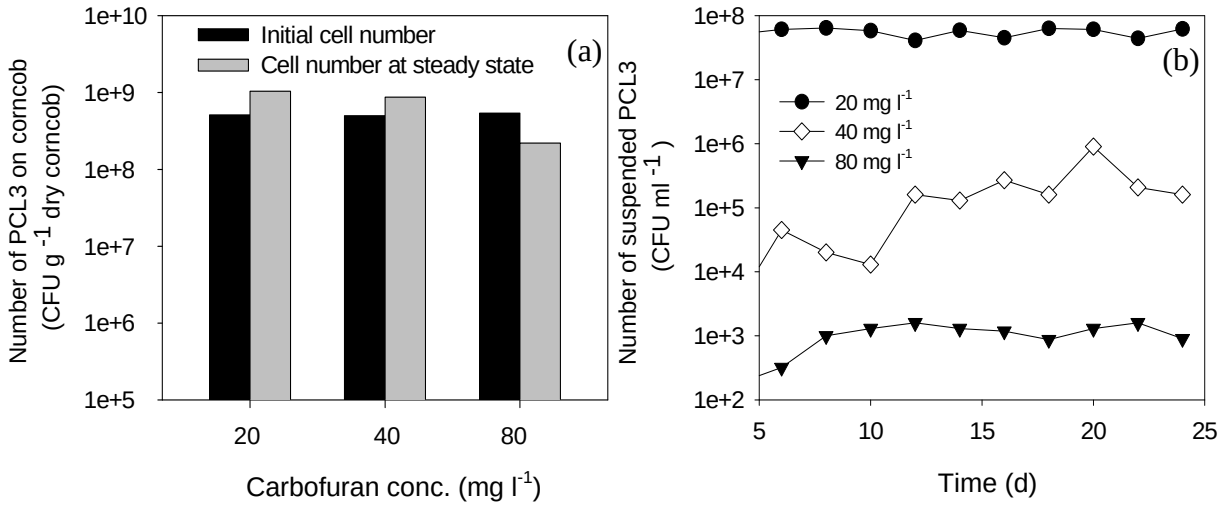

Figure 6-8. Number of PCL3 in immobilized (a) and suspended (b) forms in SBRs Operated with varying carbofuran concentrations.

and Reungsang, 2008; 2009). It was evident that kinetic rates of carbofuran degradation remained unchanged with the average value of 0.036 h^{-1} when the SBRs were operated at the HRT of 14 to 8 d. However, a further decrease in HRT to 6 d resulted in a decline in the kinetic rate of carbofuran degradation to 0.020 h^{-1} (Table 6-2). The degradation rates of carbofuran in SBRs were influenced by the addition of organic amendments. The addition of nitrogen sources, i.e. RB and SY resulted in an increase in carbofuran degradation rate constant to be approximately 0.043 h^{-1}. Whereas, the addition of CP and ML led to a markedly decrease in the rate constants to be 0.007 and 0.002 h^{-1}, respectively (Table 6-2). The kinetic constants of carbofuran degradation remained practically constant at 0.44 h^{-1} when the carbofuran concentration in the feed medium was increased from 20 mg l^{-1} to 40 mg l^{-1}. When the carbofuran concentration in feed medium was increased to 80 mg l^{-1}, the obviously decrease in the corresponding rate to 0.025 mg l^{-1} (Table 6-2) could be found. It could be concluded that the trends of kinetic rate observed in this study reflected the inhibitory effect of carbofuran at a high concentration on the performance of SBRs.

The half-lives of carbofuran regarding to the degradation rate constant values were calculated and showed in Table 6-2. Results indicated that SBR together with bioaugmentation and biostimulation gave approximately 3.5 times improvement of carbofuran degradation in comparison to batch reactor. The shortest half-life of

carbofuran of 15.57 h was obtained in SBRs stimulated with RB and these SBRs could remain the highest carbofuran degradation efficiency up to the carbofuran concentration of 40 mg l^{-1} in the feed medium.

Table 6-2. Degradation rate coefficients (k_1) and half-lives ($t_{1/2}$) of carbofuran in batch reactors and SBRs.

Kinetic parameter	Batch		SBR										
	Control reactor	PCL3–augmented reactor	HRT (d)				Stimulated amendment *				Carbofuran conc. (mg l^{-1})		
			14	10	8	6	CP	ML	RB	SY	20	40	80
First-order rate, k_1 (h^{-1})	0.0006	0.013	0.037	0.035	0.34	0.020	0.007	0.002	0.044	0.042	0.044	0.044	0.025
Half-life, $t_{1/2}$ (h)	1,155.00	52.11	18.63	19.63	20.03	34.65	99.00	346.50	15.57	16.50	15.57	15.57	27.72
r^2	0.97	0.97	0.98	0.99	0.98	0.99	0.97	0.99	0.99	0.99	0.99	0.99	0.98

*CP is cassava pulp (10 g l^{-1}), ML is molasses (10 g l^{-1}), RB is rice bran (0.1 g l^{-1}) and SY is spent yeast from beer fermentation process (0.1 g l^{-1}).

CHAPTER VII

CONCLUSIONS AND RECCOMMENDATIONS

7.1 Conclusions

A carbofuran degrader was identified base on 16s rDNA sequences as *Burkholderia* sp. PCL3 (GenBank accession number of EF990634). Both free and immobilized cell forms of the isolate PCL3 could effectively degrade carbofuran at the initial carbofuran concentration of 5-100 mg l^{-1} with no significantly different in the half-lives. The isolate PCL3 in free cell forms was inhibited by carbofuran concentration greater than 100 mg l^{-1} thus the substrate inhibition model was used to explain its biodegradation kinetic. Immobilization technique was able to reduce the inhibitory effect of carbofuran on the isolate PCL3 at high concentration which indicated by the survival of the immobilized PCL3 at the concentration up to 200 mg l^{-1}. Carbofuran removal efficiency from soil and aqueous phases by immobilized PCL3 in a larger scale was explored by the soil slurry phase sequencing batch reactors and the sequencing batch reactors (SBRs), respectively. Bioaugmentation of the immobilized PCL3 was the most efficient method to remove carbofuran from contaminated soil in the soil slurry phase reactors. Desorption of carbofuran from the soil to liquid phase played the important role for the biological degradation process of carbofuran in the soil slurry phase reactors. Successful removal of carbofuran in the SBRs by the immobilized PCL3 was obtained at the optimum HRT of 8 d. Biostimulation technique by the addition of rice brand could enhance the efficiency of carbofuran removal up to 100% at the maximum carbofuran of 40 mg l^{-1}. Results indicated that the SBRs together with bioaugmentation and biostimulation gave approximately 3.5 times improvement of carbofuran degradation in comparison to batch reactor.

7.2 Recommendations

The results demonstrated a successful application of *Burkholderia cepacia* PCL3 to bioremeidate carbofuran in soil and aqueous phases as well as in batch and continuous modes. For bioremediation of carbofuran in the contaminated

environments, we suggest to use PCL3 in immobilized cell form. The *in situ* bioaugmentation of carbofuran by directly putting the immobilized PCL3 to the contaminated area should be conducted with the advantage of cost effective. This technique can be applied to remove carbofuran from contaminated agricultural area as well as surface runoff through agricultural area. In the case of high carbofuran concentration, such wastewater discharged from manufacturing plants, the leakage from the storage containers and rinsate as well as the accidental spills, the bioreactor augmented with immobilized PCL3 was recommended for efficient remediation of carbofuran. SBR augmented with immobilized PCL3 was a suitable technology to remove carbofuran from the contaminated aqueous. The organic amendments containing mainly nitrogen source such as rice brand and spent yeast powder from the beer manufacturing process can be used to stimulate carbofuran degradation ability of PCL3. The soil slurry phase reactor technology augmented with immobilized PCL3 can be applied to bioremediate carbofuran contaminated in soil and sediments. However, the soil to liquid ratio should be taken into a consideration in order to minimize the volume of contaminated matrices as well as to reduce the cost for reactor operation.

The future experiments that should be conducted in order to fill in the gaps of knowledge on the application of the isolate PCL3 are recommended as follows:

1) The experimental field study in order to obtain the suitable method for applying the immobilized PCL3 to the carbofuran contaminated site.
2) The soil column experiment in order to determine the fate and transport of carbofuran in soil.
3) The cloning of the lux (*luciferase*) gene into the isolate PCL3 in order to monitor the isolate PCL3 after its application to soil.
4) The protein engineering of the isolate PCL3 in order to increase its capability to degrade carbofuran and metabolites.

REFFERENCES

Aiba, S., Shoda, M., and Nagatami, M. 1968. Kinetics of product inhibition in alcohol fermentation. Biotechnol. Bioeng. 10: 845-864.

Adamson, D.T., McDade, J.M., and Hughes, J.B. 2003. Inoculation of DNAPL source zone to initiate reductive dechlorination of PCE. Environ. Sci. Technol. 37: 2525-2533.

Akhtar, N., Iqbal, M., Azfar, S.I., and Iqbal, J. 2008. Biosorption characteristics of unicellular green alga *Chlorella sorokiniana* immobilized in loofa sponge for removal of Cr(III). J. Environ. Sci. 20: 231-239.

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

Ambrosoli, R., Negre, M., and Gennari, M. 1996. Indication of the occurrence of enhanced biodegradation of carbofuran in some Italian soil. Soil. Bio. Biochem. 28: 1749-1752.

Andreoni, V., Baggi, G., Colombo, M., Cavalca, L., Zangrossi, M., and Bernasconi, S. 1998. Degradation of 2,4,6-trichlorophenol by a specialised organism and by indigenous soil microflora: bioaugmentation and self-remediability for soil restoration. Lett. Appl. Micro. 27: 86-92.

Arshad, M., Hussain, S., and Saleem, M. 2008 Optimization of environmental parameters for biodegradation of alpha and beta endosulfan in soil slurry by Pseudomonas aeruginosa. J. Appl. Microbiol. 104: 364-370

Arvin, E. 1991. Biodegradation kinetics of chlorinated aliphatic hydrocarbon with methane oxidizing bacteria in anaerobic fixed biofilm reactor. Wat. Res. 25: 873-881.

Atagana, H.J. 2003. Biodegradation of phenol, *o*-cresol, *m*-cresol and *p*cresol by indigenous soil fungi in soil contaminated with cresolate. World J. Micro. Biotechnol. 20: 851–858.

Balfanz, J., and Rehm, H.J. 1991. Biodegradation of 4-chlorophenol by adsorptive immobilized *Alcaligenes* sp A 7-2 in soil. Appl. Micro. Biotechnol. 35: 662-668.

Bano, N., and Musarrat, J. 2004. Characterization of a novel carbofuran degrading *Pseudomonas* sp. with collateral biocontrol and plant growth promoting potential. FEMS Microbiol. Lett. 231: 13-17.

Barahona, L.M., Vazquez, R.R., Velasco, M.H., Jarquin, C.V., Perez, O.Z., Cantu, A.M., and Albores, A. 2004. Diesel removal from contaminated soils by biostimulation and supplementation with crop residues. Appl. Soil. Ecol. 27: 165-175.

Barbotin, J.N., and Nava Saucedo, J.E. 1998. Bioencapsultaion of living cells by entrapment in polysaccharide gel. Polysaccharide structural diversity and functional versatility. New York: Marcel Dekker.

Bardi, E.P., and Koutinas, A.A. 1994. Immobilization of yeast on delignified cellulosic material for room temperature and low-temperature wine making. J. Agri. Food. Chem. 42: 221-226.

Baron, R.L. 1991. Handbook of pesticide toxicology. New York: Academic Press.

Beaudet, R., Levesque, M.J., Villemur, R., Lanthier, M., Chenier, M., Lepine, F., and Bisaillon, J.G. 1998. Anaerobic biodegradation of pentachlorophenol in a contaminated soil inoculated with a methanogenic consortium or with Desulfitobacterium frappieri strain PCP-1. Appl. Micro. Biotechnol. 50: 135-141.

Bekatorou, A., Kourkoutas, Y., Banat, I.M., Marchant, R., and Koutinas, A.A. 2004. Immobilization technologies and support materials suitable in alcohol beverages production: a review. Food Microbiol. 21: 377-397.

Benimeli, C.S., G Castro, .R. A., Chaile, P., and Amoroso, M.J. 2008. Lindane uptake and degradation by aquatic *Streptomyces* sp. strain M7. Int. Biodeter. Biodegrad. 59: 148-155.

Bento, F.M., Camargo, F.A.O., Okeke, B.C., and Frankenberger, W.T. 2005. Comparative bioremediation of soils contaminated with diesel oil by natural attenuation biostimulation and bioaugmentation. Bioresour. Technol. 96: 1049-1055.

Beshay, U., Abd-El-Haleem, D., Moawad, H., and Zaki, S. 2002. Phenol biodegradation by free and immobilized *Acinetobacter*. Biotechnol. Lett. 24: 1295-1297.

Beunink, J., and Rehm, H.J. 1988. Synchronous anaerobic and aerobic degradation of DDT by an immobilized mixed culture system. Appl Micro Biotechnol 29: 72 -80.

Beunink, J., and Rehm, H.J. 1990. Coupled reductive and oxidative degradation of 4-chloro-2-nitrophenol by a co-immobilized mixed culture system. Appl. Micro. Biotechnol. 34: 108-115.

Bickerstaff, G.F. 1997. Immobilization of enzyme and cell. Humanan Press. Titowa. New Jersey.

Birnbaum, S., Pendleton, R., Larsson, P.O., and Mosbach, K. 1981. Covalent stabilization of alginate gel for entrapment of living whole cells. Biotechnol. Lett. 3: 393-400.

Boon, N., Top, E.M., Verstraete, W., and Siciliano, S.D. 2003. Bioaugmentation as a tool to protect the structure and function of an activatedsludge microbial community against a 3-chloroaniline shock load. Appl. Environ. Microbiol. 69: 1511-1520.

Braud, A., Jézéquel, K., and Lebeau, T. 2007. Impact of substrates and cell immobilization on siderophore activity by *Pseudomonads* in a Fe and/or Cr, Hg, Pb containing-medium. J. Hazardous. Mater. 144: 229-239.

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

Carrera, J. Vicent, T., and Lafuente, J. 2004. Effect of influent COD/N ratio on biological nitrogen removal (BNR) from high-strength ammonium industrial wastewater. Process. Biochem. 39: 2035–2041.

Carvalho, M.F., Ferreira Jorge, R., Pacheco, C.C., De Marco, P., and Castro, M.L. 2005. Isolation and properties of a pure bacterial strain capable of fluorobenzene degradation as sole carbon and energy source. Environ. Microbiol. 7: 294–298.

Celis, E., Elefsiniotis, P., and Singhal, N. 2008. Biodegradation of agricultural herbicide in sequencing batch reactors under aerobic or anaerobic conditions. Wat. Res. 42: 3218-3224.

Chang, H.K., and Zylstra, G.J. 1999. Novel organization of the genes for phthalate degradation from *Burkholderia cepacia* DBO1. J. Bacteriol. 180: 6529-6537.

Chapman, R.A., and Cole, C.M. 1982. Observations on the influence of water and soil pH on the persistence of pesticides. J. Environ. Sci. Health 17: 487-504.

Chapatwala, K.D., Babu, G.R.V., and Wolfram, J.H. 1993. Screening of encapsulated microbial cells for the degradation of inorganic cyan- ides. J. Ind. Microbiol. 11: 69-72.

Chaudhry, G.R., and Ali, A.N. 1988. Bacterial metabolism of carbofuran. Appl. Environ. Microbiol. 54: 1414-1419.

Chaudhry, G.R., Mateen, A., Kaskar, B., Sardessai, M., Bloda, M., Bhatti, A.R., and Walia S.K. 2002. Induction of carbofuran oxidation to 4-hydroxycarbofuranby *Pseudomonas* sp. 50432. FEMS Microbiol. Lett. 214: 171-176.

Chen, H.J., Guo, G.L., Tseng, D.H., Cheng, C.L., and Huang, S.L. 2006. Growth factors, kinetics and biodegradation mechanism associated with *Pseudomonas nitroreducens* TX1 grown on octylphenol polyethoxylates J. Environ. Manage. 80: 279-286.

Chin, H., Elefsiniotis, P., and Singhal, N. 2005. Biodegradation of 2,4-dicholophenoxyacetic acid using an acidogenic anaerobic sequencing batch reactor. J. Environ. Eng. Sci. 4: 57-63.

Chirnside, A. E.M., Ritter, W.F., and Radosevich, M. 2007. Isolation of a selected microbial consortium from a pesticide-contaminated mix-load site soil capable of degrading the herbicides atrazine and alachlor. Soil Biol. Biochem. 39: 3056-3065.

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

Chu, W., Lau, T.K., and Fung, S.C. 2006. Effects of combined and sequential addition of dual oxidants ($H_2O_2/S_2O_8^{2-}$) on the aqueous carbofuran photodegradation. J. Agric. Food. Chem. 54: 10047-10052.

Cohen, S.Z. 1996. Pesticides in ground water in the United States: monitoring modeling and risks form the U.S. perspective. Environ. Sci. Health 31: 345-352.

Dams, R.I., Paton, G.I., and Killham, K. 2007. Rhizoremediation of pentachlorophenol by Sphingobium chlorophenolicum ATCC 39723. Chemosphere 68: 864-870.

Danne, L.L., and Ha blom, M.M. 1999. Earthworm egg capsules as vectors for the environmental introduction of biodegradative bacteria. Appl. Environ. Microbiol. 65: 2376-2381.

Da Silva, M.L.B., and Alvarez, P.J.J. 2004. Enhanced anaerobic biodegradation of benzene-toluene-ethyl-benzene-xylene-ethanol mixtures in bioaugmented aquifer columns. Appl. Environ. Microbiol. 70: 4720-4726.

Department of Agriculture (DOA). 2003. Statistics of imported pesticides to Thailand [Microsoft Word file]. Ministry of Agriculture and Cooperatives: Bangkok; Thailand [2004, November 20]

Desaint, S., Hartmann, A., Parekh, N. R., and Fournier, J.C. 2000. Genetic diversity of carbofuran-degrading soil bacteria. FEMS Microbiol. Ecol. 34: 173-184.

Detomaso, A., Mascolo, G., and Lopez, A. 2005. Characterization of carbofuran photodegradation by-products by liquid chromatography/hybrid quadrupole time-of-flight mass spectrometry. Rapid Communicat Mass Spectromet 19: 2193–2202.

De Melo Plese, L.P., Paraiba, L.C., Foloni, L.L., and Trevizan, L.R.P., Kinetics of carbosulfan hydrolysis to carbofuran and the subsequent degradation of this last compound in irrigated rice fields. Chemosphere 60: 149-156.

De Vries, S., Evans, O. 1999. Carbofuran [Online]. Available from: http://wvlc.uwaterloo.ca/biology447/Assignments/Assignment1Submissions/On-Campus/carbofuran/Carbofuran.html. [2003, May 4]

DPR Ecotox database. 2002. Pesticide Chemistry Database. Environmental Monitoring Branch, California, Department of Pesticide Regulation.

Deuel, L.E. JR., Price, J.D., Turner, F.T., and Brown, K.W. 1979. Persistence of carbofuran and its metabolites, 3-keto and 3-hydroxy carbofuran, under flooded rice culture. J. Environ. Qual. 8: 23-26.

Duquenne, P., Parekh, N.R., Catroux, G., and Fournier, J.C. 1996. Effect of inoculant density formulation dispersion and soil nutrient amendment on the removal of carbofuran residues from contaminated soil. Soil Bio. Biochem. 28: 1805-1811.

Dybas, M.J., Hyndman, D.W., Heine, R., Tiedje, J., Linning, K., Wiggert, D., Voice, T., Zhao, X., Dybas, L., and Criddle, C.S. 2002. Development, operation, and long-term performance of a full-scale biocurtain utilizing bioaugmentation. Environ. Sci. Technol. l36: 3635-3644.

Edwards, V.H. 1970. The influence of high substrate concentrations on microbial kinetics. Biotechnol. Bioeng. 12: 679-712.

Eisler, R. 1985. Carbofuran hazards to fish, wildlife, and invertebrates: a synoptic review. U.S. Fish and Wildlife Service Biological Report 85: 36.

EPA. 2006. Interim Registration Eligibility Decision (IRED), Document for Carbofuran: Special review and re-registration division [Online]. Available from: http://www.epa.gov/oppsrrd1/reregistration/REDs/carbofuran_ired.pdf [2008, June 30]

Ernst, C., and Rehm, H.J. 1995. Development of a continuous system for the degradation of a cyanuric acid by adsorbed *Pseudomonas* sp. NRRL B-12228. Appl. Microbiol. Biotechnol. 43: 150-155.

Evert, S. 2002. Environmental fate of carbofuran [Online]. Available from: http://www.cdpr.ca.gov/docs/empm/pubs/fatememo/carbofuran.pdf [2004, March 22]

Extoxnet (Extension Toxicology Network). 1996. Pesticide Information Profiles: Carbofuran. [Online] Available from: http://extoxnet.orst.edu/pips/carbofur. Htm [2003, April 24]

Fantroussi, S.E., and Agathos, S.N. 2005. Is bioaugmentation a feasible strategy for pollutant removal and site remediation? Curr. Opinion Microbiol. 8: 268-275.

Fantroussi, S., Verschuere, L., Verstraete, W., and Top, E.M., 1999. Effect of phenylurea herbicides on soil microbial communities estimated by analysis of 16S rRNA gene fingerprints and community level physiological profiles. Appl. Environ. Microbiol. 65: 982-988.

Feng, X., Ou, L.T., and Ogram, A. 1997. Plasmid-mediated mineralization of carbofuran by Sphingomonas sp. strain CFO6. Appl. Environ. Microbiol. 63: 1332–1337.

Fennell, D.E., Underhill, S.E., and Jewell, W.J. 1992. Methanotrophic attached-film reactor development and biofliter characteristics. Biotechol. Bioeng. 40: 1218-1232.

Fennell, D.E., Underhill, S.E., and Jewell, W.J. 1993. TCE degradation in methanotrophic attached-film bioreactor Biotechnol. Bioeng. 40: 859-872.

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

Ferrell, M.A., and Aagard, S.D. 2003. Pesticide storage facility design and management plan: pesticide education program fact sheet [Online]. Available from: http://www.uwyo.edu/CES/PUBS/MP93_Series/mp-93.21_facility .pdfpatein [2004, June 24]

Ferschl, A., Loidl, M., Ditzelmuller, G., Hinteregger, C., and Streichs-bier, F. 1991. Continuous degradation of 3-chloroaniline by calcimnalginate-entrapped cells of *Pseudomonas acidovorans* CA28: influence of additional substrates. Appl. Microbiol. Biotechnol. 35: 544- 550.

FMC corporation. 2008. Thailand imported Furan 3G [Microsoft PowerPoint file]. FMC Corporation: 1735 Market Street Philadelphia, PA 19103, USA. [2008, 25 November]

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

Gentili, A.R., Cubitto, M.A., Ferrero, M., and Rodriguez, M.S. 2006. Bioremediation of crude oil polluted seawater by hydrocarbon-degrading bacteria strain immobilized on chitin and chitosan fakes. Int. Biodeteriorat. Biodegrad. 57: 222-228.

