SELECTION OF SUPPORT MATERIALS FOR *Burkholderia cepacia* PCL3 IN TREATMENT OF CARBOFURAN

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การคัดเลือกวัสดุพยุงสำหรับ *Burkholderia cepacia* PCL3 เพื่อบำบัดคาร์โบฟูราน

นางสาว สุชีรา เหล่าเจริญ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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สุธีรา เหล่าเจริญ: การคัดเลือกวัสดุพยุงสำหรับ *Burkholderia cepacia* PCL3 เพื่อบำบัดคาร์โบฟูราน (SELECTION OF SUPPORT MATERIALS FOR *Burkholderia cepacia* PCL3 IN TREATMENT OF CARBOFURAN) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. อลิศรา เรืองแสง, 123 หน้า.

วัสดุเหลือทิ้งทางการเกษตร 4 ชนิดได้แก่ กาบมะพร้าว ต้นกก กาบกล้วย และต้นผักตบชวา ที่ผ่านและไม่ผ่านกระบวนการกำจัดลิกนิน ถูกนำมาใช้เป็นวัสดุพยุงสำหรับตรึงเซลล์ *Burkholderia cepacia* PCL3 เพื่อใช้ในการบำบัดคาร์โบฟูรานที่ความเข้มข้น 5 มิลลิกรัมต่อลิตรในน้ำเสียสังเคราะห์ และ 5 มิลลิกรัมต่อกิโลกรัมในดิน โดยพบว่า กาบมะพร้าวที่ไม่ผ่านกระบวนการกำจัดลิกนิน เป็นวัสดุพยุงที่เหมาะสมที่สุดสำหรับ PCL3 ในการบำบัดคาร์โบฟูรานที่ปนเปื้อนในน้ำเสียสังเคราะห์และดิน จากผลการทดลอง พบว่า ในน้ำเสียสังเคราะห์ PCL3 ที่ถูกตรึงบนวัสดุพยุงชนิดนี้มีความคงตัว 78.72 เปอร์เซ็นต์และสามารถย่อยสลายคาร์โบฟูรานให้ค่าครึ่งชีวิต ($t_{1/2}$) เท่ากับ 3.40 วัน ซึ่งสั้นกว่าการบำบัดด้วยเซลล์อิสระประมาณ 2.8 เท่า และสามารถนำเซลล์ตรึงนี้กลับมาใช้ซ้ำได้ อย่างน้อย 3 ครั้ง โดยที่ PCL3 ไม่มีการสูญเสียความสามารถในการย่อยสลายคาร์โบฟูราน ($t_{1/2}$ = 2.58-4.41 วัน) ส่วนการบำบัดคาร์โบฟูรานที่ปนเปื้อนในดิน เซลล์ตรึงสามารถย่อยสลายคาร์โบฟูรานให้ค่า $t_{1/2}$ เท่ากับ 18.96 วัน และสามารถนำกลับมาใช้ซ้ำได้ โดยให้ค่า $t_{1/2}$ เท่ากับ 20.06 วัน จากการศึกษาผลของความเข้มข้นของคาร์โบฟูรานต่อการเจริญ และการย่อยสลายคาร์โบฟูรานโดย PCL3 ในรูปของเซลล์ตรึงบนกาบมะพร้าวที่ไม่ผ่านการกำจัดลิกนิน เปรียบเทียบกับ PCL3 ในรูปของเซลล์อิสระ ทั้งในน้ำเสียสังเคราะห์ และดิน ที่ระดับความเข้มข้นของคาร์โบฟูราน 5 ถึง 280 มิลลิกรัมต่อลิตร และ 5 ถึง 250 มิลลิกรัมต่อกิโลกรัม ตามลำดับ ผลการทดลอง พบว่า ในน้ำเสียสังเคราะห์ ระดับความเข้มข้นคาร์โบฟูรานสูงกว่า 100 มิลลิกรัมต่อลิตร มีผลยับยั้งการเจริญและการย่อยสลายคาร์โบฟูรานของเซลล์อิสระ แต่ไม่มีผลยับยั้งต่อเซลล์ตรึง สำหรับการทดลองในดิน การเจริญของเซลล์อิสระถูกยับยั้งที่ความเข้มข้นสูงกว่า 100 มิลลิกรัมต่อกิโลกรัม แต่ไม่มีผลยับยั้งการเจริญของเซลล์ตรึงในทุกระดับความเข้มข้น แบบจำลองของลองซ์ (Luong's model) ถูกนำมาใช้อธิบายจลนพลศาสตร์การเจริญ และการย่อยสลายคาร์โบฟูรานของเซลล์อิสระ ในขณะที่แบบจำลองของโมนอด (Monod's model) ถูกนำมาใช้ในการอธิบายจลนพลศาสตร์ของเซลล์ตรึง จากค่าจลนพลศาสตร์ที่ได้ พบว่าความสามารถในการเจริญ และการย่อยสลายคาร์โบฟูรานของ PCL3 ในรูปเซลล์อิสระจะถูกยับยั้งโดยสมบูรณ์ที่ระดับความเข้มข้นคาร์โบฟูรานเท่ากับ 250 มิลลิกรัมต่อลิตรในน้ำเสียสังเคราะห์ และ 284 มิลลิกรัมต่อกิโลกรัมในดิน และพบว่าการใช้เซลล์ตรึงจะให้ค่า μ_{max} และค่า K_s ที่ต่ำกว่า เมื่อเปรียบเทียบกับการใช้เซลล์อิสระ นอกจากนี้ยังพบว่าที่ความเข้มข้นของคาร์โบฟูรานในดินเท่ากับ 1-150 มิลลิกรัมต่อกิโลกรัม ประสิทธิภาพการย่อยคาร์โบฟูรานของเซลล์อิสระและเซลล์ตรึง ไม่มีความแตกต่างกัน อย่างไรก็ตามที่ความเข้มข้นของคาร์โบฟูรานเท่ากับ 200-250 มิลลิกรัมต่อกิโลกรัมดิน ค่า $t_{1/2}$ ของคาร์โบฟูรานในดินที่เติมเซลล์ตรึง สั้นกว่าในดินที่เติมเซลล์อิสระถึง 1.5 เท่า จากผลการทดลองแสดงให้เห็นถึงความเป็นไปได้ในการใช้เทคนิคการตรึงเซลล์ในการปรับปรุงประสิทธิภาพการบำบัดทางชีวภาพของดินและน้ำที่ปนเปื้อนคาร์โบฟูราน โดย *B. cepacia* PCL3

สาขาวิชา การจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต .....

ปีการศึกษา 2552 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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KEYWORDS: AGRICULTURAL RESIDUES/ BIOAUGMENTATION/ *Burkholderia cepacia* PCL3/ CARBOFURAN/ IMMOBILIZATION

SUCHEERA LAOCHAROEN: SELECTION OF SUPPORT MATERIALS FOR *Burkholderia cepacia* PCL3 IN TREATMENT OF CARBOFURAN: ASSOC. PROF. ALISSARA REUNGSANG, 123pp.

Four types of agricultural residue i.e., coir, bulrush, banana stem and water hyacinth stem in delignified and undelignified forms were used as support materials for the immobilization of *Burkholderia cepacia* PCL3 in carbofuran remediation. The bioremediation experiments were conducted at the initial carbofuran concentration of 5 mg l^{-1} in synthetic wastewater (SWW) and 5 mg kg^{-1} in soil. Results indicated that undelignified coir was the most suitable support material for PCL3 immobilization in carbofuran remediation in SWW and soil. In SWW, the immobilized cell on coir possessed a high stability of 78.72%, high carbofuran degradation ability with the short $t_{1/2}$ of 3.40 days (2.8 times shorter than the treatments with free cells of PCL3). In addition, it could be reused at least three times with the remaining of high carbofuran degradation ability ($t_{1/2}$ of 2.58-4.41 days). In soil, immobilized PCL3 on undelignified coir gave the high carbofuran degradation efficiency with the short $t_{1/2}$ of 18.96 days and showed a potential to be reused with the short $t_{1/2}$ of 20.06 days. The effect of initial carbofuran concentration on growth and carbofuran degradation ability of immobilized PCL3 on coir in comparison to free cells was investigated in SWW and soil with the concentrations ranged between 5 and 250 mg l^{-1} and 5 and 250 mg kg^{-1} soil, respectively. In SWW, the growth and degradation ability of free cells were inhibited at the initial carbofuran concentrations of greater than 100 mg l^{-1}. The inhibitory effect of carbofuran on PCL3 could not be found in the immobilization treatments. In soil, the growth of free cells was inhibited at the concentration of greater than 100 mg kg^{-1} while the growth of the immobilized cells was not inhibited in any range of carbofuran concentration. Substrate inhibition model, Loung model, was used to explain the growth and degradation kinetic of PCL3 in free cell form while the Monod model was used to predict the kinetic behavior of the immobilized cells. The results indicated that the concentration of carbofuran of approximately 250 mg l^{-1} could completely inhibit growth and degradation activity of free cells of PCL3 in SWW and the carbofuran concentration of approximately 284 mg kg^{-1} could completely inhibit growth of PCL3 in soil. The smaller μ_{max} and K_s values were obtained when the immobilized cells were used as compared to free cells. At the initial carbofuran concentration of 1-150 mg kg^{-1} soil, the carbofuran degradation efficiency of PCL3 in free and immobilized cell forms was not significant different. However, at the high carbofuran concentrations of 200-250 mg kg^{-1} soil, the $t_{1/2}$ of carbofuran in soil augmented with immobilized PCL3 were approximately 1.5 times shorter than in the treatments with free cells. The results indicated a great potential of using immobilization technique to improve the overall efficiency of carbofuran bioremediation in water and soil by *B. cepacia* PCL3.

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

Academic Year : 2009 Advisor's Signature: A. Reungsang

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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 (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; EPA, 2006). In 2008, 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 cause a risk to contaminate soil and groundwater in the applied area resulting in a sub-lethal or chronic effects to aquatic organisms through cholinesterase inhibition, neurotoxicity and reproductive effects (De Melo Plese et al., 2005). Thus, a removal of carbofuran from contaminated area is necessary.

One of bioremediation techniques to remediate a contamination of pesticide is bioaugmentation which is an addition of microorganisms to aid a dissipation of pesticide. Successful bioaugmentation of pesticides using the isolated degraders had been reported (Piutti et al., 2003; Rousseaux et al., 2001; Franzmann et al., 2000; Struthers et al., 1998; Ruberto 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 natural conditions, low recovery and low capability of 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 of works. The support materials may act as protective agents against the effects of pH, temperature, solvent, heavy metals or even substrate and product inhibitions enhancing the cell survival (Bekatorou et al., 2004).

The support materials used for immobilization could be either synthetic polymers or natural materials. Several advantages of synthetic polymer such as polyvinyl alcohol and polyurethane foam including high mechanical strength, resistant to organic solvents and microbial attack, easy handling and regenerability (Patil et al., 2006). However, they were considered to be expensive for use in large-scale outdoor system such as in the fields. In addition, the disposal of synthetic polymer is of concern due to its non-biodegradable characteristic, unlike the natural materials such as agricultural residues which are biodegradable. Therefore, collateral research on cell immobilization using natural support 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. Adsorption is the major process for cell immobilization on agricultural support materials. The structure and material of the support material directly influence microbial adhesion and its degradation ability, therefore a selection of an appropriate support material is an important step for efficient immobilization system (Pattanasupong et al., 2004). In the previous literature, agricultural residues such as coconut fiber (Pattanasupong et al., 2004), corncob powder (Labana et al., 2005), wheat straw and maple woodchips (Shin et al., 2002) showed the potential to be used as cell support materials for bioremediation treatment. However, there is a limited information on the use of Thai agricultural residues for the immobilization of carbofuran degrader to bioremediate carbofuran.

Many types of agricultural residue are different in structure, physical and chemical properties including fiber length and width, and cell wall architecture. The major physical difference are water sorption, free volume, permeability, and strength. Chemically, they are differed in lignin, cellulose, and hemicellulose content (Kelley et al., 2004). In this study, four natural fibers, viz., banana stem, waterhyacinth stem, bulrush and coir, with differences in characterization were selected from the agriculture sector and examined whether they were suitable support material to immobilize a carbofuran degrader. Banana stem (*Musa acuminate*) formed from the bases of overlapping leaves. It contains lignin, cellulose and hemicellulose about 9, 43.46 and 38.54 % wt, respectively, (Jústiz-Smith et al., 2008). Water hyacinth stem (*Eichhornia crassipes*) is a free floating aquatic weed. It is a cellulose natural fiber in

which the fiber consists of a bundle of rod-like fibrils, and its texture is harsh and coarse. It has lignin, cellulose and hemicellulose content about 7, 31 and 22% wt, respectively (Bolenz et al., 1990). Bulrush (*Scirpus grossus* L.f.) found in swampy or flooded localities, pools, ditches and rice fields. Lignin and cellulose contents are about 26.12 and 61.78 % wt, respectively, (Joedodibroto et al., 1983). Coconut coir fiber (*Cocos nucifera*) has high content of lignin that made the fiber tougher and stiffer compared to other fiber. This fiber has lignin, cellulose and hemicellulose content about 59.0, 32.65 and 7.95 % wt, respectively (Jústiz-Smith et al., 2008). The individual fiber cells are narrow and hollow, with thick walls made of cellulose. The coir fiber is relatively waterproof and is the only natural fiber resistant to damage by salt water (Harish et al., 2009).