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

Goux, S., Shapir, N.E., Fantroussi, S., Lelong, S., Agathos, S.N., and Pussemier, L. 2003. Long term maintenance of rapid atrazine degradation in soils inoculated with atrazine degraders. Wat Air Soil Pollut Focus 3: 131-142.

Gu, Y.X., Hu, Z.C., and Korus, R.A. 1994. Effect of adsorption on mass transfer in immobilization matrices. Chem. Eng. J. Biochem. Eng. J. 54: B1-B8.

Guiot, S.R., Tartakovsky, B., Lanthier, M., Levesque, M.J., Manuel, M.F., Beaudet, R., Greer, C.W., and Villemur, R. 2002. Strategies for augmenting the pentachlorophenol degradation potential of UASB anaerobic granules. Wat. Sci. Technol. 45: 35-41.

Gupta, K.V., Suhas, A.I., and Saini, V.K. 2006. Adsorption of 2,4-D and carbofuran pesticides using fertilizer and steel industry wastes. J. Colloid. Interface. Sci. 299: 556-563.

Hance, R.J. 1973. Effect of pH on the degradation of atrazine, dichlorprop, linuron and propyzamide in soil. Pesticide Sci. 10: 83-86.

Haldane, J.B.S. 1930. Enzymes, Longmans, Green and Co., Ltd, Great Britain. (Reprinted in 1965 by the Massachusetts Institute of Technology).

Halden, R.U., Tepp, S.M., Halden, B.G., and Dwyer, D.F. 1999. Degradation of 3-phenoxybenzoic acid in soil by *Pseudomonas pseudoalcaligenes* POB310 (pPOB) and two modified Pseudomonas strains. Appl. Environ. Microbiol. 65: 3354-3359.

Hallas, L.E., Adams, W.J., and Heitkamp, M.A. 1992. Glyphosate degra- dation by immobilized bacteria: field studies with industrial wastewater effluent. Appl. Environ. Microbiol. 58: 1215-1219.

Hayatsu, M., Hirano, M., and Tokuda, S. 2000. Involvement of two plasmids in fenitrothion degradation by *Burkholderia* sp. strain NF100. Appl. Environ. Microbiol. 66: 1737-1740.

Head, I.M., Cain, R.B., and Suett, D.L. 1992. Characterization of a carbofuran-degrading bacterium and investigation of the role of plasmids in catabolism of the insecticide carbofuran. Archives Microbiol. 158: 302-308.

Heinze, U., and Rehm, H.J. 1993. Biodegradation of dichloroacetic acid by entrapped and adsorptive immobilized *Xanthobacter auto-trophicus* GJ10. Appl. Microbiol. Biotechnol. 40: 158-164.

Heitkamp, M.A., Camel, V., Reuter, T.J., and Adams, W.J. 1990. Biodegradation of p-nitrophenol in an aqueous waste stream by immobilized bacteria. Appl. Environ. Microbiol. 56: 2967-2973.

Hong, Q., Zhang, Z., Hong, Y., and Li, S. 2007. A microcosm study on bioremediation of fenitrothion-contaminated soil using *Burkholderia* sp. FDS-1. Int. Biodeterior. Biodegrad. 59: 55–61.

Howard, P.H. 1991. Handbook of environmental fate and exposure data for organic chemicals: Pesticides Chelsea. Miami: Lewis Publishers.

Hu, Z.C., Korus, R.A., Levinson, W.E., and Crawford, R.L. 1994. Adsorption and biodegradation of pentachlorophenol by polyurethane immobilized *Flavobacterium.* Environ. Sci. Technol. 28: 491-496.

IDPID (Information Division of the Plant Industry Directorate). 2003. Special Review of Carbofuran Insecticide: Effects on Avian Fauna and Value to Agriculture [Online]. Available from: http://www.hcsc.gc.ca/pmra-arla/English/pdf/prdd/prdd_d9302-e.pdf [2004, April 2]

Iqbal, M., Saeed, A., Edyvean, R.G.J., Sullivan, B.O., and Styring, P. 2005. Production of fungal biomass immobilized loofa sponge (FBILS)-discs for the removal of heavy metal ions and chlorinated compounds from aqueous solution. Biotechnol. Lett. 27: 1319-1323.

Jasper, D.A. 1994. Bioremediation of agricultural and forestry soils with symbiotic microorganisms. Aust. J. Soil Res. 32:1301-1319.

Jiang, J., Zhang, R., Li, R., Gu, J.D., and Li, S. 2007. Simultaneous biodegradation of methyl parathion and carbofuran by a genetically engineered microorganism constructed by mini-*Tn 5* transposon. Biodegradation 18: 403-412.

Jimoh, A. 2004. Effect of immobilized materials on *Saccharomyces cerevisiae.* Assumption U. J. Technol. 8: 62-68.

Jinan, Y.L., and Speers, R.A. 1998. Flocculation of *Sacharomyces cerevisiae*. Food Res. Int. 31: 421-440.

Jitnuyanont, P., Sayavedra-Soto, L.A., and Semprini, L. 2001. Bioaugmentation of butane-utilizing microorganisms to promote cometabolism of 1,1,1-trichloroethane in groundwater microcosms. Biodegradation 12: 11-22.

Karns, J.S., Mulbry, W.W., Nelson, J.O, and Kearney, P.C. 1986. Metabolism of carbofuran by a pure bacterial culture. Pesticide Biochem. Physiol. 25: 211-217.

Karpouzas, D.G., Morgan, J.A.W., and Walker, A. 2000. Isolation and characterization of 23 carbofuran-degrading bacteria from soils from distant geographical areas. Lett Appl. Microbiol. 31: 353-358.

Katzbauer, B., Narodoslawsky, B., and Moser, A. 1995. Classification system of immobilized techniques. Bioprocess Eng. 12: 173-179.

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

Kourkoutas, Y., Bosnea, L., Taboukos, S., Baras, C., Lambrou, D., and Kanellaki, M. 2006. Probiotic cheese production using *Lactobacillus casei* cells immobilized on fruit pieces. J. Dairy. Sci. 89: 1439-1451.

Kumar, N., and Das, D. 2001. Continuous hydrogen production by immobilized *Enterobactor cloacae* IIT-BT 08 using lignocellulosic materials as solid matrices. Enzyme Microbial. Technol. 29: 280-287.

Kumar, K., Devi, S.S., Krishnamurthi, K., Kanade, G.S., Chakrabarti, T. 2007. Enrichment and isolation of endosulfan degrading and detoxifying bacteria. Chemosphere 68: 488-497.

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

Lalah, J.O., Wandiga, S.O., and Dauterman, W.C. 1996. Mineralization, volatilization, and degradation of carbofuran in soil samples from Kenya. Bull. Environ. Contam. Toxicol. 56: 37-41.

Lanthier, M., Tartakovsky, B., Villemur, R., DeLuca, G., and Guiot, S.R. 2002. Microstructure of anaerobic granules bioaugmented with *Desulfitobacterium frappieri* PCP-1. Appl. Environ. Microbiol. 68: 4035-4043.

Lee, W., Wood, T.K., and Chen, W. 2006. Engineering TCE-degrading rhizobacteria for heavy metal accumulation and enhanced TCE degradation. Appl. Environ. Microbiol. 72: 7503-7509.

Lee, C.M., Lu, C.J., and Chuang, M.S. 1994. Effects of immobilized cells on the biodegradation of chlorinated phenols. Wat. Sci. Technol. 30: 87-90.

Lendvay, J.M., Loffler, F.E., Dollhopf, M., Aiello, M.R., Daniels, G., Fathepure, B.Z., Gebhard, M., Heine, R., and Helton, R. 2003. Bioreactive barriers: a comparison of bioaugmentation and biostimulation for chlorinated solvent remediation. Environ. Sci. Technol. 37: 1422-1431.

Li, H., Liu, Y.H., Luo. N., Zhang, X.Y., Luan, T.G., Hu, J.M., Wang, Z.Y., Wu, P.C., Chen, M.J., and Lu, J.L. 2006. Biodegradation of benzene and its derivatives by a psychrotolerant and moderately haloalkaliphilic *Planococcus* sp. strain ZD22. Res. Microbiol. 157: 629-636.

Li, H., Liu, T., Li, Z., and Deng, L. 2008. Low-cost supports used to immobilize fungi and reliable technique for removal hexavalent chromium in wastewater. Bioresour. Technol. 99: 2234-2241.

Lima, D., Viana, P, André, S., Chelinho, S., Costa C., Ribeiro, R., Sousa J.P., Fialho, A.M., and Viegas, C.A. 2009. Evaluating a bioremediation tool for atrazine contaminated soils in open soil microcosms: The effectiveness of bioaugmentation and biostimulation approaches. Chemosphere 74: 187–192.

Lin, J.E., and Wang, H.Y. 1991. Degradation of pentachlorophenol by non-immobilized, immobilized and co-immobilized *Arthrobacter* cells. J. Ferment. Bioeng. 72: 311-314.

Liu, Y.P., Zheng, P., Sun, Z.H., Ni, Y., Dong, J.J., and Zhu, L.L. 2008. Economical succinic acid productionfrom cane molasses by *Actinobacillus succinogenes*. Bioresour. Technol. 99: 1736-1742.

Lodha, B., Bhat, P., Kumar, M.S., Vaidya, A.N., Mudliar, S., Killedar, D.J., and Chakrabarti, T. 2007. Bioisomerization kinetics of γ-HCH and biokinetics of *Pseudomonas aeruginosa* degrading technical HCH. Biochem. Eng. J. 35: 12-19.

Lü, Z., Min, H., Wu, S.W., and Ruan, A.D. 2003. Phylogenetic and degradation characterization of *Burkholderia cepacia* WZ1 degrading herbicide quinclorac. J. Environ. Sci. Heath B38**:** 771–782.

Luong, J.H.T. 1987. Generalization of Monod kinetics for analyses of growth data with substrate inhibition. Biotechnol. Bioeng. 29: 242-248.

Ma, Y.L., Yang, B.L., Zhao, J.L. 2006. Removal of H2S by *Thiobacillus denitrificans* immobilized on different matrices. Bioresour. Technol. 97, 2041-2046.

Ma, Y.L., Yang, B.L., and Zhao, J.L., 2006. Removal of H_2S by *Thiobacillus denitrificans* immobilized on different matrices. Bioresour. Technol. 97: 2041-2046.

Mabury, S.A., and Crosby, D.G. 1996. Pesticides reactivity toward hydroxyl and its relationship to field persistence. J. Agric. Food. Chem. 44: 1920-1924.

Mangat, S.S., and Elefsiniotis, P. 1999. Biodegradation of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) in sequencing batch reactors. Wat. Res. 33: 861-867.

Mahalakshmi, M., Arabindoo, B., Palanichamy, M., and Murugesan, V. 2007. Photocatalytic degradation of carbofuran using semiconductor oxides. J. Hazard. Material. 143: 240-245.

Major, D.W., McMaster, M.L., Cox, E.E., Edwards, E.A., Dworatzek, S.M., Hendrickson, E.R., Starr, M.G., Payne, J.A., and Buonamici, L.W. 2002. Field demonstration of successful bioaugmentation to achieve dechlorination of tetrachloroethene to ethane. Environ. Sci. Technol. 36: 5106-5116.

Majumder, P.S., and Gupta S.K. 2009. Effect of influent pH and alkalinity on the removal of chlorophenols in sequential anaerobic–aerobic reactors. Bioresour. Technol. 100: 1881-1883 .

Mallouchos, A., Reppa, P., Aggelis, G., Kanellaki, M., Koutinas, A.A., and Komaitis, M. 2002. Grape skins as a natural support for yeast immobilization. Biomed.

Life Sci. 24: 1331-1335.

Manohar, S., Kim, C.K., and Karegoudar, T.B. 2001. Enhanced degradation of naphthalene by immobilization of *Pseudomonas* sp. strain NGK1 in polyurethane foam. App. Microbiol. Biotechnol. 55: 311-316.

Mansee, A.H., Chen, W., and Mulchandani, A. 2000. Biodetoxification of coumaphos insecticide using immobilized *Escherichia coli* expressing organophosphorus hydrolase enzyme on cell surface. Bioproc. Eng. 5: 436-440.

Mo, K., Lora, C.O., Wanken, A.E., Javanmardian, M., Yang, X., and Kulpa, C.F. 1997. Biodegradation of methyl *tert*-butyl ether by pure bacterial cultures. Appl. Microbiol. Biotechnol. 47: 69-72.

Mohammadi, A., and Nasernejad, B., 2009. Enzymatic degradation of anthracene by the white rot fungus *Phanerochaete chrysosporium* immobilized on sugarcane bagasse J. Hazard. Mater. 161: 534-537.

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

Muyima, N.Y.O., and Cloete, T.E. 1995. Phosphate uptake by immobilization *Acinetobacter* in a full scale activated sludge plant. J. Industrial Microbiol. 15: 19-24.

Nawaz, M.S., Franklin, W., and Cerniglia, C.E. 1992. Degradation of acry- lamide by immobilized cells of a *Pseudomonas* sp. and *Xanthomonas maltophilia.* Can. J. Microbiol. 39: 207-212.

Ngampongsai, A. 1990. Degradation and residues of carbofuran in soil and sweet corn. [Master's Thesis] Agriculture. Graduate School, Kasetsart University.

Ogram, A.V., Duan, Y.-P., Trabue, S.L., Feng, X., Castro, H., and Ou, L.-T. 2000. Carbofuran degradation mediated by three related plasmid systems. FEMS Microbiol. Ecol. 32: 197-203.

Olaniran, O.A., Pillay, D., and Pillay, B. 2005. Biostimulation and bioaugmentation enhances aerobic biodegradation of dichloroethanes. Chemosphere 63: 1-9.

Omar, S.H., Budecker, U., and Rehm, H.J. 1990. Degradation of oily sludge from a flotation unit by free and immobilized microorganisms. Appl. Microbiol. Biotechnol. 34: 259-263.

O'Reilly, K.T., Kadakia, R., Korus, R.A., and Crawford, R.L. 1988. Utilization of immobilized-bacteria to degrade aromatic compounds common to wood-treatment wastewaters. Wat. Sci. Technol. 20: 95-100.

O'Reilly, K.T., and Crawford, R.L. 1989. Kinetics of p-cresol degradation by an immobilized *Pseudomonas* sp. Appl. Environ. Microbiol. 55: 866-870.

Ou, L.T., Gancarz, D.H., Wheeler, W.B., Rao, P.S.C., and Davidson, J.M. 1982. Influence of soil temperature and soil moisture on degradation and metabolism of carbofuran in soils. J. Environ. Qual. 11: 293-298.

Overmeyer, C., and Rehm, H.J. 1995. Biodegradation of 2-chloro- ethanol by freely suspended and adsorbed immobilized *Pseudomonas putida* US2 in soil. Appl. Microbiol. Biotechnol. 43: 143-149.

Parekh, N.R., Hartmann, A., and Fournier, J.C. 1996. PCR detection of the mcd gene and evidence of sequence homology between the degradative genes and plasmid from diverse carbofuran-degradaing bacteria. Soil Biol. Biochem. 28: 1797-1804.

Pattanasupong, A., Nagase, H., Sugimoto, E., Hori, Y., Hirata, K., Tani, K., Nasu, M., and Miyamoto, K. 2004. Degradation of carbendazime and 2,4 dichlorophenoxyacetic acid by immobilized consortium on loofa sponge. Biosci. Bioeng. J. 98: 28-33.

Peng, X., Zhang, J.S., Li , Y.Y., Li, W., Xu, G.M., and Yan, Y.C., 2008. Biodegradation of insecticide carbofuran by *Paracoccus* sp. YM3. J. Environ. Sci. Health B43: 588 – 594.

Perez, B.A., Corral, O.L., Linares, L.F., Garcia, F.E., and Vazquez, R.R. 2004. Biostimulation of microorganisms from sugarcane bagasse pith for the removal of weathered hydrocarbon from soil. Appl. Microbiol. Lett. 38: 373-377.

Peter, D. F. 2000. Bioaugmentation of atrazine and fenamiphos impacted groundwater: Laboratory Evaluation. Biorem. J. 4: 237-248.

Phelps, T.J., Niedzielski, J.J., Schram, R.M., Herbes, S.E., and White, D.C. 1990. Biodegradation of trichloroethylene in continuous recycle expanded-bed bioreactor. Appl. Environ. Microbiol. 56: 1702-1709.

Plangklang, P. 2004. Degradation of carbofuran by rhizosphere soil microorganisms. Master’s Thesis. Environmental Management (Interdisciplinary Program), Graduate School, Chulalongkorn University.

Plangklang, P., and Reungsang, A. 2008. Effects of rhizosphere remediation and bioaugmentation on carbofuran removal from soil. World J. Microbiol. Biotechnol. 24: 983-989.

Plangklang, P., Reungsang, A. 2009. Bioaugmentation of carbofuran residues in soil using *Burkholderia cepacia* PCL3 adsorbed on agricultural residues. Int. Biodeterior. Biodegrad. (In press) doi:10.1016/j.ibiod.2009.02.003.

Portier, R.J., and Fujisaki, K. 1986. Continuous biodegradation and detoxification of chlorinated phenols using immobilized bacteria. Toxic. Assess. 1: 501-513.

Prasanna, D., Venkata-Mohan, S., Purushotham, R.B., and Sarma, P.N. 2008. Bioremediation of anthracene ontaminated soil in bio-slurry phase reactor operated in periodic discontinuous batch ode. J. Hazard. Material. 153: 244-251.

Protzman, R.S., Lee, P.H., Ong, S.K., and Moorman, B.T. 1999. Treatment of formulated atrazine rinsate by *Agrobacterium radiobacter* strain J14a in a sequencing batch biofilm reactor. Wat. Res. 33:1399-1404.

Quintero, J.C., Lú-Chau, T.A., Moreira, M.T., Feijoo, G., and Lema, J.M. 2007. Bioremediation of HCH present in soil by the white-rot fungus *Bjerkandera adusta* in a slurry batch bioreactor Int. Biodeterior. Biodegrad. 60: 319-326.

Quintero, J.C., Moreira, M.T., Feijo, G., and Lema, J.M. 2005. Anaerobic degradation of hexachlorocyclohexane isomers in liquid and soil slurry systems. Chemosphere 61: 528-536.

Quintero, J.C., Moreira, M.T., Lema, J.M., and Feijoo, G. 2006. An anaerobic bioreactor allows the efficient degradation of HCH isomers in soil slurry. Chemosphere 63: 1005-1013.

Qiu-Xiang, Y., Qing, H., Peng, H., Xiao-Jun, D., Yu-Jia, S., and Shun-Peng, L. 2007. Isolation and characterization of a carbofuran-degrading strain *Novosphingobium* sp. FND-3. FEMS Microbiol. Lett. 271: 207-213.

Rachman, M.A., Nakashimada, Y., Kakizono, T., and Nishio, N., 1998. Hydrogen production with high yield and high evolution rate by self-focculated cells of *Enterobacter aerogenes* in a packed-bed reactor. Appl. Microbiol. Biotechnol. 49: 450-454.

Raha, P., and Das, A.K. 1990. Photodegradation of carbofuran. Chemosphere 21: 99-106.

Rahman, K.S.M., Rahman, T.J., Kourkoutas, Y., Petsas, I., Marchant, R., and Banat, I.M. 2003. Enhanced bioremediation of n-alkane in petroleum sludge using

bacterial consortium amended with rhamnolipid and micronutrients. Bioresour. Technol. 90: 159-168.

Raina, V., Suar, M., Singh, A., Prakash, O., Dadhwal, M, Gupta, S.K., Dogra, C., Lawlor, K., Lal, S., van der Meer, R.J., Holliger, C. and Lal, R. 2008. Enhanced biodegradation of hexachlorocyclohexane (HCH) in contaminated soils via inoculation with *Sphingobium indicum* B90A. Biodegradation 19:27–40.

Ramakrishna, M., Venkata-Mohan, S., Shailaja, S., Narashima, R., and Sarma, P.N. 2008. Identification of metabolites during biodegradation of pendimethalin in bioslurry reactor. J. Hazard. Material. 151: 658-661.

Ratusznei, S.M., Rodrigue, J.A.D., Camargo, E.F.M., Zaiat, M., and Borzani, W. 2000. Feasibility of a stirred anaerobic sequencing batch reactor containing immobilized biomass for wastewater treatment. Bioresour. Technol. 75: 127-132.

Reddy, L.V., Reddy, Y.H.K., Reddy, L.P.A., and Reddy, V.S. 2008. Wine production by novel yeast biocatalyst prepared by immobilization on watermelon (*Citrullus vulgaris*) rind pieces and characterization of volatile compounds. Proc. Biochem. 43: 748-752.

Rittmann, B.E., and Whiteman, R. 1994 Bioaugmentation: a coming of age. Wat. Qual. Int. 1:12-16.

Roane, T.M., Josephson, K.L., and Pepper, I.L. 2001. Dual-bioaugmentation strategy to enhance remediation of cocontaminated soil. Appl. Environ. Microbiol. 67: 3208-3215.

Robles-Gonzalez, I.V., Fava, F., and Poggi-Varaldo, H.M. 2008. A review on slurry bioreactors for bioremediation of soils and sediments. Microbiol. Cell Fact. 7: 1-16.

Rouchaud, J., Gustin, F., van Steene, F.D.E., Pelerents, C., Vanparys, L., Gillet, J., Benoit, F., and Eustermans, N. 1990. Comparative soil and plant metabolism of carbosulfan, furathiocarb and carbofuran in Brussels sprouts cauliflowerand sugar beet crops. Toxicol. Environ. Chem. 25: 109-124.

Rousseaux, S., Hartmann, A., Lagacherie, B., Piutti, S., Andreux, F., and Soulas, G. 2003. Inoculation of an atrazine-degrading strain, *Chelatobacter heintzii* Cit1,

in four different soils: effects of different inoculums densities. Chemosphere 51: 569-576.

Ruberto, L., Vazqueza, C.S., and Mac Cormackb, W.P. 2003. Effectiveness of the natural bacterial flora, biostimulation and bioaugmentation on the bioremediation of a hydrocarbon contaminated Antarctic soil. Int. Biodeter. Biodegrad. 52: 115- 125.

Ryeol, P.M., Sunwoo, L., Tae-Ho, H., Byung-Tack, O., Shim, J.H., and Kim, I.S. 2006. A new intermediate in the degradation of carbofuran by *Sphingomonas* sp. Strain SB5. J. Microbiol. Biotechnol. 16: 1306-1310.

Santos, J.C., Converti, A., de Carvalho, W., Mussatto, S.I., and da Silva S.S. 2005. Influence of aeration rate and carrier concentration on xylitol production from sugarcane bagasse hydrolyzate in immobilized-cell fluidized bed reactor Proc. Biochem. 40: 113-118.

Sahasrabudhe, S.R., Modi, A.J., and Modi, V.V. 1988. Dehalogenation of 3-chlorobenzoate by immobilized *Pseudomonas* sp. B13 cells. Biotechnol. Bioeng. 31: 889-893.