Lignin is a highly crosslinked molecular complex with amorphous structure and acts as waxes and encrusting substances on fiber surfaces that form a thick outer layer to protect the cellulose inside. The presence of encrusting substances causes the fibers to have an irregular appearance. The removal of surface waxes and encrusting substances makes the fiber surface rough and improves the adhesion of fibers and polymer matrix (Mohanty et al., 2000; Saha et al., 1990). Since major process for cell immobilization on agricultural support materials is adsorption, thus a removal of lignin from support materials is one of the pretreatment methods to improve the adhesion of microbial on surface of support materials.

Burkholderia cepacia PCL3 is the effective carbofuran degrader isolated from carbofuran phytoremediated rhizosphere soil in the study of Plangklang (2004). It could effectively degrade carbofuran in Basal Salt Medium (BSM) and soil with the short $t_{1/2}$ of 3 and 12 days, respectively (Plangklang and Reungsang, 2008). It has been immobilized on corncob and sugarcane bagasse and use as the inocula for bioremediation of carbofuran in BSM and soil in which the carbofuran degradation ability of PCL3 was not worsen or improved by immobilized on these support materials (Plangklang and Reungsang, 2009). The immobilized cells on these support materials could be reused 2 times in BSM. However, they could not be reused in soil due to their structures were broken after the first time used. Therefore, the research on finding the stronger natural support materials and the natural support materials which

can enhance carbofuran degradation ability of PCL3 in both liquid and soil phase is of our interest.

The objective of this study was to search for a suitable local agricultural residues to immobilize carbofuran degrader, *B. cepacia* PCL3, for remediating carbofuran in synthetic wastewater and soil. Carbofuran degradation abilities, stabilities and reusabilities of the immobilized PCL3 cells by various local agricultural materials including coir, banana stem, bulrush and water hyacinth stem in both delignified and undelignified form were investigated. Then, effects of the carbofuran concentrations on the immobilized PCL3 cells in the suitable support material was investigated in both synthetic wastewater and soil in order to justify its effectiveness.

1.2 Objectives

The main objective of this study was to search for a suitable agricultural residues as support materials for immobilizing *B. cepacia* PCL3. In order to achieve the major goal, the main objective was divided into two sub-objectives as follows:

1.2.1 To investigate the ability, stability, and reusability of the immobilized PCL3 on delignified and un-deilignified agricultural residues to degrade carbofuran in synthetic wastewater and investigate the ability and reusability of the immobilized PCL3 on delignified and un-deilignified agricultural residues to degrade carbofuran in soil.

1.2.2 To investigate the effect of carbofuran concentration on degradation ability, growth and survival of the immobilized PCL3 on the suitable support material.

1.3 Hypotheses

Agricultural residues could be used as an effective support material for immobilization of *B. cepacia* PCL3 to remediate carbofuran in synthetic wastewater and soil.

1.4 Scopes of the Study

Four different agricultural support materials including coir, banana stem, bulrush and water hyacinth stem in delignified and un-deilignified forms were used to immobilize *B. cepacia* PCL3. The best performance support material base on its high stability, high carbofuran removal rate and high frequently use was applied in further experiment to investigate the effect of various carbofuran concentrations on the immobilized PCL3 in both synthetic wastewater and soil.

1.5 Expected results

Efficient Agricultural support material for immobilization of *B. cepacia* PCL3 to remediate carbofuran in contaminated soil and synthetic wastewater would be obtained.

CHAPTER II

LITERATURE REVIEW

2.1 Carbofuran

Carbofuran (2,3-dihydro-2,2 dimethylbenzofuran-7-yl methylcarbamate) (Fig. 2.1) is a broad-spectrum insecticide widely used in agriculture to control insects and nematodes on contact or after ingestion (EPA, 2006). It is used against soil dwelling and foliar feeding insects of field, fruit, vegetable and forest crops (Extoxnet, 1996). Products containing the active ingredient carbofuran include Furadan, Curaterr, Yaltox, Bay 70143, Carbodan and ENT 27164 (Trotter et al., 1991).

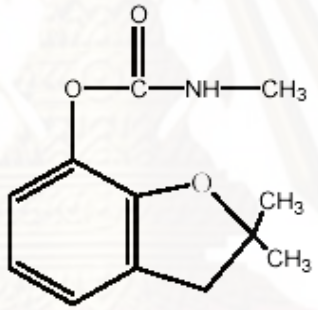

Figure 2.1 Structure of carbofuran (Evert, 2002)

2.1.1 Properties of carbofuran

Carbofuran is an odorless, crystalline solid varying from colorless to grey depending on the purity of the compound. It is soluble in water (320 mg l^{-1} at 25 ^{o}C) (DeVries and Evans, 1999) and has a low adsorption coefficient (K_{oc}= 26, Table 2.1) leading to a high potential for groundwater contamination (Howard, 1991). The physicochemical properties of carbofuran were shown in Table 2.1.

2.1.2 Use of carbofuran

In 2008, Thailand imported carbofuran in form of Furadan3G up to approximately 5,000 tons for agriculture purpose (FMC Cooperation, 2009). Carbofuran was widely applied in plants and crops growing such as rice, corn, sorghum, potato, tobacco, banana, cotton, vegetables etc. (Ngampongsai, 1990). In

rice fields, Furadan granules 3% (3G) is occasionally applied into young plants after 10 days of seeding at the rates of 1.3 to 1.6 kg ha^{-1}. In crop fields, the granular formulations of carbofuran are applied to the soil at the time of seeding. Furadan 5% (5G) can also be applied to potato, onions, turnip and carrot at the rate of 2-5 kg ha^{-1} (IDPID, 1993). Liquid carbofuran is applied by ground or aerial equipments. It was registered for use on the same crops as granular formulations (IDPID, 1993).

Table 2.1 Physicochemical properties of carbofuran (IDPID, 1993).

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

2.1.3 Sources and environmental fate of carbofuran

The widespread use of carbofuran and other pesticides in order to improve an agricultural productivity provided many possible routes of the pesticide to enter the environment. The pesticides contamination in the environment can be resulted not only from direct use of pesticide in agriculture but also from the runoff of pesticides from croplands and rinsate from cleaning pesticides containers and application equipments (Ferrell, 2003). Since carbofuran is applied directly to the soil, it may be washed off from the soil into nearby bodies of surface water or may percolate through the soil to lower soil layers and groundwater (Extoxnet, 1996). These resulted in adverse effects to human and animals exposed to those contaminated areas (Ferrell, 2003). Carbofuran is moderately persistent in soil. Its half-life in soil is approximately 30 to 120 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., 1988). 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 (EPA, 2006). Carbofuran was detected (1 to 5 μg l^{-1}) in water table aquifers beneath sandy soils in New York and Wisconsin (Howard, 1991). Carbofuran has low vapor pressure and low Henry's Law constant (Table 2.1) resulting in a low tendency to volatilize from water or moist soils (Deuel et al., 1979). Shibamoto et al. (1993) reported that 0.3 to 0.66 μg m^{-3} of carbofuran was detected after a 44-hours sampling period following an application of 44% active ingredient carbofuran.

Carbofuran and its metabolites have not been observed to accumulate significantly in any biota (Evert, 2002). In the bluegill sunfish, carbofuran and its metabolites all become conjugated and excreted in the urine and bile (Eisler, 1985). Caro et al., (1976) reported that carbofuran was absorbed by roots and transported via plant fluids to other areas such as leaves. Approximately 14% of the applied carbofuran was taken up by the crop. Carbofuran does not volatilize from water nor does it adsorb to sediment or suspended particles (EPA, 2006). The environmental fate of carbofuran was shown in Fig. 2.2.

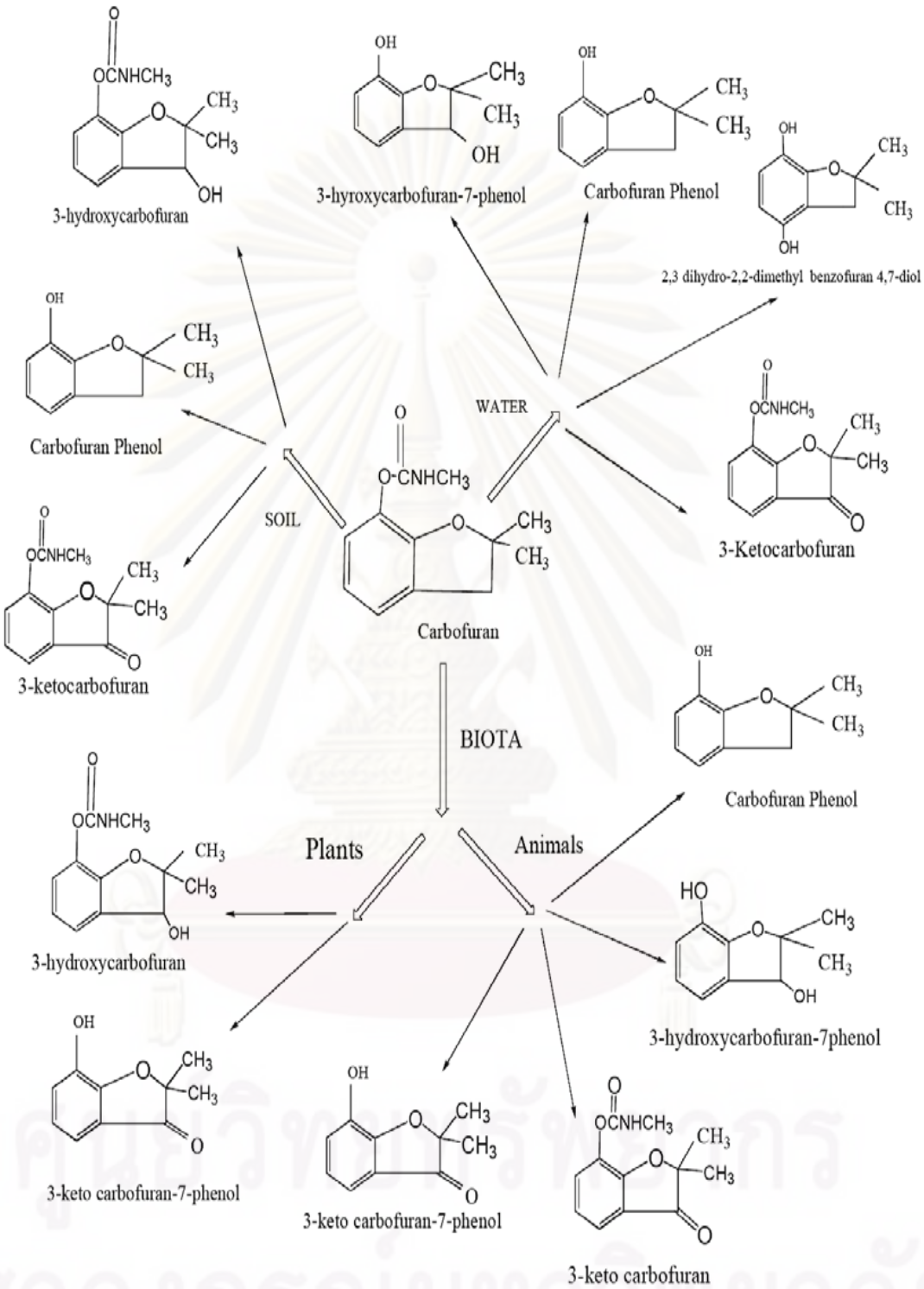

Figure 2.2 Environmental fate of carbofuran (Evert, 2002)

2.1.4 Health Effects

Carbofuran has high toxicity through inhalation and ingestion and its moderate toxicity by dermal absorption (Baron, 1991). It can cause sub-lethal or chronic effects to aquatic organisms through cholinesterase inhibition, neurotoxicity and reproductive effects (De Melo Plese et al., 2005; EPA, 2006). Carbofuran has high mammalian toxicity through cholinesterase inhibition (LD_{50} = 2 mg kg^{-1}) (EPA, 2006).

Rats given very high doses of carbofuran (5 mg kg^{-1} day) for two years showed decreases in weight. Similar tests with mice gave the same results (Baron, 1991). Carbofuran is highly toxic to birds, fish and invertebrates. One granule was sufficient to kill a small bird ingested carbofuran granules (EPA, 2006). Smith (1992) reported that red-shouldered hawks were poisoned after eating prey from carbofuran-treated fields. Carbofuran causes highly toxic to fish with the LD_{50} of 0.38 mg l^{-1} in rainbow trout and 0.24 mg l^{-1} in bluegill sunfish (Kidd and James, 1991). The ecological toxicity of carbofuran was shown in Table 2.2.

Table 2.2 Ecological toxicity of carbofuran (EPA, 2006)

Species	Ecological toxicity	Data
Mallard Duck	LD_{50}	0.40 mg/kg
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

The used of carbofuran in agriculture is prohibited in some countries such as Canada due to its ecotoxocity (De Melo Plese et al., 2005; Anonymous, 1996). 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 in the USA became effective on September 1, 1994. The ban was

established to protect birds and was not related to human health concerns (Extoxnet, 1996). There is no ban on liquid formulations of carbofuran. The formulations of carbofuran are in toxicity class I (highly toxic) or class II (moderately toxic). According to the Safe Drinking Water Act set by EPA, the Maximum Contaminant Level Goal (MCLG) and the Maximum Contaminant Level (MCL) for carbofuran were set at 40 parts per billion (ppb). The EPA believes that this level of protection would not cause any of the potential health problems (EPA, 2006).

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, 1982; Ramanand et al., 1991). The other processes involving in carbofuran degradation are oxidation, volatilization and photolysis which result in different degradation products (Deuel et al., 1979). The main carbofuran metabolites found from the degradation processes of carbofuran are 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 Fig. 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 succeed the 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, 1988; Feng et al., 1997; Karns et al., 1986). Microorganisms capable of degrading carbofuran have been isolated from contaminated natural matrices and characterized for their carbofuran

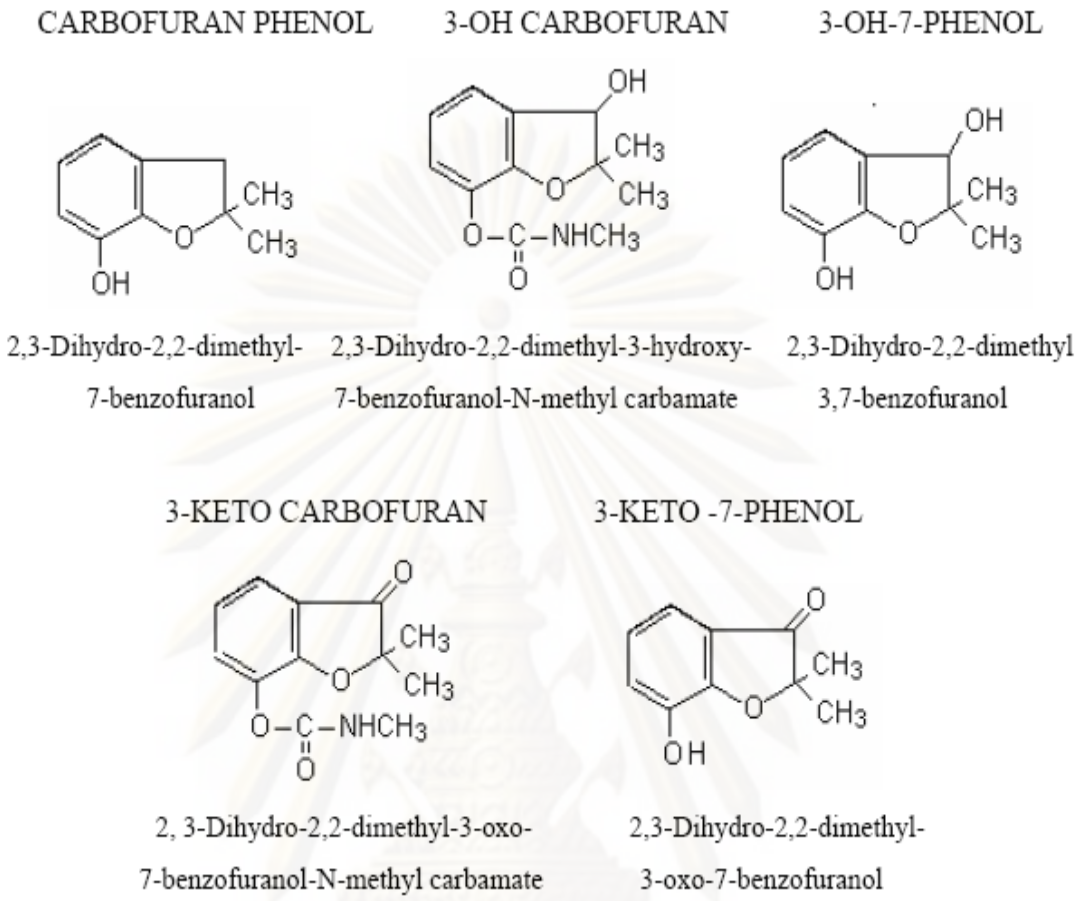

Figure 2.3 Molecular structures of carbofuran metabolites (Mora et al., 1996)

degradation abilities (Chaudhry et al., 2002; Karpouzas et al., 2000). *Pseudomonas* sp. (Felsot et al., 1981) and *Pseudomonas* sp. 50432 (Chaudhry et al., 2002) could degrade carbofuran to 4- hydroxycarbofuran 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). 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).

2.1.6.3 Chemical degradation of carbofuran

A primary mechanism of carbofuran degradation in soil and water under neutral to basic conditions is chemical hydrolysis resulting in metabolites named carbofuran phenol (Getzin, 1973; Sieber et al., 1978; Yu et al., 1974), hydroxy-7-phenolcarbofuran (Chiron et al., 1996), carbofuran phenol and *N*-methylcarbamic acid via the hydroxylation of the benzofuranyl moiety (Yu et al., 1974). The degradation of carbofuran in acidic soil is slower than in neutral and alkaline soil (Getzin, 1973; Siddaramappa and Sieber, 1979). The study by Mora (1996) 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 TiO_2 and ZnO (Mahalakshmi et al., 2007). In addition, the combination of chemical oxidation and photocatalytic process had been reported to improve the efficiency of carbofuran degradation (Wang et al., 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; Deuel et al., 1979). Volatilization rates of carbofuran were more rapid in flooded soil than in non-flooded soil because of co-evaporation with the water on the surface of soil (Lalah et al., 1996). Sunlight and high 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) (Saha 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 (Vidali, 2001). In general, bioremediation uses indigenous microorganisms in the contaminated area as the degraders. In some instances, the indigenous microbes at a contaminated sites may not display the appropriate metabolic potential for degradation and complete mineralization of the target contaminants due to the presence of very small numbers of microorganisms possessing 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).

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 pollutant repeated 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 (Tago et al., 2006) from contaminated natural matrices to be 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 2.3. 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-fold increase of atrazine mineralization capacity. Peter et al. (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 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 (Dam 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, 2007). 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

Table 2.3 Examples of bioaugmentation researches

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

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 fluorine in slurry soil by *A. cylindrospora*, fluorine degrading bacteria, inoculation. The bioaugmentation of the Long Beach soil showed the greatest degradation in the light and heavy total petroleum hydrocarbons compared to the attenuation and biostimulation techniques.

2.3 Immobilization

Previous researches, indicated a successful bioaugmentation of pesticides by the isolated degraders had been reported (Piutti et al., 2003; Rousseaux et al., 2003; Franzmann et al., 2000; Struthers et al., 1998; Rubertoa et al., 2003), in which most of pesticide degraders were used in free cell form. 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 may act as protective agents against the effects of pH, temperature, solvent, heavy metals or even substrate and product inhibition hence enhancement the cell survival (Bekatorou et al., 2004; Braud et al., 2007).

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

2.3.1 Attachment or adsorption on carrier solid

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

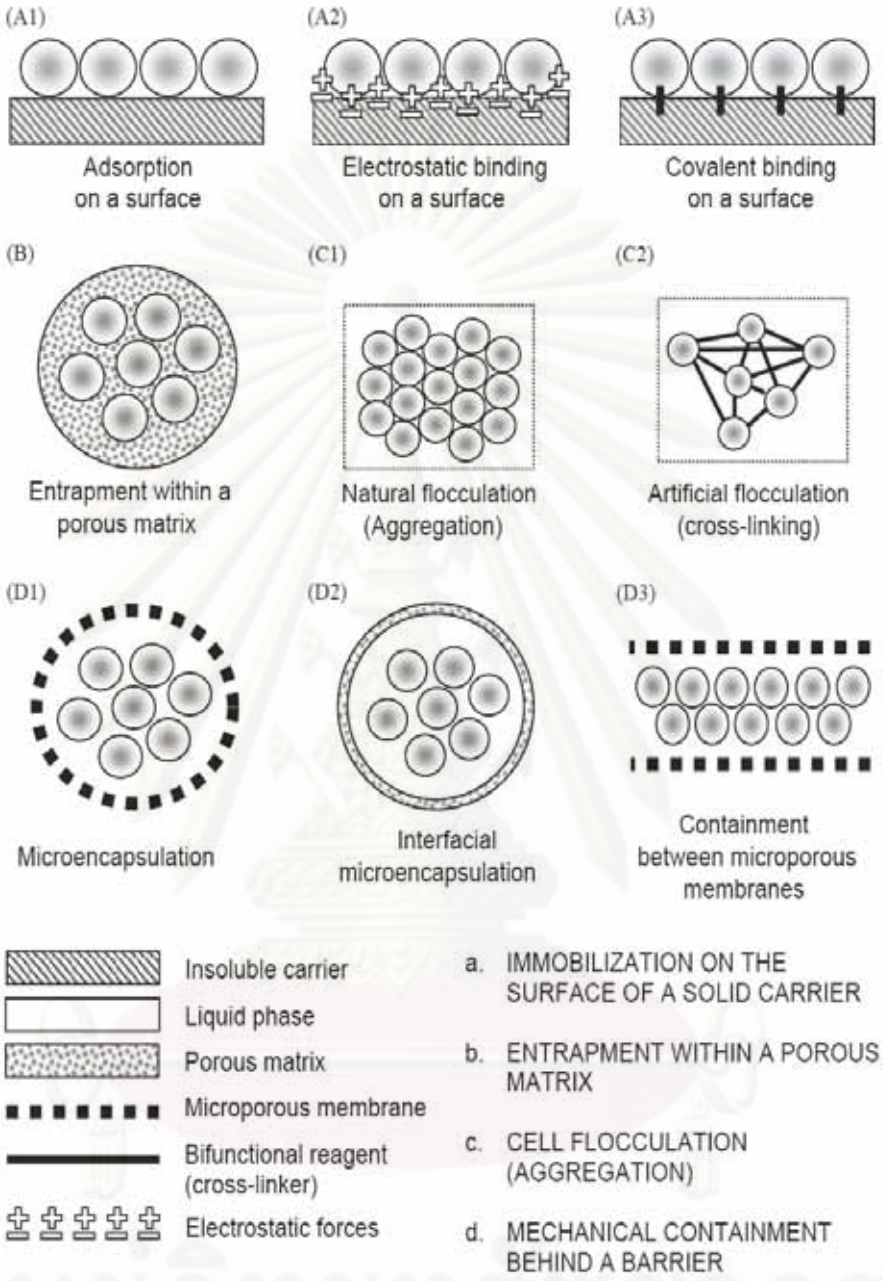

Figure 2.4 Basic methods of cell immobilization (Bekatorou et al., 2004)

2.3.2 Entrapment within a porous matrix

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

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

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

2.3.4 Cell containment behind barriers

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

The support materials used for immobilization could be either synthetic polymers (Table 2.4) or natural materials like agricultural residues (Table2.5). Natural polymeric gels such as agar, carrageenan and calcium alginate have been used as support materials for cell immobilization (Katzbauer et al., 1995). However, use of these materials is limited by their mechanical strength and the lack of open spaces to accommodate cell growth and cell release into the growth medium (Barbotin and Nava Saucedo, 1998; Kumar and Das, 2001). Calcium alginate gels were reported to be unstable when it contacts with complex anions such as phosphate and citrate which are usually used in media (Birnbaum et al., 1981). 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, 1990). 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 cells 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