Sahinkaya, E., and Dilek, F.B. 2005. Biodegradation of 4-chlorophenol by acclimated and unacclimated activated sludge-evaluation of biokinetic coefficients. Environ. Res. 99: 243-252.

Sahinkaya, E., and Dilek, F.B. 2006. Effect of biogenic substrate concentration on 4-chlorophenol degradation kinetics in sequencing batch reactors with instantaneous feed. J. Hazardous. Material. 137: 282-287.

SAS Institute. 1985. SAS User's guide, Statistics, Version 5ed. SAS Institue. North Carolina: Cary.

Saudagar, P.S., Shaligram, N.S., and Singhal, R.S. 2008. Immobilization of *Streptomyces clavuligerus* on loofah sponge for the production of clavulanic acid. Bioresour. Technol. 99: 2250-2253.

Schwartz, E., Trinh, S.V., and Scow, K.M. 2000. Measuring growth of a phenanthrene-degrading bacterial inoculum in soil with a quantitative competitive polymerase chain reaction method. FEMS Microbiol. Ecol. 34: 1-7.

Seiber, J.N., Catahan, M.P., and Barril, C.R. 1978. Loss of carbofuran from rice paddy water: chemical and physical factors. J. Environ. Sci. Health. 13: 131-148.

Seo, J., Jeon, J., Kim, S.D., Kang, S., Han, J., and Hur, H.G. 2007. Fungal biodegradation of carbofuran and carbofuran phenol by the fungus *Mucor ramannianus*: identification of metabolites. Wat. Sci. Technol. 55: 163-167.

Shapir, N., and Mandelbaum, R.T. 1997. Atrazine degradation in subsurface soil by indigenous and introduced microorganisms. J. Agric. Food. Chem. 45: 4481-4486.

Shieh, W.K., Puhakka, J.A., Melin, E., and Tuhkanen, T. 1990. Immobilized cell degradation of chlorophenols. J. Environ. Eng. 116: 683-697.

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

Siahpush, A.R., Lin, J.E., and Wang, H.Y. 1991. Effect of adsorbents on degradation of toxic organic compounds by coimmobilized systems. Biotechnol. Bioeng. 39: 619-628.

Siddaramappa, R., and Seiber, J.N. 1979. Persistence of carbofuran in flooded rice soils and water. Protect. Wat. Technol. 11: 103-110.

Silva, E., Fialho, A.M., Sá-Correia, I., Burns, R.G., and Shaw, L.J. 2004. Combined Bioaugmentation and Biostimulation To Cleanup Soil Contaminated with High Concentrations of Atrazine. Environ. Sci. Technol. 38: 632–637.

Singer, A.C., Gilbert, E.S., Luepromchai, E., and Crowley, D.E. 2000. Bioremediation of polychlorinated biphenyl-contaminated soil using carvone and surfactant-grown bacteria. Appl. Micro. Biotechnol. 54: 838-843.

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

Slaoui, M., Ouhssine, M., Berny, E., and Elyachioui, M. 2007. Biodegradation of the carbofuran by a fungus isolated from treated soil. African J. Biotechnol. 6: 419-423.

Smith, G.J. 1992. Toxicology and pesticide use in relation to wildlife: organophosphorus and carbamate compounds. Boca Raton, FL: C. K. Smoley: 3-18.

Smith, A.E., Hristova, K., Wood, I., Mackay, D.M., Lory, E., Lorenzana, D., and Scow, K.M. 2005. Comparison of biostimulation versus bioaugmentation with bacterial strain PM1 for treatment of groundwater contaminated with methyl tertiary butyl ether (MTBE). Environ. Health Perspect. 113: 317-322.

Sposito, G. 1980. Derivation of the Freundlich equation for ion exchange reactions in soils. Soil Sci. Soc. Am. J. 44: 652-654.

Steinle, P., Thalmann, P., Hohener, P., Hanselmann, K.W., and Stucki, G. 2000. Effect of environmental factors on the degradation of 2,6-dichlorophenol in soil. Environ. Sci. Technol. 34: 771-775.

Strandberg, G.W., Donaldson, T.L., and Farr, L.L. 1989. Degradation of trichloroethylene and trans-1,2-dichloroethylene by methanotrophic consortium in a fixed-film packed-bed bioreactor. Environ. Sci. Technol. 23: 1422-1425.

Streger, S.H., Vainberg, S., Dong, H., and Hatzinger, P.B. 2002. Enhancing transport of Hydrogenophaga flava ENV735 for bioaugmentation of aquifers contaminated with methyl tert-butyl ether. Appl. Environ. Microbiol. 68: 5571-5579.

Strong, L.C., McTavish, H., Sadowsky, M.J., and Wackett, L.P. 2000. Field-scale remediation of atrazine-contaminated soil using recombinant *Escherichia coli* expressing atrazine chlorohydrolase. Environ. Microbiol. 2: 91-98.

Struthers, J.K., Jayachandran, K., and Moorman, T.B. 1998. Biodegradation of atrazine by *Agrobacterium radiobacter* J14a and use of this strain in bioremediation of contaminated soil. Appl. Environ. Microbiol. 64: 3368-3357.

Tchelet, R., Meckenstock, R., Steinle, P., and van der Meer, J.R. 1999. Population dynamics of an introduced bacterium degrading chlorinated benzenes in a soil column and in sewage sludge. Biodegradation 10: 113-125.

Teerakun, M., Reungsang, A., and Virojanakud, W. 2004. Phytoremediation of carbofuran residues in soil**.** Songklanakarin J. Sci. Technol. 26: 171-176.

Tejada, A.W., and Magallona, E.D. 1985. Fate carbosulfan in a rice paddy environmental. Philipp. Entomol. 6: 255-273.

Thompson, K.T., Crocker, F.H., and Fredrickson, H.L. 2005. Mineralization of the cyclic nitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine by *Gordonia* and *Williamsia* spp. Appl. Environ. Microbiol. 71: 8265–8272.

Tomasek, P.H., and Karns, J.S. 1989. Cloning of a carbofuran hydrolase gene from *Achromobacter* strain WM111 and its expression in Gram-negative bacteria. J. Bacteriol. 171: 4038-4044.

Tongarun, R., Luepromchai, E., and Vangnai A.S. 2008. Natural attenuation, biostimulation, and bioaugmentation in 4-chloroaniline-contaminated soil. Curr. Microbiol. 56: 182–188.

Topp, E., Hanson, R.S., Ringelberg, D.B., White, D.C., and Wheatcroft, R. 1993. Isolation and characterization of an *N*-methylcarbamate insecticide-degrading methylotrophic bacterium. Appl. Environ. Microbiol. 59: 3339-3349.

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

Trotter, D., Kent, R., and Wong, M. 1991. Aquatic fate effect of carbofuran. Crit. Rev. Environ. Cont. 21: 137-176.

Tsuneda, S., Auresenia, J., Inoue, Y., Hashimoto, Y., and Hirata, A. 2002. Kinetic model for dynamic response of three-phase fluidized bed biofilm reactor for wastewater treatment. Biochem. Eng. J. 10: 31-37.

Valo, R.J., Haggblom, M.H., and Salkinoja-Salonen, M. 1990. Bioremedi- ation of chlorophenol containing simulated groundwater by immobilized bacteria. Wat. Res. 24: 253-258.

Venkata-Mohan, S., Prasanna, D., Purushotham-Reddy, B., and Sarma, P.N. 2008. *Ex situ* bioremediation of pyrene contaminated soil in bio-slurry phase reactor operated in periodic discontinuous batch mode: Influence of bioaugmentation. Int. Biodeterior. Biodegrad. 62: 162-169.

Venkata-Mohan, S., Shailaja, S., Rama-Krishna, M., Reddy, K.B., and Sarma, P.N. 2006. Bioslurry phase degradation of di-ethyl phthalate (DEP) contaminated soil in periodic discontinuous mode operation: Influence of bioaugmentation and substrate partition. Process. Biochem. 41: 644-652.

Venkata-Mohan, S., Sirisha, K., Rao, R.S., and Sarma, P.N. 2007. Bioslurry phase remediation of chlorpyrifos contaminated soil: Process evaluation and optimization by Taguchi design of experimental (DOE) methodology. Ecotoxicol. Environ. Safety. 68: 252-262.

Venkata-Mohan, S., Sirisha, K., Rao, N.C., Sarma, P.N., and Reddy, S.J. 2004. Degradation of chlorpyrifos contaminated soil by bioslurry reactor operated in sequencing batch mode: bioprocess monitoring. J. Hazard. Material. B116: 39-48.

Vidali, M. 2001. Boremediation and overview. Pure Appl. Chem. 73: 1163-1172.

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

Wang, Q., and Lemley, T.A. 2003. Oxidative degradation and detoxification of aqueous carbofuran by membrane anodic Fenton treatment. J Hazard Mater 98: 241-255.

Wang, S.G., Liu, X.W., Zhang, H.Y., Gong, W.X., Sun, X.F., and Gao, B.Y. 2007. Aerobic granulation for 2,4-dichlorophenol biodegradation in a sequencing batch reactor. Chemosphere 69: 769-775.

Wenderoth, D.F., Rosenbrock, P., Abraham, W.R., Pieper, D.H., and Hofle, M.G. 2003. Bacterial community dynamics during biostimulation and bioaugmentation experiments aiming at chlorobenzene degradation in groundwater. Microbiol. Ecol. 46: 161-176.

Westmeier, F., and Rehm, H.J. 1985. Biodegradation of 4-chlorophenol by entrapped *Alcaligenes* sp. A 7-2. Appl. Microbiol. Biotechnol. 22: 301-305.

Westmeier, F., and Rehm, H.J. 1987. Degradation of 4-chlorophenol in municipal wastewater by adsorptive immobilized *Alcaligenes* sp. A 7-2. Appl. Microbiol Biotechnol. 26: 78-83.

Wiersinga, J.W., Dessing, M.C., Kager, P.A., Cheng, A.C., Limmathurotsakul, D., Day, N.P., Dondorp, A.M., van der Poll, T., and Peacock , S. J. 2007. High-Throughput mRNA Profiling Characterizes the Expression of Inflammatory Molecules in Sepsis Caused by *Burkholderia pseudomallei.* Infec. Immun. 75: 3074-3079.

Wiesel, I., Wubker, S.M., and Rehm, H.J. 1993. Degradation of polycyclic aromatic hydrocarbons by an immobilized mixed bacterial culture. Appl. Microbiol. Biotechnol. 39: 110-116.

Wisaam, S., Rekabi, A., Qiang, H., and Qiang, W.W. 2007. Review on sequencing batch reactors. Pakistan J. Nutrition 6: 11-19.

Whyte, G.L., Claude, L.B., and Greer, W.H. 1999. Bioremediation assessment of hydrocarbon-contaminated soils from the high arctic. Biorem. J. 3: 69-80.

Wong, K.W., Toh, B.A., and Ting, O.J.P. 2005. Biodegradation of penanthrene by the indigenous microbial biomass in a zinc amended soil. Appl. Micro. Lett. 40: 50-55.

Yan, Q.X., Hong, Q., Han, P., Dong, X.J., Shen, Y.J., and Li, S.P. 2007. Isolation and characterization of a carbofuran-degrading strain *Novosphingobium* sp. FND-3. FEMS Microbiol. Lett. 271: 207-213.

Yen, J.H., Hsiao, F.L., and Wang, Y.S. 1997. Assessment of the insecticide carbofuran's potential to contaminate groundwater through soils in the subtropics. Ecotoxic. Environ. Safe. 38: 260-265.

Young, K.D. 2007. Bacterial morphology: why have different shapes? Current Opinion in Microbiol. 10: 596–600.

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

Yu, C.B., Gary, M., Hansen, D.J., and Larsen, J.R. 1974. Fate of Carbofuran in a Model Ecosystem. J. Agric. Food. Chem. 22: 431-434.

Zaiat, M., Rodrigues, J.A.D., Ratusznei, S.M., Camargo, E.F.M., and Borzani, W. 2001. Anaerobic sequencing batch reactors for wastewater treatment: a developing technology. Appl. Microbiol. Biotechnol. 55: 29-35.

Zakaria, Z.A., Zakaria, Z., Surif S. and Ahmad, W.A. 2007. Biological detoxification of Cr(VI) using wood-husk immobilized *Acinetobacter haemolyticus.* J. Hazard. Mater. 148: 164-171

Zhang, C., Hughes, J.B., Nishino, S.F., and Spain, J. 2006. Slurry-phase biological treatment of 2,4-dinitrotoulene and 2,6-dinitrotoulene: role of bioaugmentation and effects of high dinitrotoulene concentrations. Environ. Sci. Technol. 34: 2810-2816.

Zhang, Y., and Frankenberger, T.W. 2002. Factors affecting removal of selenate in agricultural drainage water utilizing rice straw. Sci. Total. Environ. 305: 207-216.

Zhon, W., Winter, B., and Zimmermann, W. 1993. Dechlorination of high-molecular-mass compounds in spent sulphite bleach effluents by free and immobilized cells of streptomycetes. Appl. Microbiol. Biotechnol. 38: 418-423.

Zilli, M., Daffonchio, D., Felice, R.D., Giordani, M., and Converti, A. 2004. Treatment of benzene contaminated airstream in laboratory-scale biofilter packed with raw and sieved sugarcane bagasse and with peat. Biodegradation 15: 87-96.

APPENDICES

APPENDIX A

SORPTION KINETIC OF CARBOFURAN TO CORNCOB AND SUGARCANE BAGASSE

Freundlich isotherm is expressed as follow:

$$q = K_f C_e^{1/n}$$

Where q is equilibrium concentration of adsorbate in the solid phase; C_e is equilibrium concentration of adsorbate in liquid phase; K_f is sorption capacity (mg kg^{-1}) and $1/n$ is adsorption intensity (kg l^{-1})

Frudich isotherm of carbofuran sorption to corncob and sugarcane bagasse are shown in Figure A-1 in which K_f for carbofuran sorption to corncob and sugarcane bagasse are 0.028 and 0.298 mg kg^{-1}, respectively.

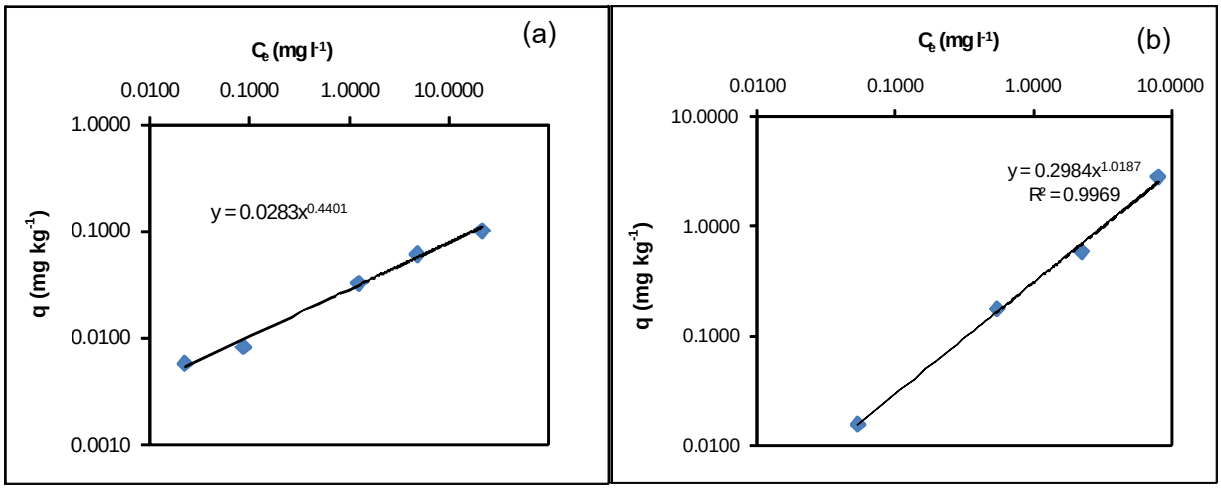

Figure A-1. Frudich isotherm of carbofuran sorption to corncob (a) and sugarcane bagasse (b)

APPENDIX B

LIST OF PUBLICATIONS

Research articles:

Plangklang, P., Reungsang, A., Bioaugmentation of carbofuran residues in soil using *Burkholderia cepacia* PCL3 adsorbed on agricultural residues, International Biodeterioration & Biodegradation (2009), doi:10.1016/j.ibiod.2009.02.003 (In press)

Plangklang, P., Reungsang, A., Effects of rhizosphere remediation and bioaugmentation on carbofuran removal from soil, World Journal of Microbiology and Biotechnology (2008), 24:983-989.

Proceedings:

Plangklang, P., Reungsang, A., Bioaugmentation of carbofuran by *Burkholderia cepacia* PCL3 in bioslurry phase sequencing batch reactor, The International Conference on Environment 2008: Environmental Management and Technologies Towards Sustainable Development, December 15-17, 2008, G Hotel, Penang, Malaysia.

Plangklang, P., Reungsang, A., Phylogenetic analysis and kinetic characterization of carbofuran degraders isolated from carbofuran phytoremediated rhizosphere soil, The 2nd International Conference on Science and Technology for Sustainable Development of Greater Mekong Sub-region (2nd STGMS), October 2-3, 2008, Faculty of Agronomy, Hanoi Agricultural University, Hanoi, Vietnam.

Plangklang, P., Reungsang, A., Bioaugmentation of carbofuran residues in soil by immobilized *Burkholderia cepacia*, PCL3, International Conference on Environmental Quality Concern, Control and Conservation, July 1-2, 2007, Tainan, Taiwan, ROC. D1-D8.

Reungsang, A., **Plangklang, P.,** Degradation of carbofuran using microorganisms isolated from phytoremediated rhizosphere soils. International Symposium 2006 on Pioneering Studies of Young Scientists on Chemical Pollution and Environmental Changes, 17-19 November, 2006, Matsuyama, Japan. 71-74.

Plangklang, P., Reungsang, A., Degradation of carbofuran residues by immobilized carbofuran degrader *Agrobacterium radiobacter* PCL3. International Symposium 2006 on Pioneering Studies of Young Scientists on Chemical Pollution and Environmental Changes, 17-19 November, 2006, Matsuyama, Japan. 75-78.

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Bioaugmentation of carbofuran residues in soil using *Burkholderia cepacia* PCL3 adsorbed on agricultural residues☆

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Immobilization

ABSTRACT

Burkholderia cepacia PCL3 (GenBank accession number of EF990634) is a carbofuran degrader isolated from phytoremediated rhizosphere soil in our laboratory. Free and the immobilized PCL3 on corncob and sugarcane bagasse were investigated for their abilities to degrade carbofuran in Basal Salt Medium (BSM) and soil microcosm. The reusability and survival of immobilized PCL3 in comparison to free cells were also examined. Short half-lives ($t_{1/2}$) of carbofuran of 3–4 d in BSM were obtained using the isolate PCL3 in both free and immobilized cell forms. Immobilized cells could survive (10^6–10^7 cfu ml^{-1}) through 30 d of incubation, while the number of free cells decreased continuously after 10 d. Immobilized *B. cepacia* PCL3 could be reused twice without loss in their abilities to degrade carbofuran in BSM, which suggested an advantage of using immobilized cell over free cell. Free and immobilized cells were augmented into soil and showed an effective capability to remediate carbofuran residues, both of which indicated by 5-folds decrease in carbofuran half-lives in augmented soil. Immobilization of PCL3 on corncob and sugarcane bagasse provided the possibilities of reusing the cells as well as improving the cell survival without decreasing carbofuran degradation activity.

1. Introduction

Carbofuran (2,3-dihydro-2,2 dimethylbenzofuran-7-yl methylcarbamate) is a broad-spectrum insecticide widely used in agriculture to control insects and nematodes on contact or after ingestion. It is of environmental concern because of its relatively high solubility of 351 mg l^{-1} at 25 °C so it is mobile in soil resulting in a high potential for groundwater contamination. Carbofuran can cause acute toxicity to mammals through cholinesterase inhibition ($LD_{50} = 2$ mg g^{-1}) (EPA, 2006).

In our previous study, thirteen different bacterial strains were isolated from carbofuran phytoremediated rhizosphere soils by an enrichment technique (Plangklang, 2004). Their abilities to degrade carbofuran were investigated in Basal Salt Medium (BSM) containing 5 mg l^{-1} of carbofuran. The shortest half-life of carbofuran, 3 d, was found in C-limited BSM cultured with an isolate named PCL3, identified by 16S rRNA as *Burkholderia cepacia* (GenBank accession number of EF990634), indicating that carbofuran was used as its sole C-source. Successful biodegradation of carbofuran using the isolated degraders has been reported (Bano and Musarrat, 2004; Jiang et al., 2007; Qiu-Xiang et al., 2007; Ryeol et al., 2006; Seo et al., 2007; Slaoui et al., 2007), in which most of the carbofuran degraders used were in free cell form. However, there are some limitations in using free cells of the isolates such as low survival ability of free cells in natural conditions, low cell recovery and low capability of cell recycling (Bekatorou et al., 2004; Manohar et al., 2001). Immobilization of cells might overcome these limitations. The use of immobilized systems offers many advantages over free cells including regeneration and reuse of immobilized cells for extend period. The supporting materials may act as protective agents against the effects of pH, temperature, solvent, heavy metals or even substrate and product inhibitions, and enhancing the cell survival (Bekatorou et al., 2004). The support materials used for immobilization could be either synthetic polymers or natural materials. Disposal of synthetic polymer is of the concern due to its non-biodegradable characteristic, unlike the agricultural residues which are natural materials and biodegradable.

Immobilized cells offer advantages over free cells, however, there is limited information on bioaugmentation of carbofuran

☆ *Scientific relevance:* Newly isolated carbofuran degrader, *Burkholderia cepacia* PCL3, was immobilized on corncob and sugarcane bagasse. Its capability in bioremediation of carbofuran in Basal Salt Media and soil was described.

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residues using immobilized carbofuran degraders on agricultural residues. Therefore, this study was designed to examine a degradation of carbofuran residues in synthetic medium and soil by *B. cepacia* PCL3 adsorbed on corncob and sugarcane bagasse in comparison to free cell. Results from these studies would provide information on the possibility of increasing the survival of *B. cepacia* PCL3 by immobilization techniques and the application of immobilized PCL3 in bioaugmentation of carbofuran residues.

2. Materials and methods

2.1. Chemicals and reagents

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

2.2. Microorganism and culture medium

The carbofuran degrader, identified by 16S rRNA as *B. cepacia* PCL3 (GenBank accession number of EF990634), was grown at 30 °C, 150 rpm in Nutrient Broth (NB) (Difco, USA) containing 5 mg l^{-1} of carbofuran for 48 h and used as inoculum for immobilization. *B. cepacia* PCL3 was previously isolated from rhizosphere soil of rice (*Oryza sativa* Linn) treated with carbofuran at a concentration of 5 mg kg^{-1}soil (Teerakun et al., 2004). This microorganism is capable of using carbofuran as a sole C-source (Plangklang, 2004).

Culture medium was a C-limited BSM (Mo et al., 1997) containing carbofuran as a sole carbon source. For BSM agar, 1.5% of bactoagar was added to BSM before sterilization. Carbofuran solution in sterile distilled water at the concentration of 5 mg l^{-1} was coated using glass spreader on BSM agar as a sole C-source prior to use.