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

Compounds degraded or reactions	Microorganisms	Carriers	References
Acrylamide	*Pseudomonas* sp *Xanthomonas* sp	alginate	[Nawaz et al., 1992]
3-Chloroaniline	*Pseudomonas acidovo-rans* CA28	alginate	[Ferschl et al., 1991]
3-Chlorobenzoate dehalogenation	*Pseudomonas* sp B13	alginate	[Sahasrabudhe et al., 1988]
4-Chloro-2-nitrophenol	Mixed culture	alginate	[Beunink and Rehm, 1990]
2-Chloroethanol	*Pseudomonas putida* US2	granulated Lecaton-particles	[Overmeyer and Rehm, 1995]
Chlorophenols	Mixed culture	alginate	[Lee et al., 1994]
Chlorophenol	*Rhodococcus* spp	polyurethane	[Valo et al., 1990]
Chlorophenols	Activated sludge	celite R-633 micro-carriers	[Shieh et al., 1990]
Chlorinated phenols	Several strains	glass, cellulose, chitin	[Portier and Fujisaki, 1986]
4-Chlorophenol	*Alcaligenes* sp A 7-2	alginate and granulated Lecaton particles	[Westmeier and Rehm, 1985; Westmeier and Rehm, 1987]
4-Chiorophenol	*Alcaligenes* sp A 7-2	granular clay	[Balfanz and Rehm, 1991]
p-Cresol	*Pseudomonas* sp	alginate	[O'Reilly et al., 1988]
p-Cresol	*Pesudomonas* sp	alginate polyurethane	[O'Reilly and Crawford, 1989]
Cyanuric acid	*Pseudomonas* sp NRRL B-12228	granular clay	[Ernst and Rehm, 1995]
DDT	Mixed culture	alginate	[Beunink and Rehm, 1988]
Dechlorination of spent sulphite bleach effluents	*Streptomycetes* spp	polyurethane	[Zhon et al., 1993]
Dichloroacetic acid	*Xanthobacter auto-trophicus*	alginate	[Heinze and Rehm, 1993]
Glyphosate	Mixed culture	diatomaceous earth pellets	[Hallas et al., 1992]
Hydrocarbons	*Candida parapsilosis*	granular clay	[Omar et al., 1990]
Inorganic cyanides	*Pseudomonas putida*	agar alginate carrageenan	[Chapatwala et al., 1993]
p-Nitrophenol	Mixed culture (*Pseudomonas* spp)	diatomaceous earth biocarrie	[Heitkamp et al., 1990]
PAHs	Mixed culture	granular clay slag of lava	[Wiesel et al., 1993]
Pentachlorophenol	*Arthrobacter* sp ATCC 33790	alginate activated carbon	[Lin and Wang, 1991]
Pentachlorophenol	*Phanerochaete chrysosporium*	alginate	[Siahpush et al., 1991]
Pentachlorophenol	*Arthrobacter* sp ATCC 33790	alginate and activated carbon	[Hu et al., 1994]
Pentachlorophenol	*Flavobacterium* sp	polyurethane	[Stormo and Crawford, 1994]
Pentachlorophenol	*Flavobacterium* spp	polyurethane	[Wu et al., 1993]
Pentachlorophenol	Mixed culture	anaerobic granules	[Hackel et al., 1975]
Phenol	*Candida*	alginate	[Bettmann and Rehm,1984]
Sodium cyanide	*Pseudomonas putida*	polyacrylamide	[Babu et al., 1992]
Pyridine	*Pseudomonas* C12B	polyacrylamide alginate	[Thomas and White, 1990; Thomas and White, 1991]

Table 2.5 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	[Huidong et al., 2008]
Itaconic acid production	*Aspergillus terreus*	Papaya wood	[Iqbal et al., 2004]
Xylitol production	*Candida guilliermondii*	Sugarcane bagasse	[Santo 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 et al., 2007]
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	[Malloucho et al., 2002]

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). 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.

Several advantages of synthetic polymer such as PVA, PUF and other support materials such as diatomaceous earth, activated carbon (Fennell et al., 1992; Fennell et al., 1993), glass (Phelps et al., 1990; Arvin, 1991) and ceramic packing material (Strandberg et al., 1989) including high mechanical strength, resistant to organic solvents and microbial attack, easy handling and regenerability have been established (Patil et al., 2006). 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. Previous research indicated successful use of various agricultural residue to immobilize microbial all in bioremediation work. Corncob and sugarcane bagasse were used to immobilize *B. cepacia* PCL3 for bioremediation of carbofuran in synthetic medium and soil in comparison to free cells. The carbofuran degradation ability between free and immobilized cells were significantly different. However, immobilized cells could survive longer than free cells and it could be reused twice without the loss in carbofuran degradation ability (Plangklang and Reungsang, 2009). In addition, PCL3 immobilized on corncob was used as inoculum to remove carbofuran in contaminated soil at the carbofuran concentration of 20 mg kg^{-1} soil by using a bioslurry phase seguencing batch reactor (SBR), the highest percentage of carbofuran removal was observed in bioaugmentation with PCL3 immobilized on corncob (96.97%) (Plangklang and Reungsang, 2010). The microbial consortium isolated from agricultural soil was immobilized on loofa sponge and the coconut fiber and used to degrade 0.2 µM carbendazim and 2,4-dichlorophenoxyacetic acid (2,4-D) in synthetic medium. After 4 days of incubation, carbendazim was degraded 95% and 80% by immobilized consortium, respectively, which were significantly higher than that of the free-living consortium, 12%. Consortia immobilized on the loofa sponge

and the coconut fiber could completely degrade 2,4-D within 1 day, while it took 2 days for complete degradation by the free-living (Pattanasupong et al., 2004).

CHAPTER III

RESEARCH METHODOLOGY

3.1 Chemicals and reagents

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

3.2 Synthetic wastewater

Basal Salt Medium (BSM) (Mo et al., 1997) was used as a synthetic wastewater in this study. It consists of (g l^{-1}); 5.57 of Na_2HPO_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 synthetic wastewater was adjusted to be 7 before autoclaved at 121 °C for 15 min before the usage.

3.3 Soil

Sandy loam soil sample, 0-15 cm depth, was collected from the rice fields of Ban Nonmuang, A. Muang, Khon Kaen Province. Carbofuran residue, organic carbon and nitrogen contents in soil and soil pH were determined. Soil was passed through a 2 mm sieve and kept in the plastic bag at 4 °C prior the usage

3.4 Microorganism preparation

Carbofuran degrader, identified by 16s rRNA as *Burkholderia cepacia* PCL3 (GenBank accession number of EF990634) was used in this experiment. This microorganism is capable of using carbofuran as a sole C-source. It was grown in 100 ml Nutrient Broth (NB) containing 5 mg l^{-1} of carbofuran at 30 °C and 150 rpm for 24

h and used as seed inoculum for immobilization. For the kinetic characterization of the isolate PCL3 in free cell form, the growing cells were harvested by centrifugation in HDPE tubes at 5,000 rpm and 4 °C for 10 min. The supernatant was discarded, and the pellets of the isolate were washed with 100 ml of 0.85% NaCl, centrifuged again and then resuspended in steriled 0.85% NaCl with a total volume of 100 ml prior the usage as seed inoculum.

3.5 Immobilization of *B. cepacia* PCL3

3.5.1 Support materials preparation

Four local agricultural wastes i.e., coir, banana stem, bulrush and water hyacinth stem were collected from Khon Kaen Province, Thailand. These support materials were evaluated if they were efficient natural support materials to immobilize *B. cepacia* PCL3. These materials were chosen because of their high matrix porosity and a pore size that could enhance the cell adsorption capability. Both delignified and un-delignified agricultural residues were evaluated for their immobilization capacity. Each material was cut into 1.0x1.0x1.0 cm using knife. In order to delignify the materials, 300 g of each natural material was boiled in 3 l of 0.25 N 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 materials were washed three times with 3 l of distilled water, soaked in distilled water overnight and then autoclaved at 121 ^{o}C for 15 min before the usage. Sorption of carbofuran to non-delignified and delignified support materials were determined.

3.5.2 Cell immobilization

Adsorption is 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 conducted by adding 90 g wet weight of each support materials into 200 ml of NB and autoclaved at 121^{o}C for 15 min. After NB is cooled down at room temperature, carbofuran solution in methanol was added to the medium at the final concentration of 5 mg l^{-1} and 10% inoculum of the isolate PCL3 (10^{6} cfu ml^{-1}) was inoculated into the medium. Then flask was incubated at 150 rpm, 30 ^{o}C, for 24 hours and harvested by filtration through Buchner filter funnel and washed with 0.85% NaCl by aseptic

technique. This process was repeated two times. The internal cell density on each support material was determined and the immobilized cells were kept at 4 ^{o}C till being used in the experiments. The structure of support materials, the attaching pattern and morphology of PCL3 on each support materials were determined using Scanning electron microscope (SEM). Stability of the immobilized cells was investigated in both synthetic wastewater and soil.

3.6 Selection of support materials for the immobilization of *B. cepacia* PCL3

3.6.1 Selection of support materials for the immobilization of *B. cepacia* PCL3 to remediate carbofuran in synthetic wastewater

Degradation of carbofuran by the immobilized cells of *B. cepacia* PCL3 was conducted in a 250 ml shake flask in bath experiment. A 100 ml of synthetic wastewater containing 5 mg l^{-1} of carbofuran was added into the flask before inoculating with the immobilized PCL3 at a final concentration of 10^6 cfu ml^{-1}. Flask was incubated at room temperature and shaken at 150 rpm. Liquid part was sampled at days 0, 3, 6, 10 and 15 to extract carbofuran and its metabolites i.e., 3-ketocarbofuran and carbofuran phenol by liquid-liquid partitioning method and analyzed for their concentrations by HPLC. Number of PCL3 in support material and in synthetic wastewater was determined by drop plate technique.

Degradation of carbofuran by *B. cepacia* PCL3 in free cell form was conducted in a 500 ml shake flask in batch experiment as a control. A 200 ml of synthetic wastewater containing 5 mg l^{-1} of carbofuran as a sole carbon source was added into the flask before inoculating with 10% inoculum of PCL3 (final cell concentration of 10^6 cfu ml^{-1}). Flasks were incubated and analyzed for carbofuran and its metabolites concentrations and number of PCL3 as previously described.

After 15 days of incubation, the immobilized cells on each support material were harvested and re-inoculated into a fresh synthetic wastewater containing 5 mg l^{-1} of carbofuran. Carbofuran degradation in synthetic wastewater, cell leakage and cell survival were determined at days 0, 3, 6, 10 and 15. This step was repeated three times to investigate a reusability of the immobilized cells. The structure of support materials was examined at day 0 and 15 of each reuse by SEM.

The suitable support material justified by the shortest half-life of carbofuran, a high stability, and a more often reuse was used to immobilize PCL3 in the next experimental step.

3.6.2 Selection of support materials for the immobilization of *B. cepacia* PCL3 to remediate carbofuran in soil.

Four sets of bioaugmentation experiment (Table 3.1) were conducted in a soil microcosm using 225 cm^3 glass jar capped with a plastic lid. Carbofuran solution at initial concentrations of 5 mg kg^{-1} dry soil was spiked into 20 g dry weight of soil in the glass jar and well mixed by hand stirring. Free cells or the immobilized cells of *B. cepacia* PCL3 on delignified and undeinlignified support materials was added into the soil at the final cell concentration of 10^6 cfu g^{-1} dry soil. The initial moisture content of 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, 20 and 30 and further extracted by an Accelerated Solvent Extractor, ASE 100 (Dionex, USA). Carbofuran and its metabolites concentrations were determined by HPLC. At each sampling date, the numbers of carbofuran degraders in soil and in support materials were counted by drop plate technique. The half-lives of carbofuran in soils were calculated by using a modified first-order kinetic model. In order to proof the capability of *B. cepacia* PCL3 to degrade carbofuran in soil in comparison to

Table 3.1 Experimental setup for the screening of the suitable support material to immobilize *B. cepacia* PCL3 for remediating carbofuran in soil.

Treatment	Experimental setup
A	Soil
B	Soil + free cells of PCL3
C	Soil + immobilized PCL3
D	Soil + autoclaved support material
E	Autoclaved soil + free cells of PCL3
F	Autoclaved soil + immobilized PCL3
G	Autoclaved soil + autoclaved support material
H	Autoclaved soil

indigenous microorganisms and the abiotic process, four sets of control were included, i.e., soil without inoculation (treatment A), autoclaved soil without inoculation (treatment H), soil mixed with autoclaved support material (treatment D), and autoclaved soil mixed with autoclaved support material (treatment G), respectively. The treatments were shown in Table 3.1.

After 30 days of incubation, the immobilized cells were harvested and re-inoculated into a fresh soil containing a final concentration of carbofuran 5 mg kg^{-1} soil. Soil samples were taken and analyzed for carbofuran concentration and numbers of carbofuran degraders in soil at days 0, 5, 10, 15, 20 and 30 to investigate a reusability of each immobilized cells.

3.7 Effect of carbofuran concentrations on carbofuran degradation ability of the immobilized PCL3 in the suitable support material.

Carbofuran degradation ability, growth and survival of the immobilized PCL3 on the suitable support material (from 3.6.1 and 3.6.2) in comparison to free cells was studied in synthetic wastewater to determine the maximum threshold of carbofuran concentration that the immobilized PCL3 could survive. One hundred ml of synthetic wastewater supplemented with carbofuran as a sole carbon source at the various concentrations of 0, 10, 50, 120, 200 and 280 mg l^{-1} was added into the 250-ml flask before inoculating with the immobilized PCL3 on the suitable support material at the final cell concentration of 10^6 cfu ml^{-1}. Flask was incubated at room temperature and shaken at 150 rpm. Synthetic wastewater was sampled at the interval time to determine number of PCL3 by drop plate technique and to extract carbofuran and its metabolites i.e., 3-ketocarbofuran and carbofuran phenol by liquid-liquid partitioning method and analyzed for the concentrations by HPLC. Number of PCL3 in support material was determined by drop plate technique.