2.3. Soils

A sandy loam soil sample, 0–15 cm depth, was collected from rice fields of Ban Nonmuang, A. Muang, Khon Kaen Province. Organic carbon and nitrogen contents of the soil were 0.89% and 0.10%, respectively. Soil pH was 6.9. Soil was passed through a 2 mm sieve and kept in a plastic bag at 4 °C for two weeks before use. The background concentration of carbofuran in the soil sample was 0.06 mg kg^{-1}.

2.4. Supporting materials

Natural supporting materials i.e., corncob and sugarcane bagasse were used to immobilize *B. cepacia* PCL3 because they have a high matrix porosity and a pore size that could enhance cell adsorption. Sugarcane bagasse and corncob were cut into $0.7 \times 0.7 \times 0.7$ cm cubes and then 300 g of each was boiled in 3 l of 1% NaOH for 3 h to remove lignin and fibers which might react with the cells (Bardi and Koutinas, 1994). The alkaline-boiled sugarcane bagasse and corncob were then washed three times with 3 l of distilled water, soaked in distilled water overnight and then sterilized at 121 °C for 15 min and kept at 4 °C prior to use.

2.5. Cell immobilization

Cell immobilization by adsorption was conducted by adding 75 g of sterile corncob and bagasse into the sterile 250 ml NB containing 5 mg l^{-1} of carbofuran before inoculation with the isolate PCL3 (10^6 cfu ml^{-1}). The medium was then incubated at 150 rpm, at room temperature, for 48 h. After incubation, supporting materials were transferred to a fresh NB containing 5 mg l^{-1} of carbofuran and incubated, as previously described, before collection by filtration through Buchner filter funnel. The support materials were washed twice with 0.85% NaCl using aseptic technique. Immobilized cells were kept at 4 °C prior use. The internal cell density on the sugarcane bagasse and corncob was approximately 10^7 cfu g^{-1} dry material. The microstructure of support materials and immobilized cells were investigated by Scanning Electron Microscope (SEM) (Rachman et al., 1998).

2.6. Microbial degradation of carbofuran by free and immobilized cells of *B. cepacia* PCL3 in BSM

Degradation of carbofuran in BSM by *B. cepacia* PCL3 in free cell form was conducted in a 500-ml shake flask in batch experiments. C-limited BSM, 200 ml, containing 5 mg l^{-1} of carbofuran as a sole carbon source, was added into the flasks before inoculation with approximately 10^6 cfu ml^{-1} of *B. cepacia* PCL3. Flasks were then incubated at room temperature and shaken at 150 rpm. Flasks were sampled at days 0, 2, 4, 6, 8, 10, 14, 18, 24, and 30 to determine the number of PCL3 in the culture media and to extract carbofuran and its metabolites i.e., 3-keto carbofuran and carbofuran phenol by liquid–liquid partitioning method. Carbofuran and its metabolites were analyzed by HPLC.

Degradation of carbofuran by the immobilized cells of *B. cepacia* PCL3 in BSM was conducted in 250-ml shake flask in batch experiments. C-limited BSM, 100 ml, containing 5 mg l^{-1} of carbofuran was added to each flask before inoculation with immobilized *B. cepacia* PCL3. This was conducted by weighing 10 g of the immobilized PCL3 with an approximate cell concentration of 10^6 cfu ml^{-1} on a sterile Petri dish and transferring to the flask using sterile forceps. Flasks were then incubated for 30 d and analyzed for carbofuran and metabolite concentrations for the number of PCL3 in support materials and in the culture medium as previously described.

After 30 d of incubation, immobilized cells on corncob and sugarcane bagasse were harvested by filtration through Buchner filter funnel and washed three times using a sterile BSM. Then 10 g of these immobilized cells were re-inoculated into a fresh BSM media containing 5 mg l^{-1} of carbofuran. Concentrations of carbofuran and its metabolites and cell leakage and survival were monitored for another 30 d. This step was repeated twice to investigate the reusability of immobilized cells.

The number of *B. cepacia* PCL3 in culture media, accounted as cells leaked from the support materials, was determined by plate count technique which modified from Zilli et al. (2004). To observe the cell growth and cell survival on the supporting materials, 10 g of wet immobilized cells were taken from culture media and washed with sterile 0.85% NaCl solution three times. The washed immobilized cells were blended to 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 the support materials. The number of *B. cepacia* PCL3 in the liquid phase was determined by plate count on the carbofuran-coated BSM agar.

2.7. Bioaugmentation of carbofuran residues in soil with free and immobilized cells of *B. cepacia* PCL3

Eight sets of bioaugmentation experiments (Table 1) were conducted in soil microcosms in 425 cm^3 glass jars capped with plastic lids. Carbofuran solution, at an initial concentration of 5 mg kg^{-1} dry soil was spiked into 20 g dry weight of soil in each

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Table 1
Soil bioaugmentation experiments.

Treatment	Experimental set up
A	Soil
B	Soil + free cells of PCL3
C	Soil + immobilized PCL3 on corncob
D	Soil + immobilized PCL3 on sugarcane bagasse
E	Autoclaved soil + free cells of PCL3
F	Autoclaved soil + immobilized PCL3 on corncob
G	Autoclaved soil + immobilized PCL3 on sugarcane bagasse
H	Autoclaved soil + autoclaved corncob
I	Autoclaved soil + autoclaved sugarcane bagasse

glass jar and well mixed by hand stirring. Either free cells or immobilized cells of *B. cepacia* PCL3 on corncob and sugarcane bagasse, was added into the soil at the initial cell concentration of 10^6 cfu g^{-1} dry soil. The initial moisture content of the soil samples was adjusted to 15–18% before incubation at an average room temperature of 29 ± 2 °C in the dark. Soil samples were sacrificed at days 0, 5, 10, 15, 25, 35 and 60 and further extracted with an Accelerated Solvent Extractor, ASE 100 (Dionex, USA). Concentrations of carbofuran and its metabolites were determined by HPLC. At each sampling date, total numbers of carbofuran degraders including bacteria and fungi in the soil were counted by spread plate technique on carbofuran-coated BSM agar. The half-lives of carbofuran in soils were calculated by fitting time-course concentration data to a modified first-order kinetic model. Three sets of control were included, i.e., soil without inoculation (treatment A) and autoclaved soil with inoculation of *B. cepacia* PCL3 with both free (treatment E) and immobilized cell forms (treatment F, G). The abiotic degradation control was sterilized soil mixed with sterilized immobilized matrices (treatment H, I). The experiment was set up as previously described.

2.8. Sorption of carbofuran to support materials

Adsorption isotherms were determined by conducting batch equilibrium experiments. De-lignified corncob and sugarcane bagasse were air-dried overnight and milled into small pieces using blender and passed through 2 mm sieve. Then a total of 0.25 g of air-dried and de-lignified corncob or sugarcane bagasse was put into a 250 ml conical flask and mixed with 50 ml of 0.01 M $CaCl_2$ solution containing carbofuran at carbofuran concentrations of 0.05, 0.1, 1.0, 5.0, 10.0, and 20.0 mg l^{-1}. Flasks were shaken at a constant speed of 100 rpm for 48 h at an average room temperature of 29 ± 2 °C. After 48 h, the solution was passed through a Whatmann filter paper No. 1 and the filtrate was extracted by liquid–liquid partitioning method and quantified for carbofuran concentration by HPLC. The data were fitted to the Freundlich equation (Sposito, 1980) to describe the kinetic of carbofuran sorption to corncob and sugarcane bagasse.

2.9. Extraction of carbofuran and its metabolites

Extraction of carbofuran from culture media using a liquid–liquid partitioning method was conducted by adding 2 ml of methanol to 2 ml of culture media and then sonicated twice for 10 min each time, 50/60 voltage cycle. After sonication, carbofuran and its metabolites were extracted from the media in separation funnel with dichloromethane. This extraction was done three times. For the first, second and third extractions, 4, 2 and 2 ml of dichloromethane, respectively, was added to the sonicated media and hand shaken for 30 s. The organic fraction of the samples from each extraction was collected and 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 before analyzing by HPLC.

Carbofuran and its metabolites were extracted from 13-g soil samples by an Accelerated Solvent Extractor ASE 100 (Dionex, USA) prior to analysis by HPLC following the conditions described by Plangklang and Reungsang (2008).

The half-lives of carbofuran in the soil were calculated by fitting to a modified first-order kinetic model using SAS program (SAS Institute Inc., 1985; Plangklang and Reungsang, 2008). Data were analyzed by SPSS program Version 10.0 (SPSS Inc., Chicago, IL). The significance of treatments was set at *p*-value less than or equal to 0.05 by the one-way ANOVA test.

2.10. PCR amplification

The oligonucleotide primer pair of mcdF1 and mcdR1 (Parekh et al., 1996) was used for specific amplification of a 590 bp fragment from the methylcarbamate degrading (*mcd*) gene region of the isolate PCL3. The *mcd* gene codes for hydrolase enzyme responsible for carbofuran degradation. Colony PCR was conducted followed the method as previously described by Parekh et al. (1996).

3. Results and discussion

3.1. Scanning Electron Microscope (SEM)

The microstructure of support materials and immobilized cells were depicted in Fig. 1. The photograph showed that corncob (Fig. 1(A)) has more porosity than sugarcane bagasse (Fig. 1(B)). It is evident from the photograph that *B. cepacia* PCL3 attached to the support materials inside their porous space which indicated that *B. cepacia* PCL3 was immobilized on corncob and sugarcane bagasse by adsorption mechanism. Cells were rod shape with approximately 5 μm in length and 1.2 μm in diameter when they were immobilized on corncob (Fig. 1(C)). When cells were immobilized on sugarcane bagasse, the size of cells was approximately 3.5 μm in length and 1.3 μm in diameter (Fig. 1(D)). Difference in cell shape could be the results of nutrient acquisitions and different attachment mechanisms to different surfaces (Young, 2007).

3.2. Degradation of carbofuran in BSM

This experiment investigated the ability of immobilized *B. cepacia* PCL3 to degrade carbofuran in BSM in comparison to free cells. Degradation of carbofuran in BSM was described by a modified first-order kinetic model (Fig. 2). The corresponding kinetic data fitted to a modified first-order kinetic model was tabulated in Table 2. The regressions coefficients, r^2, ranged between 0.80 and 0.99 indicating a good fit of the data to the first-order kinetic model (Table 2). Carbofuran was rapidly degraded by *B. cepacia* PCL3 in both free and immobilized cell forms on corncob and on sugarcane bagasse with $t_{1/2}$ of 3, 3 and 4 d, respectively (Table 2), which suggested that immobilization did not decrease carbofuran degrading activity of *B. cepacia* PCL3 in BSM. The high matrix porosity and pore size of these two materials might enhance the absorption capacity and substrate transfer to the immobilized cells (Gu et al., 1994; Jimoh, 2004). Support materials suitable for immobilization through adsorption mechanism should not be only good packing materials for bacterial growth, but they should be effective for physical adsorption of the contaminant to support a transfer of the contaminant to the cells (Ma et al., 2006).

Growth and survival of immobilized cells were determined by the numbers of carbofuran degraders in the support materials. Cell numbers on the immobilized matrices were stable in the range of

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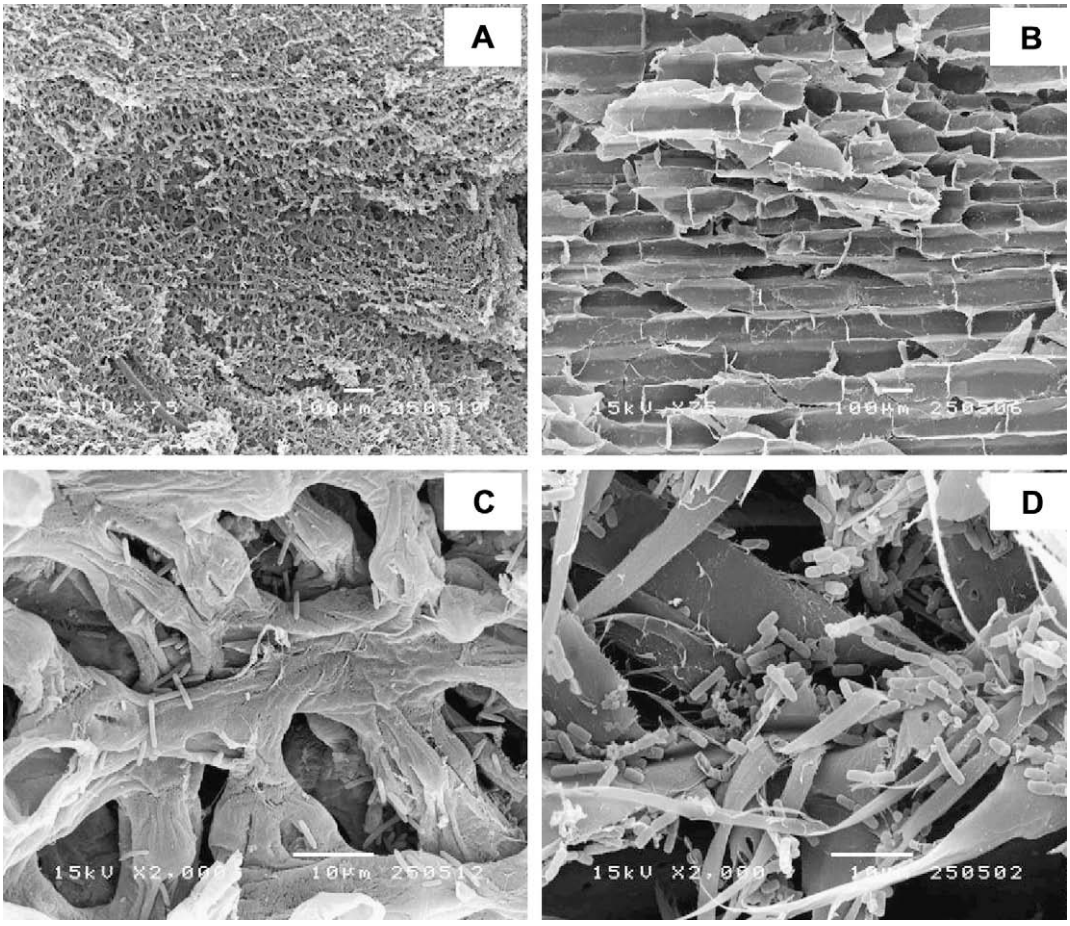

Fig. 1. Scanning electron micrographs studies of support materials i.e., (A) corncob, and (B) sugarcane bagasse and immobilized *B. cepacia* PCL3 on (C) corncob and (D) sugarcane bagasse.

10^7–10^8 cfu g^{-1} dry support material throughout 30 d of incubation (Fig. 3) while free cells of *B. cepacia* PCL3 were found to increase from 10^6 to 10^8 cfu ml^{-1} at day 8 and then decreased continuously over time (Fig. 3). These findings implied that the survival and stability of *B. cepacia* PCL3 could be improved through immobilization on corncob and sugarcane bagasse. Corncob and bagasse might act as protective agents against limiting condition such as substrate and product inhibition during incubation or shear forces (Bekatorou et al., 2004). In addition, these data suggested that the growth of immobilized *B. cepacia* PCL3 was limited by the supporting materials. Space in the immobilization matrix could limit cell density (Muyima and Cloete, 1995).

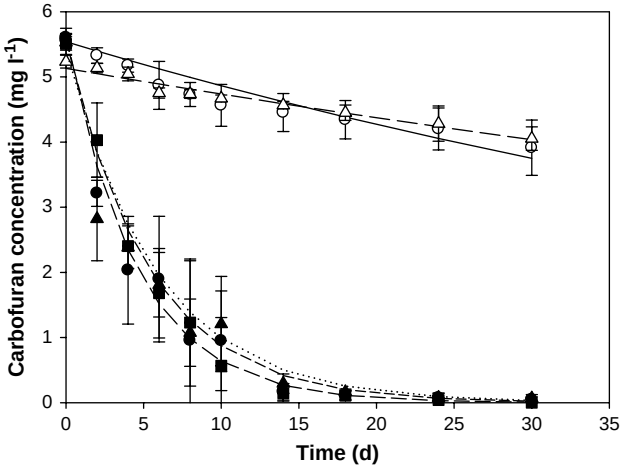

Fig. 2. Dissipation of carbofuran in BSM inoculated with *B. cepacia* PCL3 (■ = free cell, ● = immobilized cell on corncob, ▲ = immobilized cell on sugarcane bagasse) and autoclaved support materials without PCL3 (○ = corncob, △ = sugarcane bagasse). Lines indicated the carbofuran degradation fitting to the first-order kinetic model.

Leakage of cells from the support materials was represented by a cell concentration observed in culture media during incubation. At day 0, number of the PCL3 cells in the liquid phase in BSM was negligible. After day 10, number of the PCL3 in the liquid phase of the BSM was 10^5 cfu ml^{-1} (Fig. 2) (10% of the number of cells immobilized in the support materials), whilst the number of *B. cepacia* PCL3 in the support materials was not reduced (Fig. 3). These results indicated that the cells were leaking out from the porous media of corncob and bagasse due to a limited space and

Table 2
Degradation rate coefficients (k_1) and half-lives ($t_{1/2}$) of carbofuran in BSM.

Treatment	k_1 (/d)	$t_{1/2}$ (d) [a]	r^2 [b]
BSM + Autoclaved corncob	0.013a ± 0.004	55a ± 15.3	0.88
BSM + Autoclaved sugarcane bagasse	0.028a ± 0.002	87a ± 17.4	0.90
BSM + Free cells	0.230b ± 0.056	3b ± 0.8	0.91
BSM + Immobilized PCL3 on corncob	0.280b ± 0.093	3b ± 1.2	0.90
BSM + Immobilized PCL3 on sugarcane bagasse	0.200b ± 0.051	4b ± 1.0	0.97

[a] Comparison between treatment in column are significantly different (Duncan, $p \leq 0.05$) if mark different small letters.
[b] Coefficients of determination for non-linear regressions.

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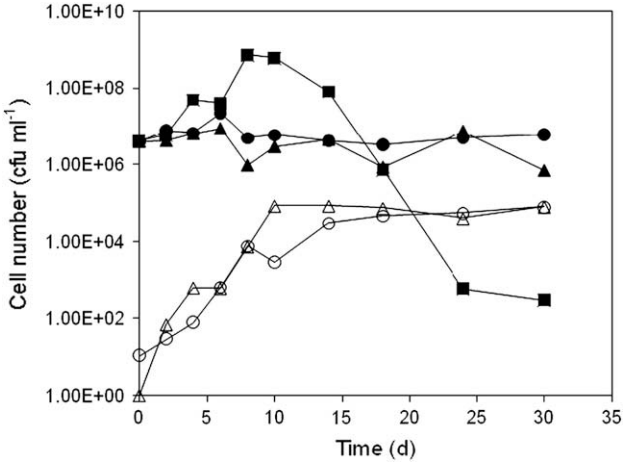

Fig. 3. Cell numbers of *B. cepacia* PCL3 (■ = free cells, ● = immobilized cells on corncob, ▲ = immobilized cells on sugarcane bagasse) and cell leaking from ○ = corncob, △ = sugarcane bagasse.

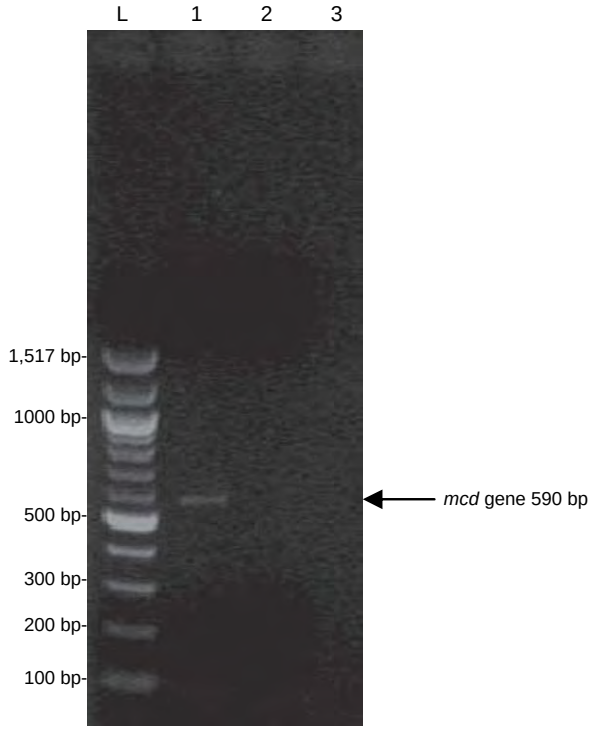

Fig. 4. Agarose gel showing PCR products obtained from cell of *B. cepacia* PCL3 using primer specific for the *mcd* gene. L is molecular weight ladder (100 bp, New England BioLab Inc.). Lanes 1–3 are PCR products from PCL3, *E. coli* DH5α and deionized water, respectively.

they continued to grow in the medium causing the increase in cell concentration. The profile of cell leakage was similar to that of the growth curve of *B. cepacia* PCL3 in BSM (Fig. 3) indicating viability of the leakage cells which could increase the cell number in culture media. Cell detachment might be resulted from the fact that there are no barriers between the cells and the media which led to a possibility of cell detachment and relocation with potential establishment of cell-equilibrium inside the supports and culture media (Bekatorou et al., 2004). In the adsorption process, cells were immobilized on a supporting material by physical adsorption due to electrostatic forces or by covalent binding between the cell membrane and the supporting materials (Bekatorou et al., 2004). Previous research by Kumar and Das (2001) reported a similar finding in which the daughter cells produced from binary fission of the immobilized cells of *Enterobactor cloacae* IIT-BT 08 leaked to the culture media when there was no free space on the porous support materials.

The major metabolite from biodegradation in BSM was carbofuran phenol (Table 3) suggesting that *B. cepacia* PCL3 may release hydrolase enzyme for degrading carbofuran (Eisler, 1985; Head et al., 1992). Therefore, the investigation on the presence of methylcarbamate degrading gene (*mcd* gene) which encodes hydrolase enzyme in the PCL3 was conducted. The PCR product of 590 bp was obtained (Fig. 4) thus assured that PCL3 produces hydrolase enzyme. Not only carbofuran phenol but also 3-keto carbofuran were the metabolites detected in the study. 3-keto carbofuran was the metabolite that was detected in all treatments including in the abiotic controls (Table 3). This metabolite is normally detected when the oxidation of carbofuran is taken placed.

Loss of carbofuran in the abiotic control might be resulted from the oxidation, the volatilization process occurred during the incubation and a continuous shaking, as well as the sorption of carbofuran to the support materials. Although the oxidation and volatilization are not as important as microbial degradation to carbofuran dissipation, they were contributing to dissipation processes which could be found in abiotic control as reported in previous studies (Lalah et al., 1996). Percentage of carbofuran dissipated in the abiotic controls i.e., BSM with autoclaved corncob and sugarcane bagasse were 12.7% and 26.7%, respectively, which might be caused by the sorption of carbofuran onto the surface and porous of the support materials during the incubation. Sorption coefficients (K_f) of carbofuran to corncob and sugarcane bagasse were found to be 0.03 and 0.30 $l\,g^{-1}$, respectively. Low K_f values may be resulted from a delignification treatment of corncob and sugarcane bagasse by NaOH before used in the sorption

Table 3
Carbofuran metabolites found in BSM augmented with free cell of PCL3 (FC), the immobilized PCL3 on corncob (IMC-CC) and sugarcane bagasse (IMC-SCB), autoclaved corncob (AC) and autoclaved sugarcane bagasse (ASCB).

Time (d)	Carbofuran phenol conc. ($mg\,l^{-1}$)					3-Keto carbofuran conc. ($mg\,l^{-1}$)				
	AC	ASCB	FC	IMC-CC	IMC-SCB	AC	ASCB	FC	IMC-CC	IMC-SCB
0	nd	nd	nd	nd	nd	nd	nd	nd	0.06	nd
2	nd	nd	0.49	0.60	0.64	0.17	0.21	0.50	0.25	0.51
4	nd	nd	0.95	1.20	1.73	0.20	0.18	0.58	0.33	0.47
6	nd	nd	0.10	1.88	1.52	0.22	0.22	0.44	0.44	0.43
8	nd	nd	1.67	0.75	1.56	0.17	0.25	0.43	0.21	0.34
10	nd	nd	2.22	1.40	2.01	0.19	0.27	0.20	0.22	0.27
14	nd	nd	0.33	nd	1.37	0.19	0.18	0.45	0.19	0.12
18	nd	nd	1.20	1.29	0.61	0.19	0.10	0.37	0.10	0.32
24	nd	nd	0.51	0.56	0.70	0.10	0.28	0.17	0.17	0.12
30	nd	nd	0.13	nd	nd	0.17	0.25	0.25	0.18	0.28

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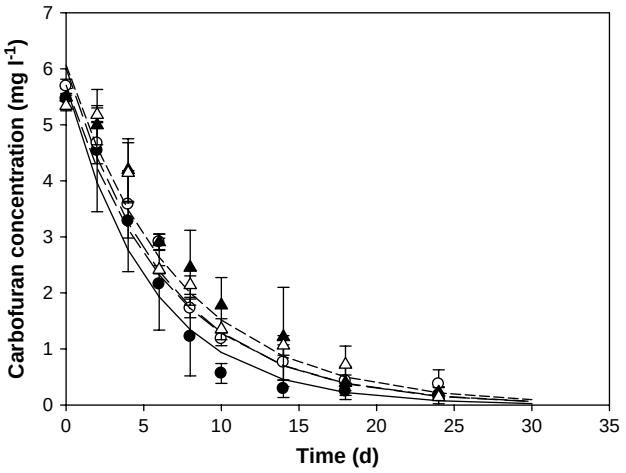

Fig. 5. Degradation of carbofuran in BSM by reused immobilized *B. cepacia* PCL3 on corncob (● = first reuse, ○ = second reuse) and on sugarcane bagasse (▲ = first reuse, △ = second reuse). Lines indicated the carbofuran degradation fitting to the first-order kinetic model.

experiment. K_f values suggested that sugarcane bagasse could be a better sorbent than corncob and facilitate the dissipation of carbofuran.

3.3. Reusability of immobilized cells

This experiment examined a possibility of reusing the immobilized cells to degrade carbofuran in BSM. Degradation of carbofuran in BSM by reused immobilized PCL3 was depicted in Fig. 5. Results demonstrated that the immobilized cells could be reused twice while retaining carbofuran degradation ability ($t_{1/2}$ of 4–5 d in BSM) (Table 4) indicating an advantage of using immobilized cells over free cells. However, decrease in cell survival and ability of supporting materials to retain cells in their structures were observed as the number of reuse steps in BSM increased (Fig. 6). These may due to a reduction in particle size from 0.7 × 0.7 × 0.7 cm to 0.4 × 0.4 × 0.4 cm of both support materials in BSM caused by soften structures after delignification. In consequence, an increase in cell leakage from supporting materials occurred. The decay of corncob and sugarcane bagasse implied that the immobilization of cells on these support materials could be environmental friendly causing less disposal problems than would occur for synthetic materials.

3.4. Bioaugmentation of carbofuran residues in soil with immobilized *B. cepacia* PCL3

This experiment investigated an ability of the immobilized *B. cepacia* PCL3 on corncob and sugarcane bagasse to remediate carbofuran residues in soil in comparison to free cells and

Table 4
Degradation rate coefficients (k_1) and half-lives ($t_{1/2}$) of carbofuran in BSM.

Immobilized *B. cepacia* PCL3	k_1 (d^{-1})	$t_{1/2}$ (d)[a]	R^2 [b]
On corncob			
First reuse	0.18a ± 0.064	4a ± 1.2	0.94
Second reuse	0.15a ± 0.030	5a ± 1.1	0.95
On sugarcane bagasse			
First reuse	0.15a ± 0.036	5a ± 1.2	0.96
Second reuse	0.14a ± 0.003	5a ± 0.1	0.99

[a] Comparison between treatment in column are significantly different (Duncan, $p \leq 0.05$) if mark different small letters.
[b] Coefficients of determination for non-linear regressions.

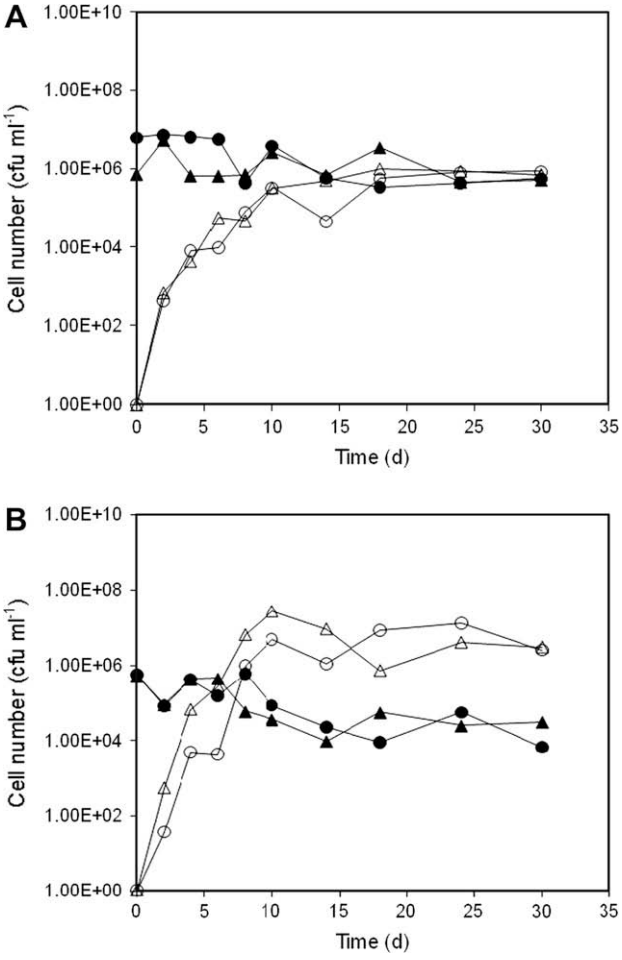

Fig. 6. Cell numbers of immobilized *B. cepacia* PCL3; ● = on corncob, ▲ = on sugarcane bagasse, leaking cells; ○ = from corncob, △ = from sugarcane bagasse: (A) = first reuse and (B) = second reuse.

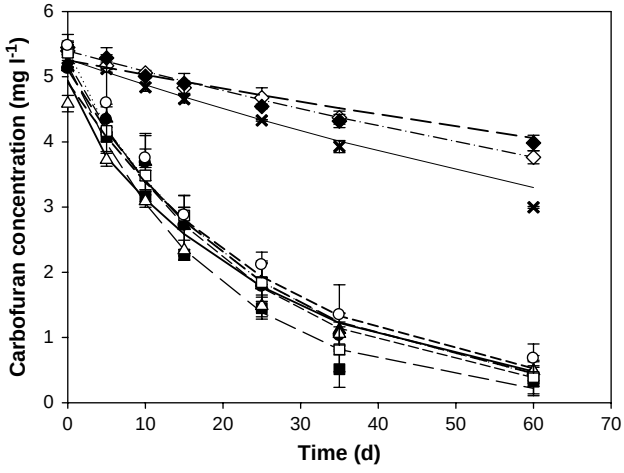

Fig. 7. Degradation of carbofuran residues in soil (closed symbols) and in autoclaved soil (open symbols) (■, □ = free cell of PCL3; ●, ○ = immobilized PCL3 on corncob; ▲, △ = immobilized PCL3 on sugarcane bagasse; × = indigenous microorganisms; ◆ = autoclaved corncob without PCL3; ◇ = autoclaved sugarcane bagasse without PCL3). Lines indicated the carbofuran degradation fitting to the first-order kinetic model.

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Table 5
Degradation rate coefficients (k_1) and half-lives ($t_{1/2}$) of carbofuran in soil.

Treatment	k_1 (d^{-1})	$t_{1/2}$ (d)[a]	r^2 [b]
Soil	0.0094a ± 0.0003	74a ± 2.0	0.88
Soil + free cells of PCL3	0.0523b ± 0.0163	13b ± 4.4	0.99
Soil + immobilized PCL3 on corncob	0.0435b ± 0.0068	16b ± 2.6	0.97
Soil + immobilized PCL3 on sugarcane bagasse	0.0401b ± 0.0097	17b ± 1.7	0.94
Autoclaved soil + free cells of PCL3	0.0462b ± 0.0107	15b ± 3.5	0.91
Autoclaved soil + immobilized PCL3 on corncob	0.0369b ± 0.0052	19b ± 2.5	0.99
Autoclaved soil + immobilized PCL3 on sugarcane bagasse	0.0416b ± 0.0039	17b ± 3.5	0.93
Autoclaved soil + autoclaved corncob	0.0058c ± 0.0003	119c ± 6.2	0.88
Autoclaved soil + autoclaved sugarcane bagasse	0.0061c ± 0.0003	114c ± 5.1	0.89

[a] Comparison between treatment in column are significantly different (Duncan, $p \leq 0.05$) if mark different small letters.
[b] Coefficients of determination for non-linear regressions.

indigenous microorganisms. Dissipation of carbofuran in soil by the immobilized PCL3 was shown in Fig. 7. The corresponding kinetic data fitting to a modified first-order kinetic model was tabulated in Table 5. Results revealed that the degradation of carbofuran in soil augmented with *B. cepacia* PCL3 in both free and immobilized cell forms on corncob and on sugarcane bagasse (treatment B, C, D; $t_{1/2}$ of 13–17 d) was more rapid than in soil without augmentation (treatment A; $t_{1/2}$ of 74 d) (Table 5) which again demonstrated the ability of *B. cepacia* PCL3 to degrade carbofuran in soil as well as the potential for its use in bioaugmentation for bioremediation of carbofuran residues in soil.

The carbofuran degradation ability of PCL3 was evident in the augmented autoclaved soil (treatment E, F, G) indicated by a much shorter half-lives of carbofuran (15–19 d) than the abiotic control (treatment H, I; 114–119 d) (Table 5). Other evidence to support this finding is that the half-lives of carbofuran in the augmented soil (13–17 d) (treatment B, C, D) (Table 5) were not significantly different ($p \leq 0.05$) from degradation in augmented autoclaved soil (15–19 d) (treatment E, F, G) (Table 5).

Carbofuran degradation by the immobilized cells on corncob (treatment C, F) and sugarcane bagasse (treatment D, G) ($t_{1/2}$ of 16–19 d) and free cell forms (treatment B, E) (13–15 d) of *B. cepacia* PCL3 were not significantly different ($p \leq 0.05$) (Table 5) suggesting that the immobilization did not worsen or improve carbofuran degradation activity of *B. cepacia* PCL3.

Carbofuran phenol was the only metabolite found in soil microcosms study with a biological activity (Table 6). The accumulation of carbofuran phenol at day 60 in the treatments with the PCL3 together with the indigenous microorganisms (treatments B, C and D) was lower than the treatments with the PCL3 or the indigenous microorganisms alone (treatments A, E, F and G). Results suggested that the PCL3 and the indigenous microorganisms might work closely to degrade carbofuran phenol which could be accounted as another advantage of the bioaugmentation.

Table 6
Carbofuran phenol detected in soil microcosms treatments.

Time (d)	Treatment								
	A	B	C	D	E	F	G	H	I
0	nd	nd	nd	nd	nd	nd	nd	nd	nd
5	0.16	0.30	0.22	0.28	0.24	0.29	0.23	nd	nd
10	0.54	2.49	2.31	2.03	2.30	1.96	2.05	nd	nd
15	0.37	2.65	2.38	2.04	2.10	2.59	2.35	nd	nd
25	0.82	3.05	2.17	2.79	2.42	1.23	2.47	nd	nd
35	1.27	2.36	1.71	1.99	2.15	2.05	2.36	nd	nd
60	1.05	0.26	0.12	0.21	1.54	1.17	1.02	nd	nd

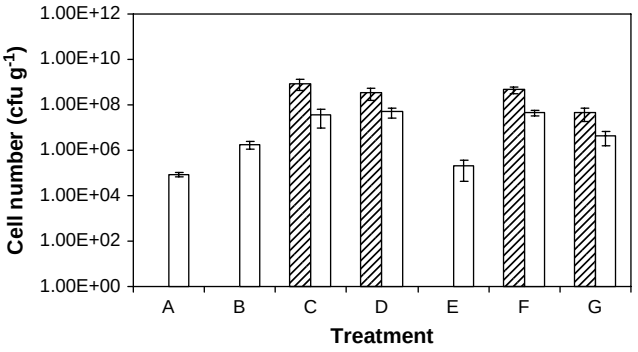

Fig. 8. Total numbers of carbofuran degraders; ▨ = in support materials (cfu g^{-1} support), □ = in soil (cfu g^{-1} soil) at day 60 of incubation. Treatment A = soil; B = Soil + free cells of PCL3; C = Soil + immobilized PCL3 on corncob; D = Soil + immobilized PCL3 on sugarcane bagasse; E = Autoclaved soil + free cells of PCL3; F = Autoclaved soil + immobilized PCL3 on corncob; G = Autoclaved soil + immobilized PCL3 on sugarcane bagasse; H = Autoclaved soil + autoclaved corncob; I = Autoclaved soil + autoclaved sugarcane bagasse.

Growth, survival and leakage of the immobilized cells at day 60 of incubation were investigated in order to evaluate the stability of immobilized cells when they were inoculated into soil. Results revealed that cell numbers of *B. cepacia* PCL3 on the immobilized matrices increased from 10^7 cfu g^{-1} dry support material to 10^8 cfu g^{-1} dry support materials at day 60 of incubation (Fig. 8). In comparison, number of inoculated free cells of *B. cepacia* PCL3 was found to decrease in soil from 10^6 cfu g^{-1} dry soil to 10^5 cfu g^{-1} dry soil at day 60 (Fig. 8). These findings indicated that immobilization technique via absorption on corncob and sugarcane bagasse could improve the survival of *B. cepacia* PCL3 when it was inoculated to the soil.

Leakage of cells from support materials was represented by a cell number observed in soil inoculated with the immobilized cells (Fig. 8). Results were similar to those obtained in the BSM experiment.

Since corncob and sugarcane bagasse are organic materials. They were degraded after 60 d of incubation in soil, therefore preventing us from reusing the immobilized PCL3 cells in soil treatment. Investigation into supporting materials with stronger structure after delignification in provision of a possibility to reuse the immobilized PCL3 cells to remediate carbofuran contaminated soils is being conducted.

Acknowledgements

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References

Bano, N., Musarrat, J., 2004. Characterization of a novel carbofuran degrading *Pseudomonas* sp. with collateral biocontrol and plant growth promoting potential. FEMS Microbiology Letters 231, 13–17.
Bardi, E.P., Koutinas, A.A., 1994. Immobilization of yeast on delignified cellulosic material for room temperature and low-temperature wine making. Journal of Agricultural and Food Chemistry 42, 221–226.
Bekatorou, A., Kourkoutas, Y., Banat, I.M., Marchant, R., Koutinas, A.A., 2004. Immobilization technologies and support materials suitable in alcohol beverages production: a review. Food Microbiology 21, 377–397.
Eisler, R., 1985. Carbofuran hazards to fish, wildlife, and invertebrates: a synoptic review. U.S. Fish and Wildlife Service Biological Report 85 (1.3) 36.
EPA, 2006. Interim Reregistration Eligibility Decision (IRED) Document for Carbofuran: Special Review and Reregistration Division Online at. http://www.epa.gov/oppsrrd1/reregistration/REDs/carbofuran_ired.pdf, pp. 44.
Gu, Y.X., Hu, Z.C., Korus, R.A., 1994. Effect of adsorption on mass transfer in immobilization matrices. Chemical Engineering Journal and the Biochemical Engineering Journal 54, B1–B8.

Head, I.M., Cain, R.B., Suett, D.L., 1992. Characterization of a carbofuran-degrading bacterium and investigation of the role of plasmids in catabolism of the insecticide carbofuran. Archives of Microbiology 158, 302–308.

Jiang, J., Zhang, R., Li, R., Gu, J.D., Li, S., 2007. Simultaneous biodegradation of methyl parathion and carbofuran by a genetically engineered microorganism constructed by mini-Tn5 transposon. Biodegradation 18, 403–412.

Jimoh, A., 2004. Effect of immobilized materials on *Saccharomyces cerevisiae*. Assumption University Journal of Technology 8, 62–68.

Kumar, N., Das, D., 2001. Continuous hydrogen production by immobilized *Enterobacter cloacae* IIT-BT 08 using lignocellulosic materials as solid matrices. Enzyme and Microbial Technology 29, 280–287.

Lalah, J.O., Wandiga, S.O., Dauterman, W.C., 1996. Mineralization, volatilization, and degradation of carbofuran in soil samples from Kenya. Bulletin of Environment Contamination and Toxicology 56, 37–41.

Ma, Y.L., Yang, B.L., Zhao, J.L., 2006. Removal of H_2S by *Thiobacillus denitrificans* immobilized on different matrices. Bioresource Technology 97, 2041–2046.

Manohar, S., Kim, C.K., Karegoudar, T.B., 2001. Enhanced degradation of naphthalene by immobilization of *Pseudomonas* sp. strain NGK1 in polyurethane foam. Applied Microbiology and Biotechnology 55, 311–316.

Mo, K., Lora, C.O., Wanken, A.E., Javanmardian, M., Yang, X., Kulpa, C.F., 1997. Biodegradation of methyl *tert*-butyl ether by pure bacterial cultures. Applied Microbiology and Biotechnology 47, 69–72.

Muyima, N.Y.O., Cloete, T.E., 1995. Phosphate uptake by immobilization *Acinetobacter* in a full scale activated sludge plant. Journal of Industrial Microbiology 15, 19–24.

Parekh, N.R., Hartmann, A., Fournier, J.C., 1996. PCR detection of the *mcd* gene and evidence of sequence homology between the degradative genes and plasmid from diverse carbofuran-degradaing bacteria. Soil Biology and Biochemistry 28, 1797–1804.

Plangklang, P., 2004. Degradation of Carbofuran by Rhizosphere Soil Microorganisms. Master thesis, Chulalongkorn University, Bangkok, Thailand, ISBN: 974-53-1222-3, pp. 98.

Plangklang, P., Reungsang, A., 2008. Effects of rhizosphere remediation and bioaugmentation on carbofuran removal from soil. World Journal of Microbiology and Biotechnology 24, 983–989.

Qiu-Xiang, Y., Qing, H., Peng, H., Xiao-Jun, D., Yu-Jia, S., Shun-Peng, L., 2007. Isolation and characterization of a carbofuran-degrading strain *Novosphingobium* sp. FND-3. FEMS Microbiology Letters 271, 207–213.

Rachman, M.A., Nakashimada, Y., Kakizono, T., Nishio, N., 1998. Hydrogen production with high yield and high evolution rate by self-focculated cells of *Enterobacter aerogenes* in a packed-bed reactor. Applied Microbiology and Biotechnology 49, 450–454.

Ryeol, P.M., Sunwoo, L., Tae-Ho, H., Byung-Tack, O., Shim, J.H., Kim, I.S., 2006. A New intermediate in the degradation of carbofuran by *Sphingomonas* sp. strain SB5. Journal of Microbiology and Biotechnology 16, 1306–1310.

SAS Institute, 1985. SAS User's Guide. Statistics, fifth ed. SAS Institute, Cary, North Carolina.

Seo, J., Jeon, J., Kim, S.D., Kang, S., Han, J., Hur, H.G., 2007. Fungal biodegradation of carbofuran and carbofuran phenol by the fungus *Mucor ramannianus*: identification of metabolites. Water Science and Technology 55, 163–167.

Slaoui, M., Ouhssine, M., Berny, E., Elyachioui, M., 2007. Biodegradation of the carbofuran by a fungus isolated from treated soil. African Journal of Biotechnology 6, 419–423.

Sposito, G., 1980. Derivation of the Freundlich equation for ion exchange reactions in soils. Soil Science Society of America Journal 44, 652–654.

Teerakun, M., Reungsang, A., Virojanakud, W., 2004. Phytoremediation of carbofuran residues in soil. Songklanakarin. Journal of Science and Technology 26, 171–176.

Young, K.D., 2007. Bacterial morphology: why have different shapes? Current Opinion in Microbiology 10, 596–600.

Zilli, M., Daffonchio, D., Felice, R.D., Giordani, M., Converti, A., 2004. Treatment of benzene-contaminated airstream in laboratory-scale biofilter packed with raw and sieved sugarcane bagasse and with peat. Biodegradation 15, 87–96.

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ORIGINAL PAPER

Effects of rhizosphere remediation and bioaugmentation on carbofuran removal from soil

Pensri Plangklang · Alissara Reungsang

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Abstract Rhizosphere soil contains important sources of nutrients for microorganisms resulting in high number of microorganisms capable of degrading various types of chemicals in the soil. Thus, this study investigated a carbofuran dissipation in rhizosphere soils of 6 weeds namely, umbrella sedge (*Cyperus iria* L.), fuzzy flatsedge (*C. pilosus* V.), small flower umbrella plant (*C. difformis* L.), tall-fringe-rush hoorah grass (*Fimbristylis miliacea* V.), cover fern (*Marsilea crenata* P.), and water primrose (*Jussiaea linifolia* V.). Rhizosphere soil of fuzzy flatsedge showed the shortest half-life ($t_{1/2}$) of carbofuran (15 days) among other soils. So, it was selected to be used in the bioaugmentation experiment using carbofuran degrader namely *Burkholderia cepacia*, PCL3, as inoculum in order to examine whether they would improve carbofuran degradation in soil. The results showed that the addition of PCL3 into rhizosphere soil did not improve carbofuran degradation suggesting that microorganisms in rhizosphere soil might be capable enough to remove carbofuran from soil. The number of carbofuran degraders in the rhizosphere soils was greater than in bulk soil 10–100 times which might be responsible to a rapid degradation of carbofuran in rhizosphere soils without the addition of PCL3. The ability of PCL3 to degrade carbofuran was evident in bulk soil ($t_{1/2}$ of 12 days) and autoclaved soils ($t_{1/2}$ 13–14 days) when compared to soils without an inoculation ($t_{1/2}$ of 58 days) indicated that the addition of a degrader was useful in improving carbofuran degradation in soil.