For free cells experiment, as a control, 200 ml of synthetic wastewater supplemented with carbofuran at the various concentrations of 0, 10, 50, 100, 150, 200 and 250 mg l^{-1} was added into the 500 ml flask before inoculating with approximately 10^6 cfu ml^{-1} (10% inoculum) of *B. cepacia* PCL3. Flask was incubated at room temperature and shaken at 150 rpm. Synthetic wastewater was sampled at the interval time to determine number of PCL3 in synthetic wastewater by drop plate

technique and to extract carbofuran and its metabolites i.e., 3-ketocarbofuran and carbofuran phenol by liquid-liquid partitioning method and analyzed for the concentrations by HPLC.

Maximum concentration of carbofuran in soil that the immobilized PCL3 on the suitable support material could survive and degrade was investigated in this study. Four sets of bioaugmentation experiment (Table 3.1) were conducted in a soil microcosm using 225 cm^3 glass jar capped with a plastic lid. Carbofuran solution at various initial concentrations of 0, 10, 50, 100, 150, 200 and 250 mg kg^{-1} 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 the immobilized cells of *B. cepacia* PCL3 on the suitable support material was added into the soil at the initial cell concentration of 10^6 cfu g^{-1} 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, 20 and 30 and further extracted by an Accelerated Solvent Extractor, ASE 100 (Dionex, USA). Carbofuran and its metabolites concentrations were determined by HPLC. At each sampling date, the numbers of carbofuran degraders in soil was counted by drop plate technique. The half-lives of carbofuran in soils were calculated by using a modified first-order kinetic model. In order to proof the capability of *B. cepacia* PCL3 to degrade carbofuran in soil in comparison to indigenous microorganisms, four sets of control were included, i.e., soil without inoculation (treatment A), autoclaved soil without inoculation (treatment H), soil mixed with autoclaved support material (treatment D). Abiotic degradation control was autoclaved soil mixed with autoclaved support material (treatment G). The maximum threshold concentration of carbofuran was justified by the maximum concentration of carbofuran that the PCL3 was able to survive with a high carbofuran removal efficiency.

3.8 Analytical methods

3.8.1 Extraction of carbofuran and its metabolites from synthetic wastewater

Extraction of carbofuran from culture media using liquid-liquid partitioning method was conducted by adding 2 ml of methanol into 2 ml of culture media and

then sonicated for 10 min, 50/60 voltage cycle, for two times. After sonication, carbofuran and its metabolites were extracted from the media by using dichloromethane in separation funnel. This extraction was done 5 times. The first, second, third, fourth and fifth times, 4, 2, 2, 2 and 2 ml of dichloromethane, respectively, were added into the sonicated media and hand shaken for 1 min. The organic fraction of the samples from each extraction was pooled and evaporated to dryness in the fume hood and then re-dissolved in 2 ml of 60% methanol and passed through a 0.45 μm nylon membrane syringe filter. The filtrate was further analyzed by HPLC

3.8.2 Extraction of carbofuran and its metabolites from soil

Carbofuran and its metabolites were extracted from 14 g soil samples by using an Accelerated Solvent Extractor ASE 100 (Dionex, USA) equipped with 11-ml stainless-steel extraction cell. The samples were extracted under the conditions which are 100 ^{o}C extraction temperature, 5 min static extraction time, 60% methanol as the extraction solvent and two extraction cycles. The ASE parameters were used according to the default settings. Volume of the extract was adjusted to 25 ml and passed through 0.45 μm nylon membrane syringe filter prior to be analyzed by HPLC.

3.8.3 Analysis of carbofuran and its metabolites concentrations by HPLC

Carbofuran and its metabolites concentrations in the extracts were analyzed by Shimadzu 10-A HPLC equipped with 4.6x150 mm-Lunar 0.5 μm C-18 column (Phenomenex, USA), a UV detector operating at 220 nm and a 20 μL injector loop. The HPLC operating parameters were: mobile phase, methanol-water (60:40); flow rate, 1 ml min^{-1} 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 was calculated by fitting to a modified first-order kinetic model; $C = C_0e^{-kt} + Ya$, using Sigmaplot programe ® 9.0, where C is the mean concentration of carbofuran as a function of time in hours (mg l^{-1} or mg kg^{-1} dry soil), C_0 is the initial carbofuran concentration (mg l^{-1} or mg kg^{-1} dry soil), k is the rate constant (day^{-1}), t is time (day) and Ya is an asymptotic estimate of the concentration of carbofuran that degrades very slowly overtime (residual carbofuran)

(mg l^{-1} or mg kg^{-1} dry soil). The mean concentrations used in the regression was weighed with inverse of the variance squared, S^{-2}. This method compensates for the non-constant variance and helps to improve the estimation of the parameters.

3.8.4 Enumeration of PCL3 by drop plate technique

Number of *B. cepacia* PCL3 in culture media and soil were determined by drop plate technique. The serial-diluted aliquots of the samples, 20 µl, was plated onto BSM agar coated with 5 mg l^{-1} of carbofuran and incubated at 30 °C for 5 days. The experiment was repeated three times.

To observe the cell growth and cell survival on the natural support materials, 10 g of wet immobilized cells was sampled from culture media and washed with 50 ml sterile 0.85% NaCl solution three times. The washed immobilized cells was blended to small particles using blender and then added to 100 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 determined by drop plate technique on the BSM agar coated with 5 mg l^{-1} of carbofuran.

3.8.5 Growth and degradation kinetic analysis

Growth of PCL3 in both free and immobilized cell forms in synthetic wastewater added with carbofuran at various concentrations was described by fitting to the first order growth kinetic model as follow (Kotturi et al., 1991):

$$\frac{dX}{dt} = \mu X \qquad (1)$$

Where X is number of carbofuran degraders (cfu ml^{-1}), t = times (days) and μ = specific growth rate (day^{-1}).

Kinetic analysis for bacteria growth was evaluated by fitting the data to the bacterial growth model using Sigmaplot programe ® 9.0. The model proposed by Luong (1987) as presented in Eq. (2), appeared to be used for representing the kinetic of substrate inhibition. (Saravanan et al., 2009).

$$\mu = \frac{\mu_{max} S}{K_s + S} (1 - S/S_m)^n \qquad (2)$$

Where: K_s is the half-saturation constant (mg l^{-1}), S is carbofuran concentration (mg l^{-1}), μ_{max} is the maximum specific growth rate (day^{-1}), S_m is maximum substrate inhibitory concentration at which no growth was observed (mg l^{-1}), and n is the constant which accounts the relationship between μ and S (Gokulakrishnan and Gummadi, 2006).

In the case of degradation kinetic, the first order degradation kinetic model was used as follow

$$- \frac{dS}{dt} = qX \qquad (3)$$

Where X is number of carbofuran degraders (cfu ml^{-1}), t = times (days) and q = specific degradation rate (day^{-1}).

Kinetic analysis for carbofuran degradation was also modeled using the same set of equation (2). However, for applying the model to the data on specific degradation rate the terms, μ and μ_{max}, were replaced with q ans q_{max} representing specific carbofuran degradation and maximum specific carbofuran degradation rate, respectively.

3.8.6 Scanning Electron Microscopy (SEM)

The attaching pattern and morphology of PCL3 on support materials were investigated using SEM. The support materials with and without the PCL3 cells was cut to a size of 5mm x 5mm x3 mm, transferred to 2.5% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer pH 7.2 and soaked for 1 h at 4 ^{o}C in order to fix the cells on solid matrix. The materials were washed two times with the same buffer and one time with distilled water for 15 min each. The samples were dehydrated three times with ethanol of increasing concentration (30, 50, 70, 90, and 100%, vol/vol). Dehydrated cells were dried with a critical point dryer. The dried samples were mounted on SEM stubs and ion sputter-coated with gold at 20 mA and then viewed with a scanning electron microscope (JEOL JSM-5410LV) at 15–20 kV.

3.8.7 Sorption of carbofuran to support materials and soil

Adsorption isotherm was determined by conducting batch equilibrium experiments. Support materials was air-dried overnight and milled into small pieces using blender and passed through 2 mm sieve. Then a total of 0.125 g of powder

support material was put into 100 ml glass tubes and mixed with 25 ml of 0.01 M $CaCl_2$ solution containing carbofuran at carbofuran concentrations of 0.1, 1.0, 5.0, 10.0 and 20.0 mg l^{-1}. Flasks were horizontal 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 the liquid-liquid partitioning method and quantified for carbofuran concentration by HPLC. Adsorption isotherm of carbofuran to soil was determined by conducting a batch equilibrium experiment at carbofuran concentrations of 0.1, 1.0, 5.0, 10.0 and 20.0 mg l^{-1}. All solutions were prepared in 0.01 M $CaCl_2$. A total of 3-g of air-dried soil was put into 100 ml glass tubes and mixed with 9 ml of 0.01 M $CaCl_2$ solution containing carbofuran. Tubes were shaken on a horizontal shaker for 48 hours at 100 rpm. After centri-fugation at 5,000 x g, supernatant was analyzed for carbofuran concentration using HPLC (Teerakul et al., 2004). The data were fitted to the Freundlich isotherm which it is a nonlinear isotherm and was originally developed as an empirical expression that encompasses the heterogeneity of the surface. The Freundlich equation is expressed in Eq. 4

$$q_e = K_f * C_e^{1/n} \quad (4)$$

where q_e is the amount of carbofuran sorbed per unit weight of sorbent (mg kg^{-1}), K_f is Freundlich sorption coefficient (l kg^{-1}), C_e is the equilibrium solution concentration of carbofuran (mg l^{-1}), and 1/n is an empirical constant. The Freundlich equation could be decribed by the linearized form as presented in Eq. 5

$$\ln q_e = \ln K_f + 1/n (\ln C_e) \quad (5)$$

The values of K_f are calculated from the intercept of the linear plots between ln q_e and ln C_e whereas, the values of 1/n are computed from the slope of the linear plots (Zheng et al., 2009)

3.8.8 Stability of immobilized cells attached on support material

Since cells are adsorbed to the surface and porous of the support material which might be resulted in a susceptible to any shear or attrition due to the relative motion of the support material and medium during incubation. Therefor, a stability of the cells attached on the support material was determined. The stability test was conducted according to the method of Wang et al. (2001). Briefly, 10 g of each immobilized cells was rinsed with sterile BSM and then placed to 250-ml flask

containing 100-ml sterile BSM. The flask was shaken at 150 rpm, 30 ^{o}C for 30 min. The number of PCL3 in the support material and washed out from the support material to BSM were counted by drop plate technique. The amount of cells immobilized or washed out were expressed as cfu per g support material. The cell stability (%) was calculated according to Eq. (6).

$$\text{Stability (\%)} = \left[\frac{\text{IMC}}{\text{WC + IMC}} \right] \text{x } 100 \qquad (6)$$

Where, WC is number of cells observed in BSM (cfu g^{-1} support material), and IMC is number of cells observed in support material (cfug^{-1} support material).

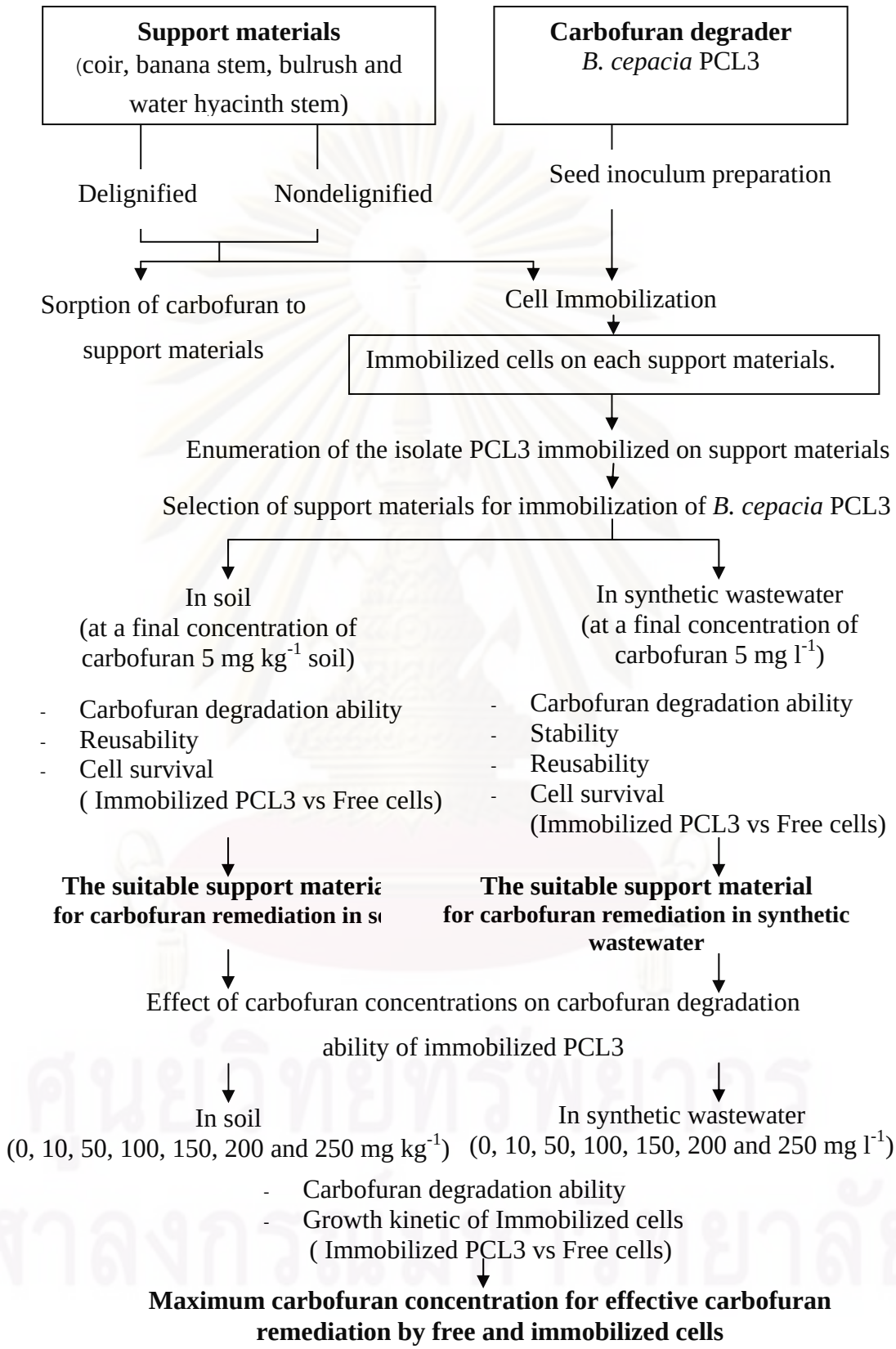

Figure 3.1 Experimental steps of the research

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Characterization of the support materials