Keywords Bioaugmentation · *Burkholderia cepacia* · Carbofuran · *Cyperus pilosus* · Rhizodegradation

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Introduction

Carbofuran (2,3-dihydro-2,2 dimethylbenzofuran-7-yl methylcarbamate) is a broad-spectrum insecticide widely used in agriculture to control insects and nematodes on contact or after ingestion (EPA 2006). Carbofuran is of environmental concern because it is soluble in water and highly mobile in soil resulting in a high potential for groundwater contamination (Howard 1991) and can cause acute toxicity to mammals through cholinesterase inhibition (EPA 2006). In 2003, Thailand imported carbofuran in solid and liquid forms up to 826.6 and 45.5 m, respectively, for use in agriculture especially in rice fields (Department of Agriculture 2003).

The heavy use of carbofuran in rice field may cause contamination risk to soil and groundwater in the area. Remediation in the contaminated land is thus necessary and the most important route for pesticide removal from soil is through microbial degradation. The number of microorganisms in the soil and their pesticide degradation abilities could affect the rate of pesticide degradation (Yu et al. 2003). The soil in the root zone or so-called rhizosphere soil generally consists of 10–100 times greater number of indigenous microorganisms than in bulk soil and pesticide degradation ability was found to be increased in this soil

(Anderson et al. 1994). This might due to carbon containing compounds exudated from plant root such as sugars, carbohydrate, alcohol and amino acids, which could stimulate the microbial activities and increase number of rhizosphere microorganisms (Schnoor et al. 1995; Stottmeister et al. 2003). Published studies reported the degradation/removal of pesticides (Singh et al. 2004; Yu et al. 2003; Sun et al. 2004), others organic hydrocarbons (Biryukova et al. 2007; Chaudhry et al. 2005; Nakamura et al. 2004; Jordahl et al. 1997), and heavy metals (Gaur and Adholeya 2004) by rhizosphere remediation. Their results also evidenced the enhancement of the contaminant removal in the rhizosphere zone as compared to in bulk soil.

Addition of microbial cultures capable of degrading pesticide as a bioaugmentation technique is effective approach to improve pesticide degradation in soil which possesses the lack of microbial activities (Thompson et al. 2005). The successive bioaugmentation of pesticides (Rousseaux et al. 2003; Franzmann et al. 2000) and hydrocarbons (Ruberto et al. 2003) with isolated degraders was reported.

Many reports have indicated a promise of rapid degradation of pesticide by microorganisms in rhizosphere soil and an addition of degraders as described earlier. Furthermore, it might be possible to enhance the degradation of pesticide residues by inoculating microorganisms capable of degrading pesticides into rhizosphere soil. To date, the information of carbofuran removal by these two approaches was limit. Therefore, this study presents the investigation on rhizosphere degradation and/or bioaugmentation effect on carbofuran degradation. Microcosms consisted of carbofuran-spiked rhizosphere soils collected from various types of weeds were used to evaluate the carbofuran degradation efficiency of rhizosphere microorganisms and the efficiency of carbofuran degrader to improve the carbofuran degradation in rhizosphere soil.

Materials and methods

Microorganisms and culture medium

The carbofuran degrader, *Burkholderia cepacia*, PCL3, used as an inoculum in this study was previously isolated from carbofuran phytoremediated rhizosphere soil (Teerakun et al. 2004). It can utilize carbofuran as a sole carbon source. A single colony of *B. cepacia*, PCL3 was grown at 30°C, 150 rpm in Nutrient Broth (NB) containing 5 mg/l of carbofuran for 48 h. Cell pellets were harvested by centrifugation at 10,000 rpm, 4°C for 10 min and resuspended in sterile 0.85% NaCl to the final cell concentration of 4×10^6 c.f.u./ml prior inoculation to soils. Cell concentration was determined by measuring the optical density at 600 nm (OD_{600}) and converting it to c.f.u./ml by using a standard calibration curve.

Culture medium used in this study was C-limited Basal Salt Medium (BSM) (Mo et al. 1997), pH 7, containing (in g/l): 5.57, $NaHPO_4$; 2.44, KH_2PO_4; 2.00, NH_4Cl; 0.20, $MgCl_2{\cdot}6H_2O$; 0.0004, $MnCl_2{\cdot}4H_2O$; 0.001, $FeCl_3{\cdot}6H_2O$; and 0.001, $CaCl_2$.

Soil sampling and preparation

The rhizosphere soils (sandy loam texture) of 6 weeds, namely, umbrella sedge; *Cyperus iria* Linn., fuzzy flatsedge; *C. pilosus* Vohl., small flower umbrella plant; *C. difformis* Linn., tall-fringe-rush hoorah grass; *Fimbristylis miliacea* Vahl., cover fern; *Marsilea crenata* Presl., and water primrose; *Jussiaea linifolia* Vahl., growing in rice fields of Ban Nonmuang, A. Muang, Khon Kaen Province, Thailand, were collected on November, 2003, and used throughout this study. The rice fields have a history of carbofuran application, thus we expected that the rhizosphere microorganisms at the root zone of these weeds would have adapted themselves to degrade carbofuran. Rhizosphere soils were collected by digging around and close to the plant roots with the depth to approximately 15 cm below ground. The extra soil around the root zone was shaken out and the rhizosphere soil which was soil attached to the root was then collected and passed through a 2 mm sieve. Small and hairy roots were carefully sorted out by hand. Bulk soil was taken from the same site and then passed through 2 mm sieve. Both rhizosphere and bulk soils were placed in plastic bags and kept at 4°C prior to usage in the experiment.

Soil microcosm construction

A 20 g of each rhizosphere soil was spiked with carbofuran (Sigma-Aldrich, USA) at an initial concentration of 5 mg/kg dry soil in a 425 cm^3 glass jar capped with a plastic lid. The initial moisture content was adjusted to 15–18% by sterile distilled water. The jars were incubated at room temperature for 100 days. At intervals of 0, 7, 14, 21, 35, 70 and 100 days, samples were taken and extracted by using Accelerate Solvent Extractor, ASE 100 (Dionex, USA) and then the concentrations of carbofuran and its metabolites were analyzed by HPLC. The numbers of indigenous carbofuran degraders which consisted of bacteria and fungi were counted on the BSM agar coated with 5 mg/l of carbofuran. The $t_{1/2}$ of carbofuran in rhizosphere soils were calculated by fitting to a modified first-order kinetic model: $C = C_0e^{-kt} + Ya$ using SAS program (SAS

Institute, Inc. 1982), where C was the mean concentration of carbofuran as a function of time in hours (mg/kg), C_0 was the initial carbofuran concentration (mg/kg), k was the rate constant (/days), t was time (days) and Ya was an asymptotic estimate of the concentration of carbofuran that degrades very slowly overtime (residual carbofuran) (mg/kg). Three control sets of this experiment were conducted in similar manners but the soil samples were changed from the rhizosphere soils to autoclaved rhizosphere soil, bulk soil and autoclaved bulk soil. All treatments were done in triplicates. The rhizosphere soil of the plant that possessed the shortest $t_{1/2}$ of carbofuran was further used in the bioaugmentation study.

Microcosms of bioaugmentation study were constructed in similar manners using the rhizosphere soil of the weed that possessed the shortest $t_{1/2}$ of the carbofuran. About 1 ml of *B. cepacia*, PCL3 prepared as described earlier was added dropwise, while hand mixing, into the soil to obtain the final cell density of 10^5 c.f.u./g soil. The soil was spiked with carbofuran at a concentration of 5 mg/kg dry soil prior to the inoculation. Samples were sacrificed and collected at the intervals of 0, 5, 10, 15, 25, 35 and 50 days to extract and analyze carbofuran and its metabolites concentrations using HPLC. Three control sets of soil microcosms were autoclaved rhizosphere soil, bulk soil and autoclaved bulk soil which were constructed in similar manners. All treatments were done in triplicates.

Extraction and analysis of carbofuran and its metabolites concentrations in soil

Extraction and analysis methods were modified from Teerakun et al. (2004). Carbofuran and its metabolites were extracted from 13-g soil samples by using an Accelerate Solvent Extractor, ASE 100 (Dionex, USA) equipped with 11-ml stainless-steel extraction cell. The samples were extracted under the conditions which are 100°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, i.e., the maximum extraction pressure was set not to exceed 1,500 psi, the flush volume was 60% of the extraction cell volume, and the N_2-purge time was set to 1 min. Volume of the extract was adjusted to 25 ml and passed through 0.45 μm nylon membrane syringe filter prior to analysis by Shimadzu 10-A HPLC equipped with 4.6 × 150 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 (60:40); flow rate, 1 ml/min at the ambient temperature. The detection limits of the HPLC for analyzing carbofuran, carbofuran phenol and 3-keto carbofuran were 0.07, 0.01 and 0.01 mg/l, respectively.

Results and discussion

Dissipation of carbofuran in rhizosphere soils

The dissipation of carbofuran was investigated in rhizosphere soils from six weeds growing in rice fields with a history of carbofuran application. The disappearance profiles of carbofuran in each rhizosphere soil and bulk soil were shown in Fig. 1. The degradation of carbofuran in soils was described by a modified first-order kinetic model. The coefficients of determination, r^2, ranged between 0.95–0.99 indicating a good fit of the data to the first-order kinetic model (Table 1). Results indicated that carbofuran rapidly dissipated in rhizosphere soils with $t_{1/2}$ of 15–19 days, which were approximately 3, 4-folds faster than in bulk soil (58 days) (Table 1). This might be resulted from the number of carbofuran degraders in the rhizosphere soils were greater than in bulk soil 10–100 times (Fig. 2) leading to the rapid degradation of carbofuran in rhizosphere soils. Similar findings were reported previously by many authors (Singh et al. 2004; Sun et al. 2004; Yu et al. 2003) in which the degradation of pesticides was improved in rhizosphere soil of various plants. Degradation of aldicarb and oxime, carbamate insecticides, in the rhizosphere soil of corn, mung bean and cowpea was found to be more rapid than in unplanted sterile and non-sterile soil (Sun et al. 2004). Two times shorter $t_{1/2}$ of butachlor was observed in

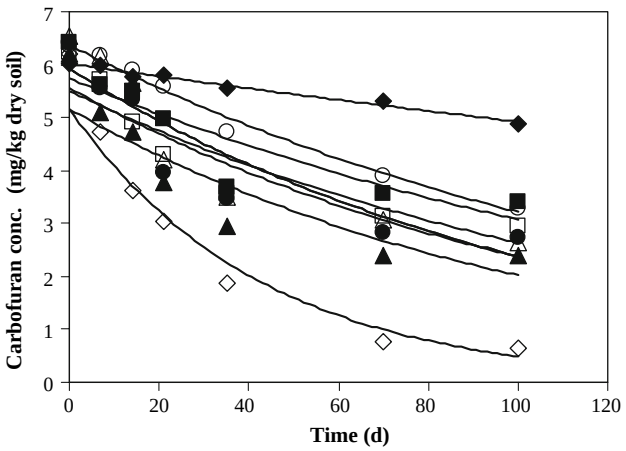

Fig. 1 Dissipation of carbofuran in rhizosphere soils of weeds grown in rice fields with a history of carbofuran application, (◆ = autoclaved rhizosphere soil of fuzzy flatsedge, ◯ = bulk soil, ■ = umbrella sedge, ▲ = water primrose, ◇ = fuzzy flatsedge, □ = small flower umbrella plant, ● = tall-fringe-rush hoorah grass, △ = cover fern; lines = carbofuran concentrations in each soil fitted to the first-order kinetic model)

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

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

[a] Coefficients of determination for non-linear regressions

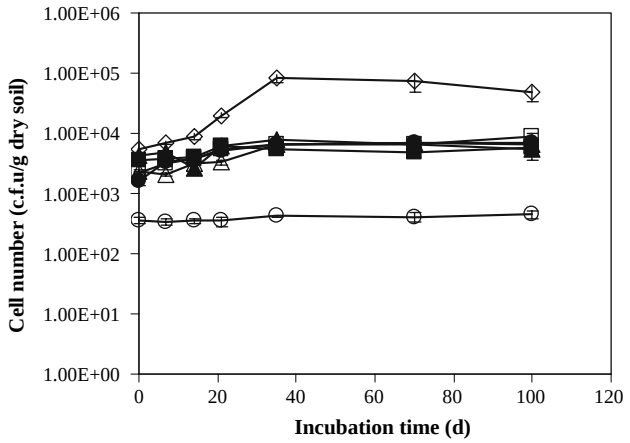

Fig. 2 Total number of indigenous carbofuran degraders which consisted of bacteria and fungi over time in rhizosphere soils of weeds grown in rice fields with a history of carbofuran application (◯ = bulk soil, ■ = umbrella sedge, ▲ = water primrose, ◇ = fuzzy flatsedge, □ = small flower umbrella plant, ● = tall-fringe-rush hoorah grass, △ = cover fern)

rhizosphere soil in comparison to in non-planted soil indicating the enhancement of butachlor degradation by wheat rhizosphere soil (Yu et al. 2003). The removal of atrazine and simazine, 45% and 25%, respectively, in soil planted with pennisetum (*Pennisetum clandestinum*) within 80 days were higher than in the unplanted soil i.e., 22% and 20%, respectively, (Singh et al. 2004) which might be resulted from a higher number of microorganisms in rhizosphere soil than in unplanted soil. In addition, microbial biomass and soil dehydrogenase activity were significantly increased (7-folds) in soil planted with *P. clandestinum* higher than that in unplanted soil. Anderson et al. (1994) revealed that the order of magnitude higher microbial numbers of 4.2×10^5 c.f.u./g in *Kochia* sp. rhizosphere soil compared to bulk soil (3.5×10^4 c.f.u./g) resulting in a significantly ($P \leq 0.10$) enhanced degradation of atrazine, metolachlor and trifluralin mixed in the soil after 14 days of incubation. The incidents of more rapid degradation of pesticides in planted soils than in unplanted soil might be due to the fact that the rhizosphere soils have carbon containing compounds such as sugars, alcohols and acids exudated from plant roots that supported the growth of microorganisms (Schnoor et al. 1995). This resulting in a larger number of microorganisms and more rapid degradation of pesticides (Sun et al. 2004; Singh et al. 2004) in rhizosphere soil than in bulk soil.

Carbofuran phenol was the main degradation product in the rhizosphere soils. The highest concentration of carbofuran phenol was found in the rhizosphere soil of fuzzy flatsedge at day 35 (Table 2) which was in correlation to the shortest $t_{1/2}$ of carbofuran (15 days) (Table 1) in this soil. The carbofuran degraders in fuzzy flatsedge might be responsible for this trend, in which the numbers of carbofuran degraders in the rhizosphere soils of fuzzy flatsedge were highest among others i.e., 10^5 c.f.u./g dry soil (Fig. 2). The rice field from which soil samples were collected had a history of carbofuran application, therefore microorganisms in the soils would be able to adapt themselves to use carbofuran as their energy source. Since carbofuran phenol was the major metabolite detected in this study without other unidentified peak from HPLC analysis, this indicated that rhizosphere microorganisms might contain the hydrolase enzyme (Tomasek and Karns 1989).

Carbofuran was degraded very slowly in the autoclaved rhizosphere soil of fuzzy flatsedge (Fig. 1) with the $t_{1/2}$ of 111 days. This result showed a similar trend to that of the autoclaved rhizosphere soils of the other plants and autoclaved bulk soil (data not shown). The results suggested that there was abiotic degradation process involved in carbofuran dissipation in the soils but this process did not

Table 2 Concentrations of carbofuran phenol in the rhizosphere soils of weeds grown in rice fields with a history of carbofuran application

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

R1 = rhizosphere soil of umbrella sedge
R2 = rhizosphere soil of water primrose
R3 = rhizosphere soil of fuzzy flatsedge
R4 = rhizosphere soil of small flower umbrella plant
R5 = rhizosphere soil of tall-fringe-rush hoorah grass
R6 = rhizosphere soil of cover fern

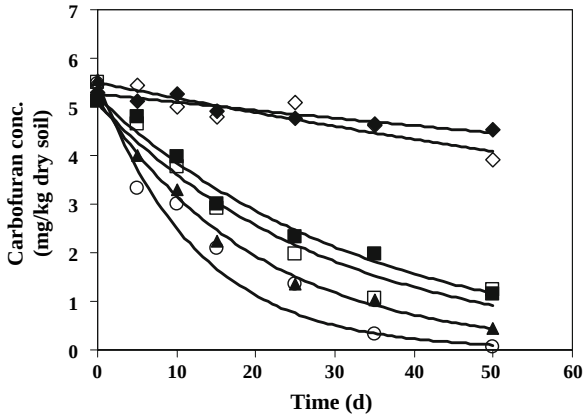

Fig. 3 Degradation of carbofuran in the soils inoculated with isolate PCL3, (■ = autoclaved bulk soil, ▲ = bulk soil, □ = autoclaved fuzzy flatsedge rhizosphere soil, ◯ = fuzzy flatsedge rhizosphere soil) and without isolate PCL3, (◆ = autoclaved bulk soil, ◇ = autoclaved fuzzy flatsedge rhizosphere soil; lines = carbofuran concentrations in each soil fitted to the first-order kinetic model)

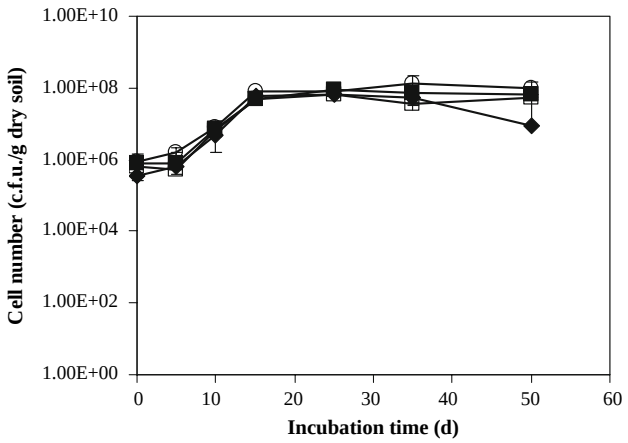

Fig. 4 Total number of carbofuran degraders which consisted of bacteria and fungi over time in the soils inoculated with isolate PCL3, (◆ = autoclaved bulk soil, ■ = bulk soil, □ = autoclaved fuzzy flatsedge rhizosphere soil, ◯ = fuzzy flatsedge rhizosphere soil)

play a significant role in this study. An abiotic degradation of carbofuran could occur by the hydrolysis of carbofuran at the carbamate linkage (Revilla et al. 1996).

Results from this experiment indicated that fuzzy flatsedge rhizosphere soil was the most effective soil in remediating carbofuran contaminated soil. Therefore, it was chosen to be used in the bioaugmentation experiment.

Bioaugmentation of carbofuran

Bioaugmentation of carbofuran by inoculation of *B. cepacia*, PCL3, into fuzzy flatsedge rhizosphere soil was conducted. Dissipation profiles of carbofuran in the soils were presented in Fig. 3. The degradation of carbofuran in all kinds of soils was described by a modified first-order kinetic model with r^2 ranged between 0.97–0.99.

There were no differences in $t_{1/2}$ of carbofuran (12–14 days) (Table 3) in all soils inoculated with the isolate PCL3 which corresponded with the numbers of carbofuran degraders (PCL3) in all soils were not markedly different (Fig. 4). In addition, the $t_{1/2}$ of carbofuran in inoculated soils of 12–14 days (Table 3) were not obviously different from the $t_{1/2}$ of carbofuran in all kinds of non-inoculated rhizosphere soils (15–19 days) (Table 1). These results indicated that bioaugmentation by adding the isolate PCL3 into the rhizosphere soil of fuzzy flatsedge did not improve the degradation of carbofuran and only rhizosphere remediation of carbofuran might be sufficient to remediate carbofuran residues in soil. However, our findings were in contrast to the findings of Yu et al. (2003) in which the degradation of butachlor in wheat rhizosphere soil at the initial concentration of 10 mg/kg was 5-times improved by butachlor degrader (Yu et al. 2003).

The $t_{1/2}$ of carbofuran, 12 days, in bulk soil inoculated with isolate PCL3 (Table 3) was shorter than in bulk soil without the inoculation (58 days) (Table 3) demonstrating that an inoculation of isolate PCL3 into bulk soil enhanced the degradation of carbofuran. Autoclaved soils with an addition of isolate PCL3 had a short half-life of carbofuran, 13–14 days (Table 3), which was not different from fuzzy flatsedge rhizosphere soil inoculated with isolate PCL3, confirming the ability of PCL3 to degrade carbofuran and indicating that bioaugmentation is useful in removal of carbofuran from soil.

Successful bioaugmentation of pesticides and organic hydrocarbons was discovered in the previous studies. For example, a shorter $t_{1/2}$ of butachlor in inoculated wheat rhizosphere soil than in non-inoculated rhizosphere soil and in bulk soil was reported by Yu et al. (2003). The inoculation of atrazine degrading bacteria into the soil treated with atrazine accelerated the atrazine

Table 3 Degradation rate constants (k) and half-lives ($t_{1/2}$) of carbofuran in inoculated soils with *B. cepacia*, PCL3

Samples	k_1 (/days)	$t_{1/2}$(days)	r^2*
Autoclaved bulk soil + PCL3	0.049	14	0.98
Bulk soil + PCL3	0.059	12	0.99
Autoclaved fuzzy flatsedge rhizosphere soil + PCL3	0.055	13	0.97
Fuzzy flatsedge rhizosphere soil + PCL3	0.060	12	0.98
Bulk soil	0.012	58	0.99

*Coefficients of determination for non-linear regressions

Table 4 Concentrations of carbofuran phenol in the soils with and without inoculation of isolate PCL3

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

B = bulk soil

BC = autoclaved bulk soil

R = rhizosphere soil of fuzzy flatsedge

RC = autoclaved rhizosphere soil of fuzzy flatsedge

mineralization in soil (Struthers et al. 1998; Topp 2001; Rousseaux et al. 2003). Strain B-14 at 10^6 c.f.u./g inoculated to soil mixed with 35 mg of chlorpyrifos/kg soil resulted in a higher degradation rate than was observed in non-inoculated soils (Singh et al. 2004). Three-folds increase in atrazine mineralization was found in soil inoculated with *Chelatobater heintzii* Cit1 (Rousseaux et al. 2003).

Carbofuran phenol was the only degradation product in all inoculated soils (Table 4). This result was coincided with the finding of Lalah et al. (1996) who reported that carbofuran phenol was the main degradation product in soil treated with carbofuran.

Our findings indicated that rhizosphere remediation as well as bioaugmentation is the effective bioremediation techniques to remove carbofuran residues from soil. In addition, only rhizosphere degradation is sufficient to remove carbofuran from soil.

Conclusion

Burkholderia cepacia, PCL3, is effective carbofuran degrader which its degradation ability was evident in bulk and autoclaved soils. However, addition of PCL3 into rhizosphere soil did not improve carbofuran removal efficiency of the indigenous rhizosphere microorganisms. The results suggested that rhizodegradation itself is the effective technique to remediate carbofuran from soil.

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References

Anderson JA, Kruger EL, Coats JR (1994) Enhanced degradation of a mixture of three herbicides in the rhizosphere of a herbicide-tolerant plant. Chemosphere 28:1551–1557

Biryukova OV, Fedorak PM, Quideau SA (2007) Biodegradation of naphthenic acids by rhizosphere microorganisms. Chemosphere 67:2058–2064

Chaudhry Q, Blom-Zandstra M, Gupta SK, Joner E (2005) Utilising the synergy between plants and rhizosphere microorganisms to enhance breakdown of organic pollutants in the environment. Environ Sci Pollut Res 12:34–48

Department of Agriculture (2003) Statistics of imported pesticides to Thailand. Ministry of Agriculture and Cooperatives, Bangkok, Thailand

EPA (2006) Interim Reregistration Eligibility Decision (IRED) Document for carbofuran: special review and reregistration division. www.epa.gov/oppsrrd1/reregistration/REDs/carbofuran_ired.pdf. Cited 15 Jan 2007

Franzmann PD, Zappia LR, Tilbury AL, Patterson BM, Davis GB, Mandelbaum RT (2000) Bioaugmentation of atrazine and fenamiphos impacted groundwater: laboratory evaluation. Biorem J 4:237–248

Gaur A, Adholeya A (2004) Prospects of arbuscular mycorrhizal fungi in phytoremediation of heavy metal contaminated soils: review article. Curr Sci 86:528–534

Howard PH (1991) Handbook of environmental fate and exposure data for organic chemicals: pesticides chelsea. Lewis Publishers, MI

Jordahl JL, Foster L, Schnoor JL, Alvarez PJJ (1997) Effect of hybrid poplar trees on microbial populations important to hazardous waste bioremediation. Environ Toxicol Chem 16:1318–1321

Lalah JO, Wandiga SO, Dauterman WC (1996) Mineralization, volatilization, and degradation of carbofuran in soil samples from Kenya. Bull Environ Contam Toxicol 56:37–41

Mo K, Lora CO, Wanken AE, Javanmardian M, Yang X, Kulpa CF (1997) Biodegradation of methyl *tert*-butyl ether by pure bacterial cultures. Appl Microbiol Biotechnol 47:69–72

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

Revilla E, Mora A, Cornejo J, Hermosin MC (1996) Persistence and degradation of carbofuran in Spanish soil suspensions. Chemosphere 32:1585–1598

Rousseaux S, Hartmann A, Lagacherie B, Piutti S, Andreux F, Soulas G (2003) Inoculation of an atrazine-degrading strain, *Chelatobacter heintzii* Cit1, in four different soils: effects of different inoculums densities. Chemosphere 51:569–576

Ruberto L, Vazqueza CS, Mac Cormackb WP (2003) Effectiveness of the natural bacterial flora, biostimulation and bioaugmentation on the bioremediation of a hydrocarbon contaminated Antarctic soil. Int Biodeter Biodegr 52:115–125

Schnoor JL, Licht LA, McCutcheon SC, Wolfe NL, Carreira LH (1995) Phytoremediation of organic and nutrient contaminants. Environ Sci Technol 29:318–323

Singh N, Megharaj M, Kookana RS, Naidu R, Sethunathan N (2004) Atrazine and simazine degradation in pennisetum rhizosphere. Chemosphere 56:257–263

Stottmeister U, Wieszner A, Kuschk P, Kappelmeyer U, Kastner M, Bederski O, Muller RA, Moormann H (2003) Effects of plants and microorganisms in constructed wetlands for wastewater treatment. Biotechnol Advan 22:93–117

Struthers JK, Jayachandran K, Moorman TB (1998) Biodegradation of atrazine by *Agrobacterium radiobacter* J14a and use of this strain in bioremediation of contaminated soil. Appl Environ Microbiol 64:3368–3357

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

Teerakun M, Reungsang A, Virojanakud W (2004) Phytoremediation of carbofuran residues in soil. Songklanakarin J Sci Technol 26:171–176

Thompson IP, van der Gast CJ, Ciric L, Singer AC (2005) Bioaugmentation for bioremediation: the challenge of strain selection: minireview. Environ Microbiol 7:909–915

Tomasek PH, Karns JS (1989) Cloning of a carbofuran hydrolase gene from *Achromobacter* sp. strain WM111 and its expression in gram-negative bacteria. J Bacteriol 171:4038–4044

Topp E (2001) A comparison of three atrazine-degrading bacteria for soil bioremediation. Biol Fertil Soils 33:529–534

Yu YL, Chen YX, Luo YM, Pan XD, He YF, Wong MH (2003) Rapid degradation of butachlor in wheat rhizosphere soil. Chemosphere 50:771–774

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O-04

PHYLOGENETIC ANALYSIS AND KINETIC CHARACTERIZATION OF CARBOFURAN DEGRADERS ISOLATED FROM CARBOFURAN PHYTOREMEDIATED RHIZOSPHERE SOIL

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Partial sequences of 16s rDNA and phylogenetic patterns of carbofuran degraders isolated from carbofuran phytoremediated rhizosphere soil were determined. The most effective isolated carbofuran degrader named PCL3 was 96% identical to *Burkolderia cepacia*. The 16s rRNA sequences of PCL3 was deposited to GenBank database as *B. cepacia* PCL3 (GenBank accession number of EF990634). PCL3 was investigated for its carbofuran degradation activity and grown at various carbofuran concentrations. Results revealed that isolate PCL3 could grow in BSM with the initial carbofuran concentration up to 150 mg/L. The growth kinetic of PCL3 could be described by Haldane equation. The Haldane parameters of the isolate PCL3 grown in BSM containing carbofuran as a sole carbon source, obtained by using nonlinear least square regression analysis, were μ_{max} = 0.0097 /day, K_s = 1.15 Mg/L and K_i = 96.82 mg/L. The values of yield coefficient were in the range of 0.24-0.38. The concentration of carbofuran which provided the maximum specific growth rate of PCL3 (S_{max}), base on the obtained K_i and K_s values, was 10.55 mg/L. Results implied that the concentration of carbofuran at a contaminated site should not be greater than 10.55 mg/L in order to obtain an effective bioaugmentation treatment using PCL3.

Keywords: carbofuran, carbofuran degraders, growth kinetic, phylogenetic analysis.

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BIOAUGMENTATION OF CARBOFURAN BY *Burkholderia cepacia* PCL3 IN BIOSLURRY PHASE SEQUENCING BATCH REACTOR

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ABSTRACT

Effectiveness of bioremediation technology in removal of carbofuran in contaminated soil using bioslurry phase sequencing batch reactor (SBR) was investigated. Two-liter blue bottle was used as a bioreactor with a working volume of 1.5 L at room temperature (27±2 °C). One total cycle period of SBR comprises of 1 h of fill, 82 h of react and 1 h of decant phases. Carbofuran concentration in soil was 20 mg/kg soil. Carbofuran degrader isolated from carbofuran phytoremediated soil namely *Burkholdera cepacia* PCL3 immobilized on corncob was used as the inoculum. Results revealed that biougmentation treatment (addition of PCL3) had the highest percentage of carbofuran removal of 89.7 followed by bioaugmentation together with biostimulation (addition of molasses) treatment of 87.9%, suggesting that bioremediation was an effective technology to remove carbofuran in contaminated soil. Abiotic experiments i.e. autoclaved soil slurry with corncob and no addition of PCL3 treatment and autoclaved soil slurry without PCL3 treatment could adsorb 19.67 and 7.8% of carbofuran, respectively, which implied that soil and corncob could act as sorbent for removal of carbofuran.

Keywords: Bio-slurry phase reactor; *Burkholderia cepacia* PCL3; Carbofuran; Sequencing batch reactor (SBR); Soil slurry

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BIOAUGMENTATION OF CARBOFURAN RESIDUES IN SOIL BY IMMOBILIZED *Burkholderia cepacia*, PCL3

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ABSTRACT

An ability of immobilized *B. cepacia*, PCL3, on corncob and sugarcane bagasse to remediate carbofuran in soil in comparison to free cell and indigenous microorganisms was investigated. Degradation of carbofuran in soil inoculated with PCL3, in both free and immobilized cell forms on corncob and sugarcane bagasse ($t_{1/2}$ 13-17 days) was more rapid than in soil without inoculation (74 days). An evident of PCL3 to degrade carbofuran was revealed in autoclaved soil in which a short $t_{1/2}$ of carbofuran (15-19 days) compared to 119 days in abiotic control was obtained. Half-lives of carbofuran in PCL3 amended soil (13-17 days) and autoclaved soil (15-19 days) were not significantly different ($p \leq 0.05$) indicating an effectiveness of the bioaugmentation technique to enhance carbofuran degradation in soil. Carbofuran degradation by immobilized PCL3 in corncob and sugarcane bagasse with $t_{1/2}$ of 16-19 days and free cell forms of 13-15 days were not significantly different ($p \leq 0.05$) suggesting that immobilization did not decrease carbofuran degradation activity of PCL3 as well as carbofuran transferring to the cells in support materials. Cell numbers of PCL3 on the immobilized matrices were observed to be stable at above 10^7 CFU/g dry support materials while inoculated free cells of PCL3 was found to decrease in soil after 25 of incubation. These findings implied that immobilization technique via an absorption on corncob and sugarcane bagasse improved the survival of *B. cepacia*, PCL3.

Keywords: Bioaugmentation, carbofuran, carbofuran degrader, corncob, immobilization, sugarcane bagasse

1. INTRODUCTION

Carbofuran (2,3-dihydro-2,2 dimethylbenzofuran-7-yl methylcarbamate) is a broad-spectrum insecticide widely used in paddy field to control insects and nematodes on contact or after ingestion (Extension Toxicology Network (Extoxnet), 1996). It is moderately persistent in soil with the half-life of 30 to 120 days depending on temperature, moisture content, pH and the numbers of microorganisms present (Devries and Evans, 1999). Carbofuran is of environmental concern because it is soluble in water and highly mobile in soil resulting in a high potential for groundwater contamination (Howard, 1991). Carbofuran is highly acute toxic to mammalian through cholinesterase inhibition (LD_{50} = 2 mg/ kg) (EPA, 2003).

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Bioaugmentation of Carbofuran Residues in Soil by Immobilized *Burkholderia cepacia*, PCL3

Microorganisms capable of degrading specific contaminants could be isolated from contaminated natural matrices and used to bioremediate the contaminant. These degraders are capable of using the contaminant as their energy sources, i.e., C- or N- or C and N-sources. Bioaugmentation is one of bioremediation techniques conducted by adding microbial cultures into the environmental matrices of concern to reduce contaminant concentrations, remediation time and capital cost. The successive bioaugmentation of pesticides (Rousseaux et al., 2003) and hydrocarbon (Rubertoa et al., 2003) by the isolated degraders had been reported (Chaudhry et al., 2002; Piutti et al., 2003) in which most of these degraders were used in free cell form. However, these free cells have some limitations for being used in the bioremediations system such as low survival ability in natural conditions, low recovery and low recycling capabilities (Kourkoutas, 2004). These limitations might be overcome by immobilization technique.

Immobilization is the action of physically confining cells in a defined region or space with retention of their activities for repeated and continue use. The use of immobilized systems offers many advantages over free cells including regeneration and reuse of immobilized cells for extend period of works. The supporting materials 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 (Kourkoutas et al., 2004).

Our previous study used immobilized PCL3 on corncob and sugarcane bagasse to degrade carbofuran spiked in Basal Salt Medium (BSM). We found that carbofuran was rapidly degraded in BSM by immobilized cells as well as by free cell (Plangklang and Reungsang, 2007). In addition, the survival of immobilized cells was evident in comparison to free cells. However, those experiments were conducted in the synthetic media which is not a natural matrix. Therefore, we are interested in applying the immobilized *B. cepacia*, PCL3, in carbofuran contaminated soil in order to investigate carbofuran degradation capability of immobilized degrader in mimic environment (soil) in comparison to free cell and indigenous microorganisms (soil without inoculation). Results from these studies will provide the information on the possibilities of prolong the life and increase the survival of *B. cepacia*, PCL3, as well as its application in a natural environmental matrix (soil).

2. MATERIALS AND METHODS

2.1 Microorganism and culture media

Carbofuran degrader, *B. cepacia*, PCL3, previously isolated from phytoremediated rhizosphere soil was grown at 30°C, 150 rpm, in Nutrient Broth (NB) containing 5 mg/L of carbofuran for 48 h and used as inoculum for immobilization. This microorganism is capable of using carbofuran as a sole C-source.

Culture medium used in this study was C-limited BSM containing (in g/L): 5.57 of $NaHPO_4$, 2.44 of KH_2PO_4, 2.00 of NH_4Cl, 0.20 of $MgCl_2.6H_2O$, 0.0004 of $MnCl_2.4H_2O$, 0.001 of $FeCl_3.6H_2O$ and 0.001 of $CaCl_2$. pH of the media was adjusted to be 7 before autoclaved at 121 °C for 15 min. For the BSM agar, 1.5% of bactoagarose was added to BSM before sterilization. Carbofuran solution in sterile distilled water at the concentration of 5 mg/L was coated on BSM agar as a sole C-source prior the usage.

2.2 Soil

A sandy loam soil sample, 0-15 cm depth, used in this study was collected from a rice field of Ban Nonmuang, A. Muang, Khon Kean Province. It was passed through a 2 mm sieve and kept in plastic bags at 4 °C before used. Background carbofuran in soil sample, detected by HPLC was 0.06 mg/kg.

2.3 Supporting materials

Sugarcane bagasse and corncob were used as the supporting materials for immobilization of carbofuran degrader because they are natural supporting materials presenting high matrix porosity and pore size to enhance the cell adsorption capability of immobilization. Sugarcane bagasse and corncob were cut into 0.7x0.7x0.7 cm and then boiled in 1% NaOH for 3 h to remove the lignin and fibers inside the materials which might react with the cells. The alkaline-boiled sugarcane bagasse and corncob were then washed using distilled water and then sterilized by autoclaving at 121°C for 15 min and kept at 4 °C prior to be used.

2.3 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 easy to operate (Bickerstaff, 1997). The immobilization technique was done by adding 75 g of each sterile corn cob and bagasse into the sterile 250 ml NB containing 5 mg/L of carbofuran before inoculating with isolate PCL3 (10% inoculum size). Flask was then incubated at 150 rpm, room temperature, for 48 hours. After incubation, supporting materials were then transferred to the fresh NB containing 5 mg/L of carbofuran and incubated, as previously described, before harvesting by filtration and washed with 0.85% NaCl under sterile condition. This process was repeated two times. Immobilized cells were kept at 4°C prior to be used in the experiment. The internal cell density on the sugarcane bagasse and corncob was then observed to be approximately 10^6 CFU/g dry materials.

2.4 Bioaugmentation of carbofuran residues in soil with *B. cepacia*, PCL3

Bioaugmentation of carbofuran in soil was conducted in a soil microcosm using 425 cm^3 glass jar capped with a plastic lid. Carbofuran solution at an initial concentration of 5 mg/kg dry soil was spiked into 20 g of soil dry weight in the glass jar and well mixed by hand stirring. Each of free cells and immobilized cell of *B. cepacia*, PCL3, on corncob and sugarcane bagasse was then added into the soil at the initial cell concentration of 10^5 cells/g dry soil. The initial moisture content of the soil samples was adjusted to 15-18% before incubating at room temperature with avoidance of sunlight. The soil samples were sacrificed at days 0, 5, 10, 15, 25, 35 and 60 and further extracted by an ASE100 Accelerate Solvent Extractor (Dionex, USA). Carbofuran and its metabolites concentrations were determined by HPLC. At each sampling date, the numbers of carbofuran degraders in soil were counted by spread plate technique. The half-lives of carbofuran in soils were calculated by fitting to a modified first-order kinetic model. In order to prove the capability of *B. cepacia*, PCL3, to degrade carbofuran in soil in comparison to indigenous microorganisms, two sets of control were included, i.e., autoclaved soil with inoculation of *B. cepacia*, PCL3, in both free and immobilized cell forms and soil without inoculation which were conducted as a described

manner. Abiotic degradation control was conducted in autoclaved soil with an inoculation of autoclaved immobilized matrices.

Number of leakage cells from immobilized matrices of *B. cepacia*, PCL3, in soil was determined by plate count technique on the BSM agar coated with 5 mg/L of carbofuran. To observe the cell growth and cell survival on the supporting materials, 10 g of immobilized materials were sampled from soil and washed with sterile 0.85% NaCl solution three times to remove soil particle attaching to the supports. The washed immobilized cells were blended to small particles using blender and then added to 50 mL sterile 0.85% NaCl solution and shaken at 250 rpm for 5 min in order to dissolve cells out from the support materials. Number of *B. cepacia*, PCL3, in the liquid part was then determined by plate count technique on the BSM agar coated with 5 mg/L of carbofuran.

2.5 Extraction of carbofuran and its metabolites from soil samples

Carbofuran and its metabolites i.e., carbofuran phenol and 3-keto carbofuran were extracted from soil samples by using an ASE 100 Accelerated Solvent Extractor (Dionex, Austria) equipped with 11-mL stainless-steel extraction cell. The extraction cell was prepared as follows: the bottom of the extraction cell was covered with a cellulose acetate membrane (16.2 mm I.D., Dionex, Austria) as a filter agent to prevent a frit blockage and fine soil breakthrough into the collection bottle. Afterwards, 13 g of dried soil sample was transferred into the extraction cell and finally, the top of the extraction cell was covered with a cellulose acetate membrane as filter agent before closed. The samples were extracted under the conditions which are 100 °C extraction temperature, 5 min static extraction time, 60% methanol (HPLC grade) as the extraction solvent and two extraction cycles. The ASE parameters were used according to the default settings, i.e., the maximum extraction pressure was set not to exceed 1,500 psi, the flush volume was 60% of the extraction cell volume, and the N_2-purge time was set to 1 min. After static extraction, the raw extracts collected in a special 200-mL glass bottle were adjusted to the volume of 25 mL in volumetric flask and passed through 0.45 μm nylon membrane syringe filter prior to analyze by HPLC. The percent recovery of this extraction method was 96%.

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

The concentrations of carbofuran, carbofuran phenol and 3-keto carbofuran in extracts were analyzed by Shimadzu 10-A HPLC equipped with 4.6x150 mm-Lunar 0.5 μm C-18 column (Phenomenex, USA), a UV detector operating at 220 nm and a 20 μL injector loop. The operating parameters were: mobile phase, methanol-water (60:40); flow rate, 1 mL/min at the ambient temperature. External standard linear calibration curves of carbofuran, carbofuran phenol and 3-keto carbofuran were used to quantify their concentrations in the aqueous phase. The observed concentration was characterized by its peak areas. The half-lives of carbofuran in the soil were calculated by fitting to a modified first-order kinetic model; $C = C_0e^{-kt} + Ya$, using SAS program (SAS Institute, Inc. 1982), where C was the mean concentration of carbofuran as a function of time in hours (mg/kg dry soil), C_0 was the initial carbofuran concentration (mg/kg dry soil), k was the rate constant (/day), t was time (days) and Ya was an asymptotic estimate of the concentration of carbofuran that degrades very slowly overtime (residual carbofuran) (mg/kg dry soil). The mean concentrations used in the regression were

weighed with inverse of the variance squared, /S^2 This method compensated for the non-constant variance and helped to improve the estimation of the parameters.

2.8 Statistical analysis

Data was analyzed by SPSS program Version 10.0 (SPSS Inc., Chicago, Il). The significance of treatments was set at *p*-value less than or equal to 0.05 by the one way ANOVA test.

3. RESULTS AND DISCUSSION

3.1 Degradation of carbofuran in soil by immobilized *B. cepacia*, PCL3

This experiment investigated an ability of immobilized *B. cepacia*, PCL3, on corncob and sugarcane bagasse to remediate carbofuran residues in soil in comparison to free cell and indigenous microorganisms. Carbofuran degradation profiles were depicted in Figure 1.

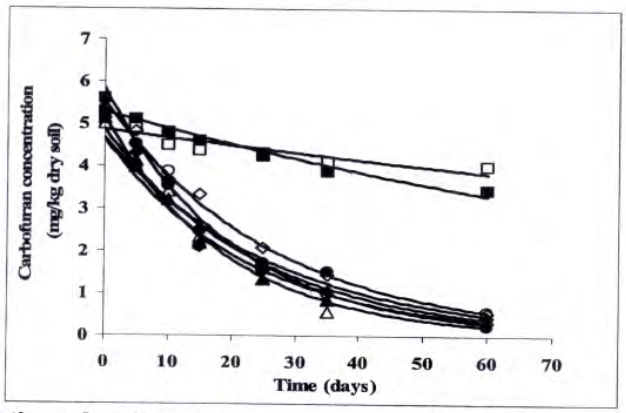

Figure 1 Degradation of carbofuran residues in soil (close) and in autoclaved soil (open) by indigenous microorganisms (■), *B. cepacia*, PCL3 in free cell form (▲ , △), immobilized cell on corncob (◆ , ◇), and immobilized cell on sugarcane bagasse (● , ○), and by abiotic degradation process (□); lines indicated the carbofuran degradation fitting to the first order kinetic model

The corresponding kinetic data fitting to a modified first-order kinetic model was tabulated in Table 1. The coefficients of determination, r^2, ranged between 0.80-0.99 indicating a good fit of the data to the modified first-order kinetic model (Table 1). Results revealed that degradation of carbofuran in soil inoculated with *B. cepacia*, PCL3, in both free cell form and immobilized cell form on corncob and on sugarcane bagasse (haft-lives of 13-17) was more rapid than in soil without inoculation (half-life of 74 days) (Table 1) which indicated a capability of *B. cepacia*, PCL3, to degrade carbofuran in soil. In addition, these results showed a promise of using bioaugmentation technique to remediate the carbofuran residue in soil. A carbofuran degradation ability of PCL3 was evident in inoculated autoclaved soil which a shorter half-life of carbofuran (15-19 days) than in an abiotic control (autoclaved soil added with autoclaved support materials) (119 days) was obtained (Table 1). Another evidence to support this finding is that half-lives of carbofuran in inoculated soil (13-17 days) (Table 1) were not significantly different ($p \leq 0.05$) from in inoculated autoclaved soil (15-19

days) (Table 1) which confirmed an effectiveness of the bioaugmentation technique to enhance carbofuran degradation in soil. An addition of degrader into soil is effective in remediation of the contaminant may be a result of degrader has a specific enzyme responsible for degrading the contaminant as its energy sources, i.e., C-, N- or C and N- sources. In this study, metabolites obtained were carbofuran phenol and 3-keto carbofuran (data not shown) suggesting that degrader and indigenous microorganisms may release hydrolase and oxidase' enzymes for degrading carbofuran (Evert, 2002). Previous research had reported a success bioaugmentation of pesticides. For example, a shorter half-life of butachlor in soil inoculated with butachlor degrader than in non-inoculated soil was reported by Yu et al. (2003). Bioaugmentation of atrazine with *Pseudomonas* sp. strain ADP resulted in a decrease in half-life of atrazine in aquifer material from 730 days to be less than 20 days in comparison to un-bioaugmented aquifer material (Rousseaux et al., 2003). Strain B-14 (10^6 cells/g) inoculated to soil mixed with 35 mg of chlorpyrifos/kg soil resulted in a higher degradation rate than that was observed in non-inoculated soils (Singh et al. 2004).