Four types of agricultural residues i.e., coir, banana stem, bulrush and water hyacinth stem in both delignified and undelignified forms were used as support materials for the immobilization of *B. cepacia* PCL3 in carbofuran remediation. Undelignified appearance structures of coir, bulrush, banana stem and waterhyacinth stem were shown in Fig. 4.1a to 4.1d. The appearance structures of coir (Fig. 4.1e), bulrush (Fig. 4.1f) and banana stem (Fig. 4.1g) were not changed after delignification. Undelignified coir (Fig. 4.1a) was stiffer as compared to its delignified form (Fig. 4.1e), while the textures of bulrush (Fig. 4.1f) and banana stem (Fig. 4.1g) were softened after delignification. Water hyacinth stem had a significant change in appearance structure after a delignification in which its bundle of cellulose fiber was loosened and its structure was ruined (Fig. 4.1h).

The microstructures of the support materials were determined by SEM and shown in Fig. 4.2. Delignified water hyacinth stem was not observed by SEM and would not be used as a support material for cell immobilization because its structure was broken as described above. From SEM, it could be seen that the support materials had complex layers and porous which were suitable for cell immobilization. Coir (Fig. 4.2a) has more complex layers than bulrush (Fig. 4.2b), banana stem (Fig. 4.2c) and water hyacinth stem, respectively (Fig. 4.2d). The micro structure of delignified coir (Fig. 4.2e) remained the same as appeared in undelignified form (Fig. 4.2a), while the complex layer of bulrush (Fig. 4.2f) and banana stem (Fig. 4.2g) had been removed after delignification. The different in structures of the support materials might result in the different efficiency of immobilized cells on each support material to degrade carbofuran.

The attachment patterns and arrangement of *B. cepacia* PCL3 on the support materials were studied under SEM and shown in Fig. 4.3. It is evident from the

Figure 4.1 Structure of undelignified (a = coir, b = bulrush, c = banana stem, d = water hyacinth stem) and delignified (e = coir, f = bulrush, g = banana stem, h = water hyacinth stem) support material.

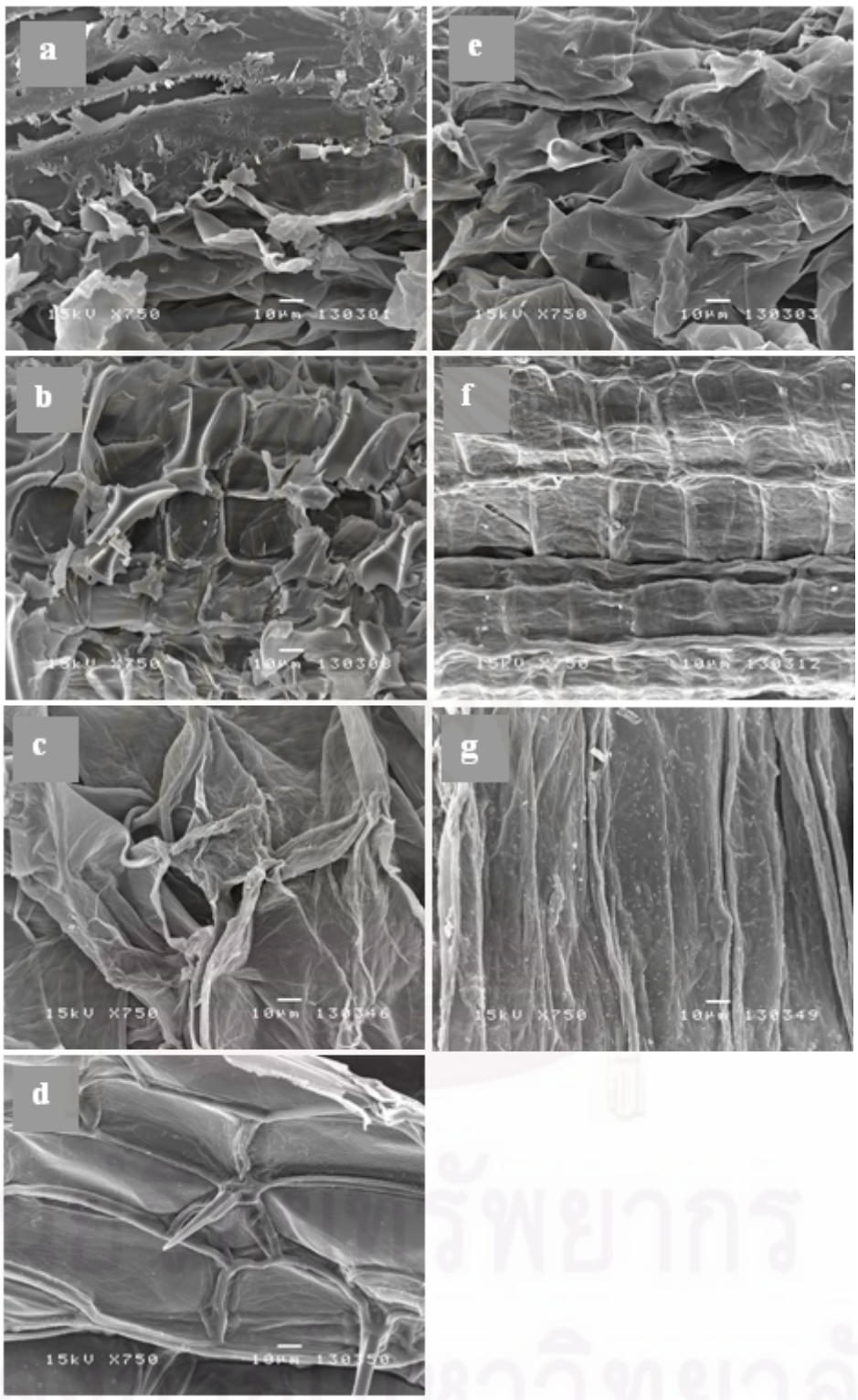

Figure 4.2 Scanning electron microscopic image of undelignified support materials (a = coir, b = bulrush, c = banana stem, d = water hyacinth stem) and delignified (e = coir, f = bulrush, g = banana stem) support material.

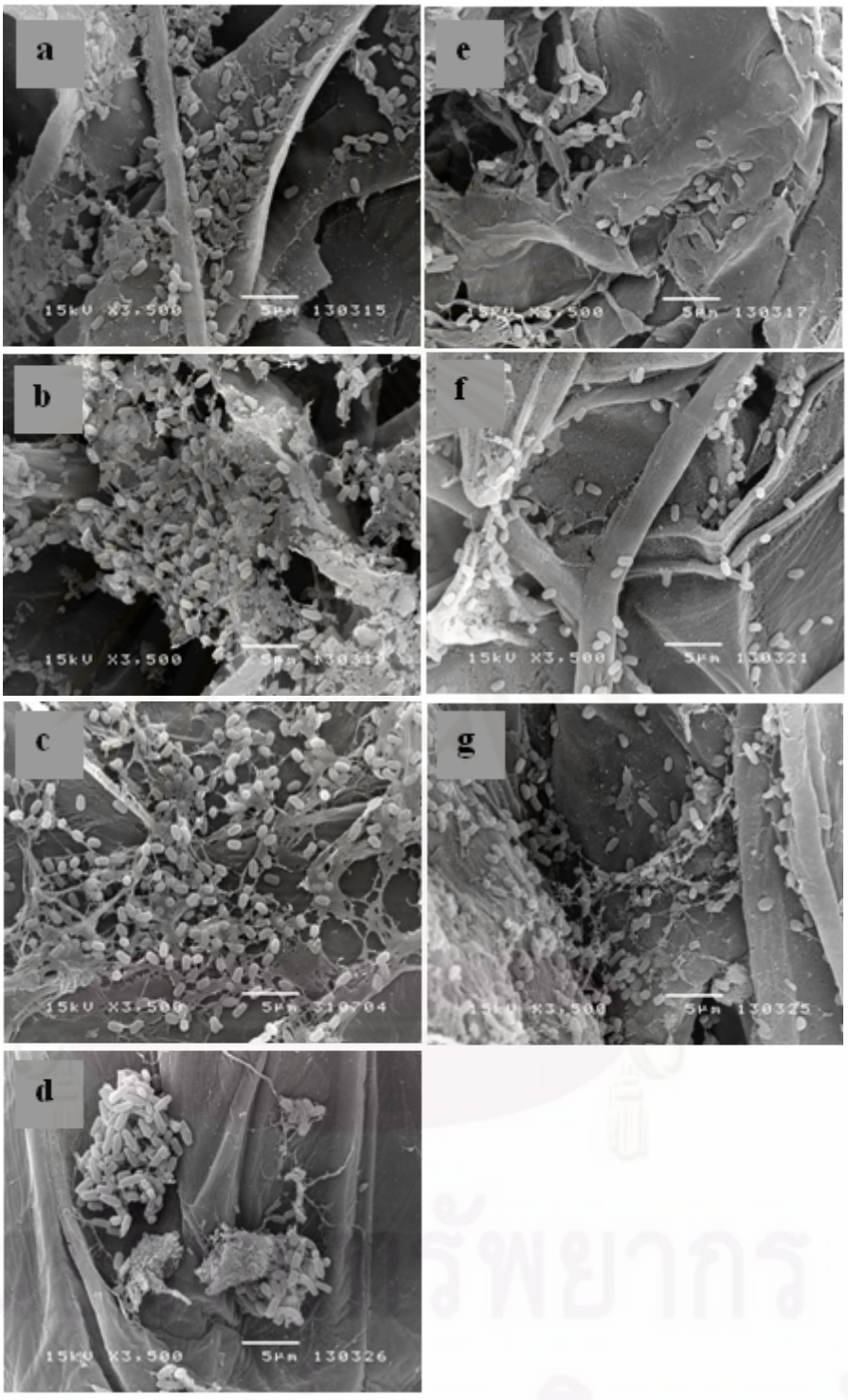

Figure 4.3 Scanning electron microscopic image of *B. cepacia* PCL3 immobilized on undelignified (a = coir, b = bulrush, c = banana stem, d = water hyacinth stem) and delignified (e = coir, f = bulrush, g = banana stem) support materials.

photograph that *B. cepacia* PCL3 attached to the support materials inside their porous and complex layers space (Fig. 4.3) which indicated that *B. cepacia* PCL3 was immobilized on support materials by adsorption mechanism. Cells were rod shape with approximately 1.6 µm in length and 0.7 µm in diameter when they were immobilized on coir (Fig. 4.3a and 4.3e), bulrush (Fig. 4.3b and 4.3f) and banana stem (Fig. 4.3c and 4.3g) in undelignified and delignified forms. When cells were immobilized on water hyacinth stem, the sizes of the cells were approximately 2.2 µm in length and 0.7 µm in diameter (Fig. 4.3d). It was obviously seen that cells were scattered in all support materials except in water hyacinth stem that the cells have clumps on the surface of support materials (Fig. 4.3d). Difference in cell shape and arrangement could be the results of nutrient acquisition and different attachment mechanisms to different surfaces (Young, 2007).

4.2 Sorption kinetic of carbofuran

The sorption isotherm of carbofuran onto support materials was investigated. Fig. 4.4 shows the linear plot of Freundlich isotherm of carbofuran on each support material at 30±2 °C. All sorption data fitted well to the Freundlich isotherm with the regression coefficient ranged between 0.91 and 0.97. The values of K_f were calculated from the intercept of the linear plots and the values of 1/n was computed from the slope of the linear plots and presented in Table 4.1. Results indicated that the sorption isotherms were not linear ($1/n \neq 1$). The 1/n values were less than 1.0 indicates a less effect of concentration change on the adsorptive capacity (Faust and Alg, 1987). The K_f values were used to describe the extent of sorption between carbofuran and the support materials and found to be correlated to the organic matter content of support materials (Table 4.1). The K_f values of carbofuran on undelignified support materials was less than that of delignified support materials which might due to less organic matter content of undelignified support materials than delignified support materials. Undelignified and delignified coir had the higher K_f values (20.88-24.23 l kg^{-1}) than the other support materials (8.13-17.25 l kg^{-1}). Using the support material with the high carbofuran sorption capacity might enhance the carbofuran degradation efficiency of PCL3 in which carbofuran could be absorbed to the support materials facilitating mass transfer between carbofuran and cells inside the support material.

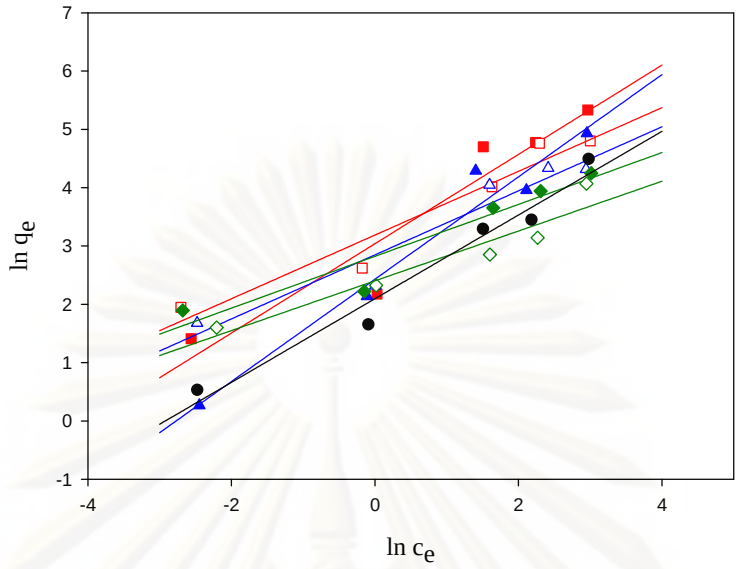

Figure 4.4 Linear plots of Freundlich isotherm of carbofuran on undelignified (■ = coir, ▲ = bulrush, ◆ = banana stem and ● = water hyacinth stem) and delignified (□ = coir, △ = bulrush and ◇ = banana stem) support materials.

Table 4.1 Organic matter content (OM), Freundlich sorption coefficient (K_f) and sorption constant (1/n)

Type of support materials	OM (%)	K_f (l kg^{-1})	1/n	r^{2*}
Undelignified coir	89.11	20.88	0.76	0.91
Delignified coir	97.41	24.23	0.55	0.94
Undelignified bulrush	80.99	11.36	0.88	0.96
Delignified bulrush	85.76	17.25	0.54	0.92
Undelignified banana stem	78.52	11.05	0.44	0.92
Delignified banana stem	79.73	16.84	0.43	0.91
Undelignified water hyacinth tem	73.63	8.13	0.72	0.97

***** Coefficients of determination for linear plots of Freundlich isotherm regression

4.3 Enumeration of the isolate PCL3 immobilized on support materials

The numbers of the PCL3 cells on support materials were in the range of $1.46x10^8$-$6.9x10^8$ cfu g^{-1}dry support material (Fig. 4.5). The delignified coir could adsorb approximately 2 times of the cells than the undelignified coir. This might due to the fact that the delignification process could remove the microcrystalline structure of lignin on the surface of coir resulting in a better sorption of the cells on to the surface of delignified coir. In the case of bulrush, the number of *B. cepacia* PCL3 on its undelignified form was greater than its delignified form which might due to the

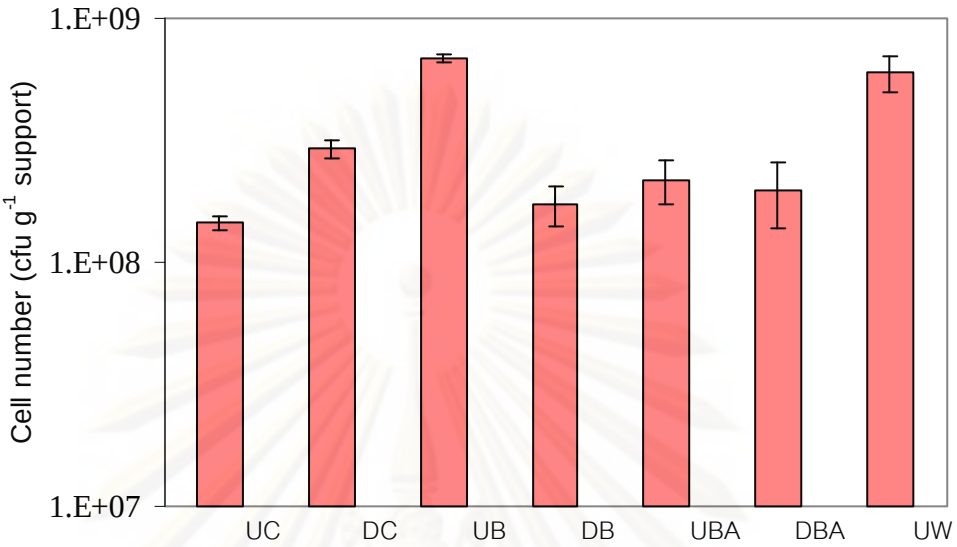

Figure 4.5 Cell number of PCL3 immobilized on each support materials (UC = undelignified coir, DC = delignified coir, UB = undelignified bulrush, DB = delignified bulrush, UBA = undelignified banana stem, DBA = delignified banana stem, UW = undelignified water hyacinth stem)

loss of complex structure and porous from the surface of bulrush after delignification. The number of PCL3 on undelignified and delignified banana stem was not significantly different which might due to the non-markedly change in physical appearance, texture and microstructure of banana stem after delignification. The number of PCL3 on undelignified waterhyacinth stem was relatively high at 6.00×10^{8} cfu ml^{-1} which could be resulting from the clumping of the cells on the surface. Using the support materials which can absorb the greater number of microorganisms for bioremediation of the contaminant would provide the higher cell density environment to increase overall degradation rate (Kampf, 2002). However, the other factors such as the stability of the immobilized cells, the contaminant degradation ability as well as the reusability of the immobilized cells should also be taken into consideration in order to select the suitable support material for cell immobilization.

4.4 Selection of support materials for the immobilization of *B. cepacia* PCL3 to remediate carbofuran in synthetic wastewater

4.4.1 Stability

Stability of the immobilized cells attached on each support material was shown in Table 4.2. Results indicated that the immobilized cells on all support materials, except for delignified bulrush and undelignified water hyacinth stem, had a percentage of stability greater than 75 (Table 4.2) which indicated a good characteristic of support material for cell immobilization. Undelignified bulrush has the highest stability of 82.36% while the delignified bulrush had the lowest stability of 69.71%. The lowest stability could be resulted from a loss of porosity and complex layers after delignification.

Table 4.2 Stability of the immobilized PCL3 on support materials

Support materials	Stability* (%)
Undelignified coir	78.72ab ± 1.84
Delignified coir	77.75ab ± 2.41
Undelignified bulrush	82.36a ± 1.37
Delignified bulrush	69.71c ± 2.42
Undelignified banana stem	77.12b ± 0.92
Delignified banana stem	75.96b ± 1.36
Undelignified water hyacinth stem	57.36d ± 3.74

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

4.4.2 Degradation of carbofuran by free and immobilized cells of PCL3

Degradation of carbofuran in synthetic wastewater was described by a modified first-order kinetic model (Fig. 4.6). The corresponding kinetic data fitted to a modified first-order kinetic model was tabulated in Table 4.3. The regression coefficients, r^2, ranged between 0.98 and 0.99 indicating a good fit of the data to the first-order kinetic model (Table 4.3). The free cells of PCL3 could degrade carbofuran in synthetic wastewater with half-life of 9.31 days. The shorter half-lives in the range of 2.85-6.85 days could be obtained when the immobilized cells were used (Table 4.3). The results suggested that the degradation ability of *B. cepacia* PCL3 was increased by the immobilization in comparison to free cells. The high matrix porosity

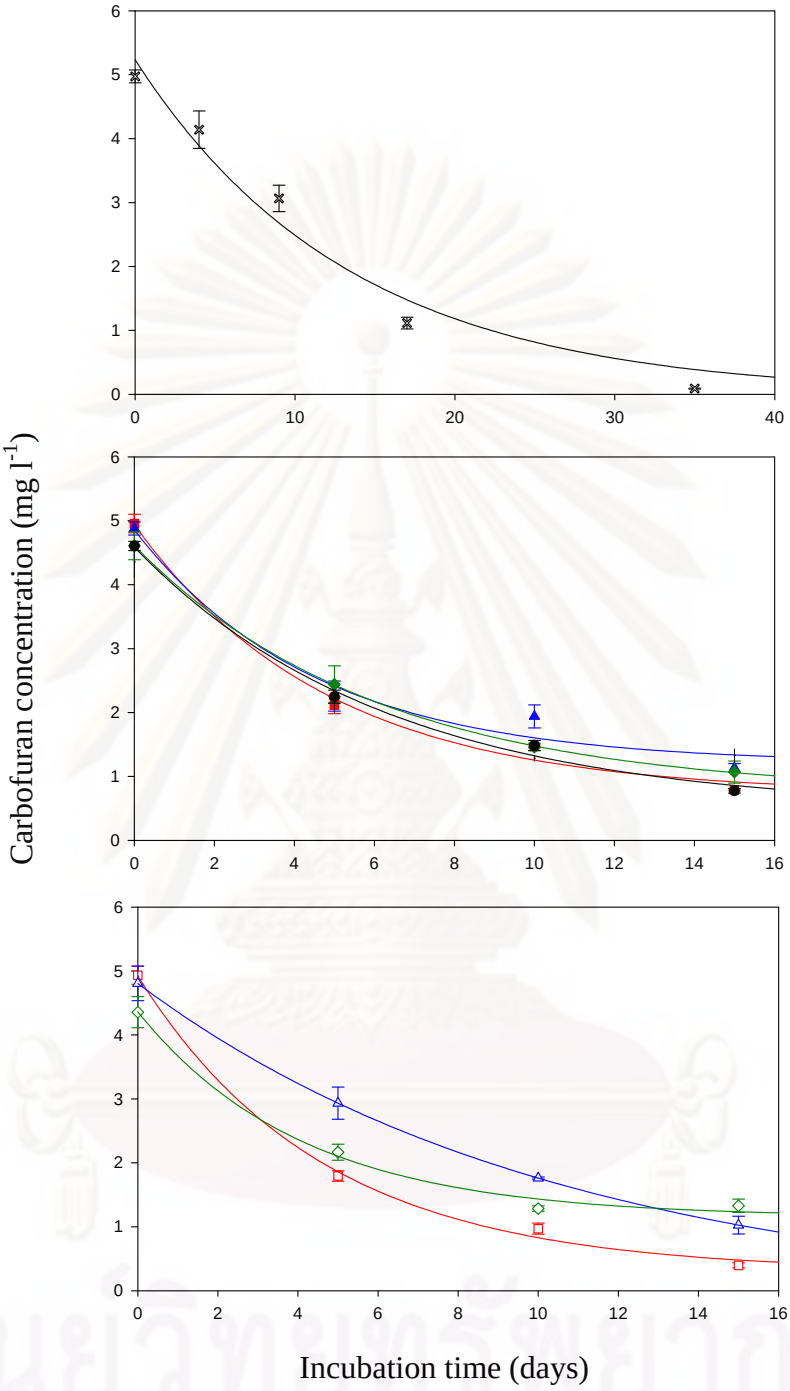

Figure 4.6 Degradation of carbofuran in synthetic wastewater by *B. cepacia* PCL3 in free cells form (x) and the immobilized cells on undelignified support materials (■ = coir, ▲ = bulrush, ◆ = banana stem, ● = water hyacinth stem) and delignified support materials (□ = coir, △ = bulrush, ◇ = banana stem); lines indicated the carbofuran degradation in synthetic wastewater fitted to the modified first-order kinetic model)

Table 4.3 Degradation rate constants (k_1) and half-lives ($t_{1/2}$) of carbofuran in synthetic wastewater by the immobilized cells on each support materials in comparison to free cells

Treatments	k_1, day^{-1}	$t_{1/2}$, days*	r^{2**}
Immobilized PCL3 cells on;			
Undelignified coir	0.2102±0.0523	3.40a±0.85	0.99
Delignified coir	0.2175±0.0030	3.19a±0.04	0.99
Undelignified bulrush	0.2229±0.0844	3.35a±1.27	0.98
Delignified bulrush	0.1012±0.0016	6.85b±0.11	0.99
Undelignified banana stem	0.1686±0.0536	4.33a±1.38	0.99
Delignified banana stem	0.2429±0.0064	2.85a±0.07	0.99
Undelignified water hyacinth stem	0.1598±0.0337	4.44a±0.94	0.99
PCL3 in free cells form	0.0749±0.0085	9.31c±1.05	0.98

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

** Coefficients of determination for non-linear regressions

and pore size of the support materials might enhance the absorption capacity and substrate transfer to the immobilized cells (Gu et al., 1994 and 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 material a transfer of the contaminant to the cells (Ma et al., 2006).