Table 1 Degradation rate constants (k_1) and half-lives ($t_{1/2}$) of carbofuran in soil

Treatments		k_1 (/day)	$t_{1/2}$ (days)**	r^2*
Soil	indigenous	0.0094	74[a]	0.88
	free cell	0.0523	13[b]	0.99
	immobilized cell in corncob	0.0435	16[b]	0.97
	immobilized cell in sugarcane bagasse	0.0401	17[b]	0.94
Autoclaved soil	without inoculation	0.0058	119[c]	0.48
	free cell	0.0462	15[b]	0.91
	immobilized cell in corncob	0.0369	19[b]	0.99
	immobilized cell in sugarcane bagasse	0.0416	17[b]	0.93

*Coefficients of determination for non-linear regressions

**Comparison between treatment in column are significantly different (Duncan, $p \leq 0.05$) if mark different small letters

Carbofuran degradation by immobilized cell in corncob and sugarcane bagasse (half-lives of 16-19 days) and free cell forms (13-15) of *B. cepacia*, PCL3, were not significantly different ($p \leq 0.05$) (Table 1). This suggested that immobilization did not decrease carbofuran degradation activity of *B. cepacia*, PCL3, as well as carbofuran transferring to the cells in support materials. High matrix porosity and pore size present in these two materials might enhance the absorption capacity and substrate transfer to the immobilized cell (Gu, 1994; Jimoh, 2004). These finding were supported by Ma et al. (2006) who found that an immobilization of *Thiobacillus denitrificans* on peat moss and wood chip effectively removed H_2S in the humidified air with removal efficiency was above 98%.

3.2 Cells growth, survival and leakage

This experiment was designed to monitor growth, survival and leakage of immobilized cells in order to evaluate the stability of immobilized cells when they were inoculated into soil. Growth and survival of immobilized cell were presented as number of carbofuran degrader in support materials. Results revealed that cell numbers of *B. cepacia*, PCL3, on the immobilized matrices increased from 10^6 CFU/g dry support material to 10^7 CFU/g dry support materials and stable at this rate after 25 days of experiment (Figure 2). In comparison, number inoculated free cells of *B. cepacia*, PCL3, was found to decrease in soil from 10^6 CFU/g dry soil to 10^5 CFU/g dry soil after 35 days of incubation (Figure 2). These findings implied that immobilization technique via an absorption on corncob and sugarcane bagasse

improved the survival of *B. cepacia*, PCL3. These support materials might act as protective agents against improper condition such as substrate and product inhibition during incubation as well as shear force (Kourkoutas et al., 2004). A similar finding was reported by Jimoh (2004) in which the immobilized cell of *Saccharomyces cerevisiae* on agricultural materials i.e., sugarcane chip, wood chip, bamboo chip, rice husk and maize coke could survive during the ethanol fermentation without the inhibitory effect from high ethanol concentration.

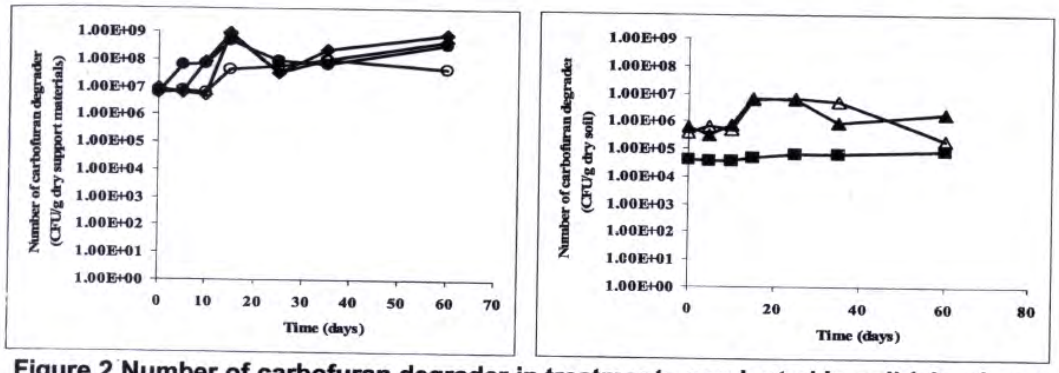

Figure 2 Number of carbofuran degrader in treatments conducted in soil (close), and in autoclaved soil (open); in immobilized cells of *B. cepacia*, PCL3, on corncob (◆ , ◇) and on sugarcane bagasse (● , ○), free cells (▲ , △) and indigenous microorganisms (■)

Leakage of cells from support materials was presented as a cell number observed in soil inoculated with immobilized cells during incubation (Figure 3). Though the number of the cells leak from support materials was found to be above 10^6 CFU/g dry soil after 25 days of incubation in all treatments (Figure 3), the number of *B. cepacia*, PCL3, in the support materials was not reduced (Figure 2). Cell leakage was observed after cell number in support materials was stable (after day 25) (Figure 2, 3), indicating that there was a cell growth with a limitation of space inside the support materials resulting in the leakage of the immobilized cells from corncob and sugarcane bagasse.

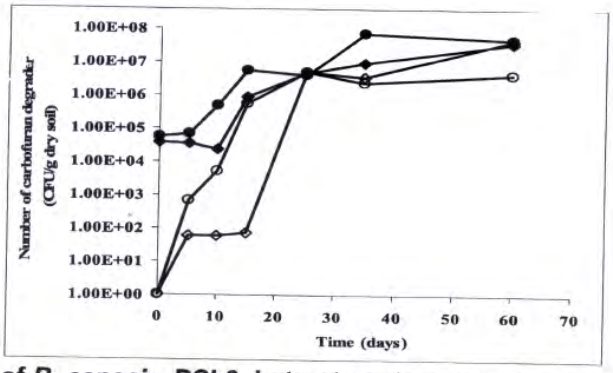

Figure 3 Number of *B. cepacia*, PCL3, in treatments conducted in soil (dark), and in autoclaved soil (transparent); leakage cell from immobilized cells of on corncob (◆ , ◇) and on sugarcane bagasse (● , ○)

Cell detachment might be resulted from the fact that there are no barriers between the cells and the media which led to a possibility of cell detachment and relocation with potential

establishment of equilibrium between adsorbed and cells. In the adsorption process, cells were immobilized on a supporting material by physical adsorption due to electrostatic forces or by covalent binding between the cell membrane and the supporting materials (Kourkoutas et al., 2004). A previous research by Kumar and Das (2001) reported a similar finding in which the daughter cells produced from binary fission of the immobilized cells of *Enterobactor cloacae* IIT-BT 08 leaked to the culture media when there was no free space on the porous support materials.

4. CONCLUSIONS

B. cepacia, PCL3, in both free and immobilized cell forms on corncob and sugarcane bagasse into soil improved carbofuran degradation efficiency indicating that bioaugmentation of *B. cepacia*, PCL3, is a success. In addition, immobilization technique improved the survival of *B. cepacia*, PCL3, by adsorbing of the cell on corncob and sugarcane bagasse.

5. ACKNOWLEDGEMENT

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6. REFERENCES

Bickerstaff G.F. (1997) Immobilization of enzyme and cell. NJ. Titowa. *Humanan press*. 367.

Chaudhry G.R., Mateen A., Kaskar B., Sardessai M., Bloda M., Bhatti A.R., Walia S.K. (2002) Induction of carbofuran oxidation to 4-hydroxycarbofuran by *Pseudomonas* sp.50432. *FEMS Microbiol. Lett.* **214**: 171-176.

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

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

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

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

Gu Y.X., Hu Z.C., Korus R. A. (1994) Effects of adsorption on mass transfer in immobilization matrices. *Chem. Eng. J. Biochem. Eng. J.* **54**: B1-B8.

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

Jimoh A. (2004) Effect of immobilized materials on *Saccharomyces cerevisiae*. *AU J. Technol.* **8**: 62-68.

Kourkoutas Y., Bekatorou A., Banat I. M., Marchant R., Koutinas A. A. (2004) Immobilization technologies and support materials suitable in alcohol beverages production: a review. *Food Microbiol.* **21**: 377-397.

Kumar N., Das D. (2001) Continuous hydrogen production by immobilized *Enterobactor Cloacae* IIT-BT 08 using lignocellulosic materials as solid matrices. *Enzyme Microbial. Technol.* **29**: 280-287.

Mar Y.L., Yang B.L., Zhao J.L. (2006) Removal of H_2S by Thiobacillus Denitrificans immobilized on different matrices. *Bioresource Technol.* **97**: 2041-2046.

Piutti S., Semon E., Landry D., Hartmann A., Dousset S., Lichtfouse E.,Topp E., Soulas G., Martin-Laurent F. (2003) Isolation and Characterisation of *Nocardiodes sp.* SP12, an atrazine-degrading bacterial strain possessing the gene trzN from bulk and maize rhizosphere soil. *FEMS Microbiol. Lett.* **211**: 111-117.

Plangklang P. Reungsang A. (2007) Degradation of carbofuran using Immobilized carbofuran degrader *Agrobacterium radiobacter* PCL3. Proceeding of the International Symposium 2006 on Pioneering Studies of Young Scientists on Chemical Pollution and Environmental Changes. Nov 17-19. Matsuyama, Japan. 75-78.

Rubertoa, L., Vazqueza, C.S., Mac Cormackb, W.P. (2003) Effectiveness of the natural bacterialfora, biostimulation and bioaugmentation on the bioremediation of a hydrocarbon contaminated Antarctic soil. *Int. Biodeteriorat. Biodegrad.* **52**: 115 – 125.

Rousseaux, S., Hartmann, A., Lagacherie, B., Piutti, S., Andreux, F., Soulas G. (2003) Inoculation of an atrazine-degrading strain, Chelatobacter heintzii Cit1, in four different soils: effects of different inoculum densities. *Chemosphere* **51**:569–576.

SAS Institute. 1985. SAS User's guide. Statistics. Version 5ed. SAS Institue. Cary, NC.

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

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Degradation of Carbofuran Residues by Immobilized Carbofuran Degrader *Agrobacterium radiobacter* PCL3

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Abstract. Carbofuran degrader, *Agrobacterium radiobacter* PCL3, previously isolated from carbofuran phytoremediated rhizosphere soil was immobilized by adsorbing on corncob and sugarcane bagasse as the environmental friendly support materials. The carbofuran degradation abilities of the immobilized cells were investigated in Basal Salt Medium (BSM) containing 5 mg/L of carbofuran as a sole C-source in comparison to free cells. The growth, leakage, survival and reusability of the immobilized cells were monitored throughout the experiment. Results indicated that carbofuran was rapidly degraded by *A. radiobacter* PCL3 in both free cell and immobilized cell forms with half-lives in the ranges of 3-4 days, indicating that there was no limitation of carbofuran transfer to the immobilized cell on the porous media of corn cob and bagasse. Immobilized carbofuran degrader could survive (10^6-10^7 CFU/ml) through 30 days of incubation while the number of free cell was observed to reduce continuously after 10 days indicating an improvement of cell survival by this immobilization technique. A high number of leakage cells i.e., 10% of initial cell on the support materials were found which might be resulting from cell growth within a limited space and the weak of adhesive bond between cells and support materials. Immobilized cell could be reused two times with an ability to degrade carbofuran (half lives of 4-5 days in BSM), which suggested an advantage of immobilized carbofuran degrader over free cells and the capabilities of corn cob and bagasse to be used as cells supporting materials.

Introduction

Carbofuran (2,3-dihydro-2,2 dimethylbenzofuran-7-yl methylcarbamate) is a broad-spectrum insecticide widely used in paddy field to control insects and nematodes on contact or after ingestion (Extoxnet 1996). It is of environmental concern because it is soluble in water and mobile in soil resulting in a high potential for groundwater contamination (Extoxnet 1996).

In our previous study, thirteen-different single colonies were isolated from carbofuran phytoremediated rhizosphere soils by an enrichment technique. Their abilities to degrade carbofuran were investigated in Basal Salt Medium (BSM) containing 5 mg/L of carbofuran. The shortest half-life of carbofuran, 3 days, was found in C-limited BSM cultured with an isolate named PCL3, *Agrobacterium radiobacter,* indicating that carbofuran was used as a sole C-source. In our point of view, it is interesting toward using isolate PCL3 to remediate carbofuran contaminated in environment as a bioaugmentation treatment, one of bioremediation techniques. The successive bioaugmentation of pesticides using the isolated degraders had been reported (Piutti et al. 2003), in which most of pesticide degraders were used in free cell form. However, there are some limitations of using free cells of the isolates such as low survival ability of free cells in a natural condition, low recovery and low recycling (Kourkoutas 2004). Immobilization of cells might overcome these limitations. The use of immobilized systems offers many advantages over free cells including regeneration and reuse of immobilized cells for extend period of works. The supporting materials may act as protective agents against the effects of pH, temperature, solvent, heavy metals or even substrate and product inhibition enhancing the cell survival (Kourkoutas et al. 2004). The support materials used for

immobilization could be either synthetic polymers or natural portions. Due to the synthetic polymer possessed the disposal problems through non-biodegradable characteristic, thus the natural materials such as agricultural residues becomes attractive to be used as environmental friendly support materials for immobilization.

Above reports revealed that immobilized cells offer the advantages over free cells. However, there is very limited information on the degradation of carbofuran by immobilized carbofuran degrader cells using agricultural residue. Thus, the objective of this study was to examine the efficiency of immobilized cell of *A. radiobacter* PCL3 on corn cob and sugar cane bagasse for carbofuran degradation.

Microorganism and Culture Media

Carbofuran degrader, *A. radiobacter* PCL3, using carbofuran as a sole C-source, previously isolated from phytoremediated rhizosphere soil was grown at 30°C, 150 rpm in Nutrient Broth (NB) containing 5 mg/L of carbofuran for 48 hrs and used as inoculum for immobilization.

Immobilization Technique

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 easy to operate (Bickerstaff, 1997). Sugarcane bagasse and corncob were used in this study as the supporting materials for immobilization of carbofuran degrader because they are natural supporting materials presenting high matrix porosity and pore size to enhance the cell adsorption capability of immobilization. Sugarcane bagasse and corncob were cut into 0.7x0.7x0.7 cm and then boiled in 0.001 M H_2SO_4 for 4 hours, 2 times to remove the lignin and fibers inside the materials, which might react with the cells. The acid-boiled sugarcane bagasse and corncob were then washed using distilled water and then sterilized by an autoclave at 121°C for 15 min. A 75-g of each sterile corn cob and bagasse were added into the sterile 250 ml NB containing 5 mg/L of carbofuran before inoculating with isolate PCL3 (10% inoculum size) in order to have the cells grown and adsorbed on the porous. Flask was then incubated at 150 rpm, room temperature for 48 hours. Supporting materials were then transferred to the fresh NB containing 5 mg/L of carbofuran and incubated as before 2 times, before harvesting by filtration and washed with 0.85% NaCl under sterile. The internal cell density on the support materials was then observed to be approximately 10^7 CFU/g dry materials.

Biodegradation of Carbofuran by *A. radiobacter* PCL3, Free and Immobilized Cell Form

Degradation of carbofuran by *A. radiobacter* PCL3 in free cell form was conducted in 500-mL shake flask in batch experiments. A 200 ml of C-limited BSM containing 5 mg/L of carbofuran as a sole carbon source was added into the flasks before inoculating with approximately 10^6 cells/ml of free cell of *A. radiobacter* PCL3. BSM, pH 7, containing (in g/L): 5.57 of $NaHPO_4$, 2.44 of KH_2PO_4, 2.00 of NH_4Cl, 0.20 of $MgCl_2.6H_2O$, 0.0004 of $MnCl_2.4H_2O$, 0.001 of $FeCl_3.6H_2O$ and 0.001 of $CaCl_2$. Flask was then incubated at room temperature and shaken at 150 rpm for 30 day. Culture media was sampled to extract carbofuran by liquid-liquid portioning method using dichloromethane as extracting organic solvent. Carbofuran concentrations were then 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 mobile phase; methanol-water (60:40), flow rate 1 mL/min at the ambient temperature. Half-lives of carbofuran in the culture media were calculated by fitting to a modified first-order kinetic model. Number of degrader in culture media was determined by plate count technique. Degradation of carbofuran by immobilized cell was conducted in 250-ml shake flask in batch experiments. A 100 ml of C-limited BSM containing 5 mg/L of carbofuran were added into the flasks before inoculating with approximately 10^6 cells/mL of immobilized cell. Previously described parameters were monitored for 30 days. Cell growth and cell survival on the supporting materials were determined by sampled 10g of wet immobilized cells from culture media and washed with sterile 0.85% NaCl solution three times, blended to small

particles and then added to 50 mL sterile 0.85% NaCl solution. Number of *A. radiobacter* PCL3 in the liquid part was then determined by plate count technique.

Results indicated that carbofuran was rapidly degraded by isolate PCL3 in both free and immobilized cell form on corncob and on sugarcane bagasse with half-lives of 3, 3 and 4 days, respectively (Table 1). This indicated that the immobilization of carbofuran degrader by cell adsorption on corn cob and bagasse did not limit carbofuran transferring to the cell. Cell numbers on the immobilized matrices were observed in the range of 10^7-10^8 CFU/g dry support material (10^6-10^7 CFU/mL) throughout the 30 days, while free cells of *A. radiobacter* PCL3 was found to decrease continuously in culture media after day 10 (Figure 1b). These findings implied that the survival and stability of *A. radiobacter* PCL3 could be improved by immobilization technique through absorbing on corncob and sugarcane bagassess. Despite the fact that an initial cell concentration used in both forms were the same at the start of the experiment i.e., 10^6 CFU/mL, though results indicated that number of free cell was 100 times higher than number of the immobilized cell (Figure 1b) indicating that the growth of immobilized *A. radiobacter* PCL3 was limited. However, even though the number of the cells leak was found to be 10^5 CFU/mL, the number of *A. radiobacter* PCL3 in the support materials was not reduced (Figure 1b), which indicated that the cells were leaking out from the porous media of corn cob and bagasse due to a limited space.

Table 1. Degradation rate constants (k_1) and half-lives ($t_{1/2}$) of carbofuran in BSM.

Carbofuran degrader	k_1 (/day)	$t_{1/2}$ (days)	R^2
Free cells	0.23	3	0.91
Immobilized cell in corncob	0.28	3	0.90
Immobilized cell in sugarcane bagasse	0.2	4	0.97

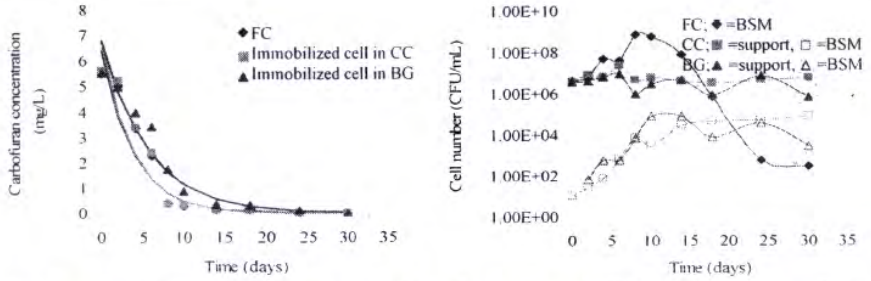

Figure 1. The correlations between: a) = degradation of carbofuran in BSM by carbofuran degrader (lines = carbofuran concentrations in C-limited BSM fitted to the first-order kinetic model) and b) = cell number of carbofuran degrader (FC = free cell, CC = corncob, SG = sugarcane bagasse).

After 30 days of incubation, immobilized corn cob and bagasse were harvested and re-inoculated into a fresh BSM media containing 5 mg/L of carbofuran. Carbofuran concentration, cells leakage and survival were monitored for 30 days. This was done to investigate a reusability of immobilized cells. At the end of day 30, immobilized corncob and bagasse were harvested again and re-inoculated into a fresh BSM media containing 5 mg/L of carbofuran. Previously described parameters were monitored for 30 days. Results demonstrated that the immobilized cell could be reused two times with the remaining of carbofuran degradation ability (half lives of 4-5 days in BSM) (Table 2) indicating an advantage of using immobilized cell over free cell. However, as the number of reuse step increased, numbers of cells survive decreased while number of cells leak increased (Figure 2). Changes in particle size from 0.7 cm to 0.4 cm of both two support materials in the second reuse step were in charge to this incident. In consequent, an increase in cell leakage occurred. A degradation of corncob and sugarcane bagasse implied that the immobilization of cell on these support materials could be environmental friendly without the disposal problems unlike the synthetic materials.

Table 2. Degradation rate constants (k_1) and half-lives ($t_{1/2}$) of carbofuran in BSM.

Immobilized carbofuran degrader		k_1 (/day)	$t_{1/2}$ (days)	R^2
Corncob:	first reuse	0.18	4	0.94
	second reuse	0.15	5	0.95
Sugarcane bagasse:	first reuse	0.15	5	0.96
	second reuse	0.14	5	0.99

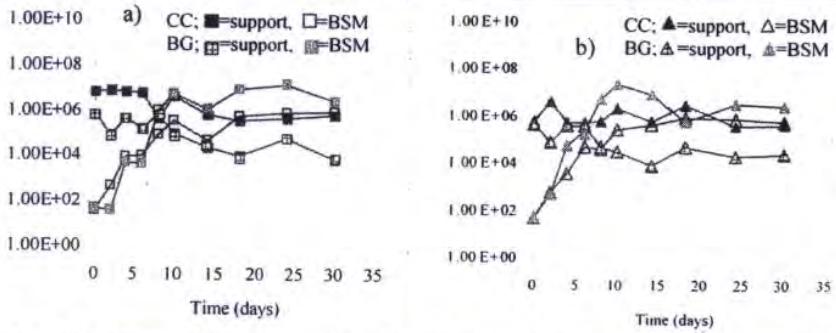

Figure 2. Cell number of immobilized carbofuran degrader; a) = first reuse and b) = second reuse (CC = corncob, SG = sugarcane bagasse).

Conclusion

In conclusions, corncob and sugarcane bagasse could be used as supporting materials to immobilize *A. radiobacter* PCL3. Carbofuran was rapidly degraded by immobilized cells as well as by free cell. Immobilized cell could survive and be reused indicating the stability and the possibility to use immobilized cell in continuous process. Corncob and bagasse were biodegradable. Thus, they could be used environmental friendly for remediating contaminant in the contaminated site as support materials for immobilization.

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References

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

Kourkoutas Y., Bekatorou A., Banat I. M., Marchant R. & Koutinas A. A. (2004) Food Microbiol. 21: 377-397.

Piutti S., Semon E., Landry D., Hartmann A., Dousset S., Lichtfouse E., Topp E., Soulas G. & Martin-Laurent F. (2003) FEMS Microbiol. Lett. 211: 111-117.

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