Delignification did not significantly affect the carbofuran degradation efficiency of the immobilized cells on coir. This suggested by non significantly different ($p>0.05$) between the $t_{1/2}$ of carbofuran in synthetic wastewater amended with delignified and undelignified forms of these support materials (3.19-3.40 days) (Table 4.3). A significant longer $t_{1/2}$ of carbofuran of 6.85 days was observed when the immobilized PCL3 on delignified bulrush was used as compared to immobilized cells on its undelignified form ($t_{1/2}$ of 3.35 days) (Table 4.3). In the case of banana stem the delignification could improve the carbofuran degradation efficiency in which the significant shorter $t_{1/2}$ of carbofuran (2.85 days) could be obtained when the immobilized cells on its delignified form was used comparing to its undelignified form ($t_{1/2}$ of 4.33 days). Delignification, in fact, could improve carbofuran sorption efficiency of the support materials (Table 4.1) by facilitating the transfer of carbofuran to the cells inside the support materials thus enhancing the carbofuran

degradation efficiency. In addition, after lignin structure was removed, the fibrous of the support material is softened and can be easier to be used by the PCL3 as the additional energy for its growth. The presence of additional energy sources in the pesticide bioremediation system might result in an increase in cell number as well as their activities to degrade the pesticides (Robles-Gonzalez, 2008). However, sometime, it could worsen the efficiency of pesticide remediation in which the degrader would prefer to use the additional energy sources than the target pesticide (Plangklang and Reungsang, 2010).

The survival and growth of free and immobilized cells were determined by the numbers of *B. cepacia* PCL3 in synthetic wastewater and on the support materials, respectively. Results indicated that free cells and the immobilized cells on all materials could survive through 15 days of incubation at the cell numbers of approximately 10^6 cfu ml^{-1} and 10^8 to 10^9 cfu g^{-1} dry support material, respectively (Fig. 4.7 and 4.8a). A rapid increase of the cell number in the treatments using delignified bulrush and water hyacinth stem could be observed (Fig. 4.8a). This might be because these support materials were easier to be used by PCL3 as the energy sources for growth in comparison to the other support materials which could either improve or worsen the carbofuran removal efficiency as described earlier.

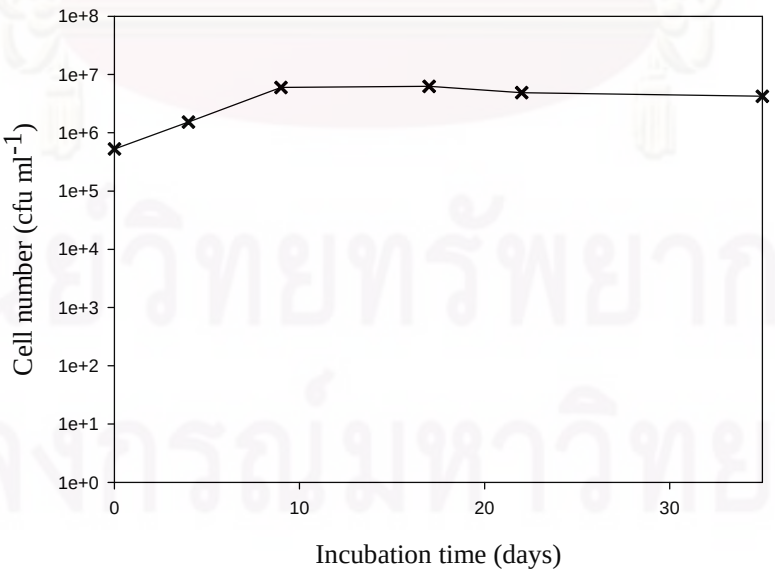

Figure 4.7 Growth of *B. cepacia* PCL3 in free cells form grown in synthetic wastewater

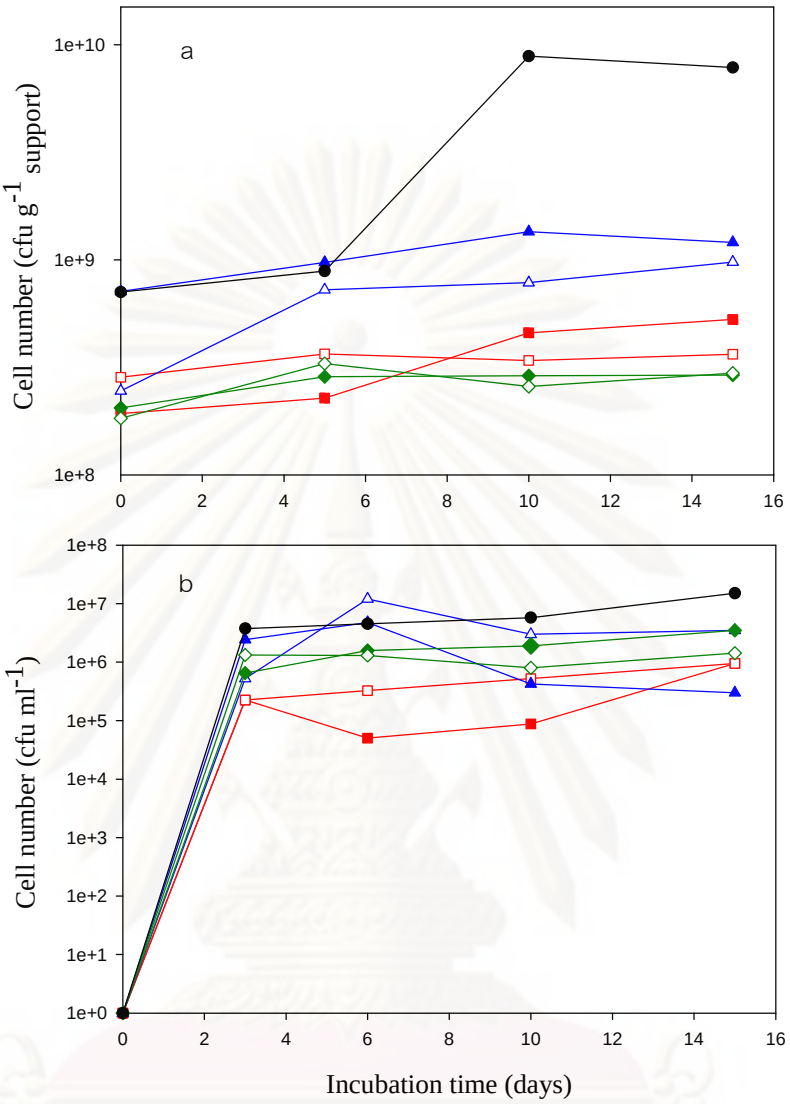

Figure 4.8 Number of *B. cepacia* PCL3 immobilized on support materials (a) and cell leaked from support materials (b) in undelignified (■ = coir, ▲= bulrush, ◆ = banana stem, ● = water hyacinth stem) and delignified (□ = coir, △ = bulrush, ◇= banana stem) forms during cultivation in synthetic wastewater

Cells leaked from the support materials was determined by a cell concentration observed in synthetic wastewater during the incubation. At day 0, number of the PCL3 cells in the liquid phase in synthetic wastewater was negligible. After day 3, number of the PCL3 in the liquid phase of the synthetic wastewater was in a range of 10^5 to 10^7 cfu ml^{-1} (Fig. 4.8b). The leaking of the cells from support materials might be resulted from the fact that there is no barrier between the cells and synthetic wastewater which led to the possibility of cells detachment and relocation

with the establishment of cells equilibrium inside the support material and synthetic wastewater (Bekatorou et al, 2004).

The dissipation of carbofuran could be observed in synthetic wastewater amended with steriled support materials (Fig. 4.9). Half-lives of carbofuran in synthetic wastewater when amended with sterile support materials (29.05-58.02 days) (Table 4.4) were significantly longer than inoculated with immobilized cells (2.85-6.85 days). This suggested that the degradation of carbofuran in synthetic wastewater was mainly from biological process. Approximately 30% of carbofuran was dissipated in synthetic wastewater with steriled support materials (Fig. 4.9). The dissipation of carbofuran might be caused by the sorption of carbofuran onto the surface and porous of the support materials during incubation. Sorption coefficients (K_f) of carbofuran to each support materials were in the range of 8.13 to 24.23 l kg^{-1} (Table 4.1) which could response to carbofuran dissipation in synthetic wastewater. The shortest $t_{1/2}$ of 2.85 days could be obtained when the immobilized cells on undelignified banana stem was used but its $t_{1/2}$ was not significantly different ($p \geq 0.05$) from the $t_{1/2}$ obtained from the immobilized cells on undelignified coir and bulrush

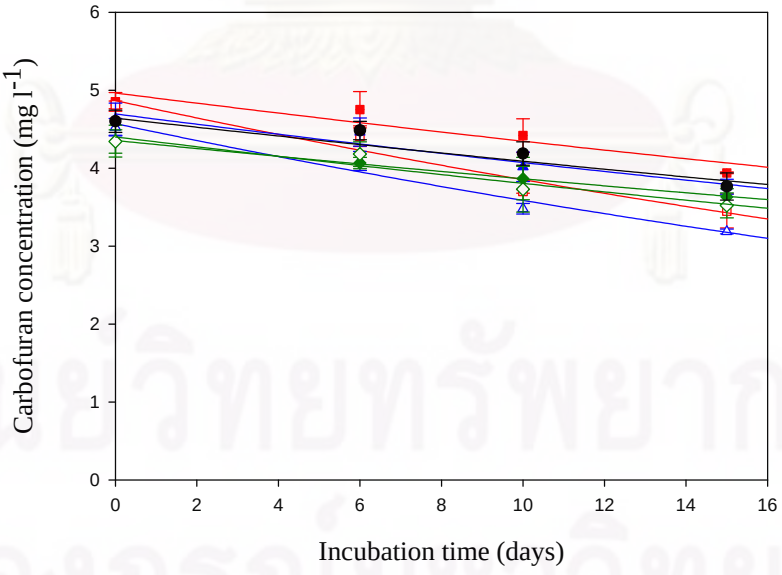

Figure 4.9 Dissipation of carbofuran in synthetic wastewater with sterile support materials in undelignified (■ = coir, ▲ = bulrush, ◆ = banana stem, ● = water hyacinth stem) and delignified (□ = coir, △ = bulrush, ◇ = banana stem) forms

Table 4.4 Dissipation rate constants (k_1) and half-lives ($t_{1/2}$) of carbofuran in synthetic wastewater with steriled support materials

Sterile support materials	k_1, day^{-1}	$t_{1/2}$, days	r^{2*}
Undelignified coir	0.0133±0.0015	52.63±5.90	0.93
Delignified coir	0.0235±0.0056	30.42±7.25	0.96
Undelignified bulrush	0.0142±0.0013	49.00±4.39	0.96
Delignified bulrush	0.0242±0.0041	29.05±4.92	0.98
Undelignified banana stem	0.0120±0.0004	58.02±1.72	0.96
Delignified banana stem	0.0147±0.0022	47.84±7.16	0.95
Undelignified water hyacinth stem	0.0130±0.0013	53.80±5.58	0.95

* Coefficients of determination for non-linear regressions

(3.40 and 3.35 days, respectively). Therefore, delignified banana stem, undelignified coir and bulrush could be the good candidates to be used as support materials for PCL3 immobilization.

4.4.3 Reusability of immobilized *B. cepacia* PCL3 for degrading carbofuran in synthetic wastewater

The immobilized cells were harvested after 15 days of incubation and re-inoculated into fresh synthetic wastewater containing 5 mg l^{-1} of carbofuran and re-incubated to examine their reusability. The immobilized cells on undelignified water hyacinth stem and delignified bulrush were not be reused because the support materials had been degraded after 15 days of incubation. Results indicated that undelignified coir could be reused 3 times, undelignified bulrush and undelignified banana stem could be reused 2 times and delignified banana stem could be reused 1 time. The profiles of carbofuran degradation in synthetic wastewater by the reused immobilized cells on each support material were depicted in Fig. 4.10. The corresponding kinetic data fitting to a modified first-order kinetic model were tabulated in Table 4.5. Results revealed that the reused immobilized cells on all support materials could effectively degrade carbofuran in synthetic wastewater with the short $t_{1/2}$ of 2.57-6.21 days (Table 4.5) which were not significantly different from the $t_{1/2}$ of carbofuran degraded by the first time usage immobilized cells (2.85-6.85 days) (Table 4.3).

Number of PCL3 on the support materials slightly decreased as the reuse step increased (Fig. 4.11). This might be because the support materials were degraded over time indicated by the reduction in size of the support materials (data not shown). The

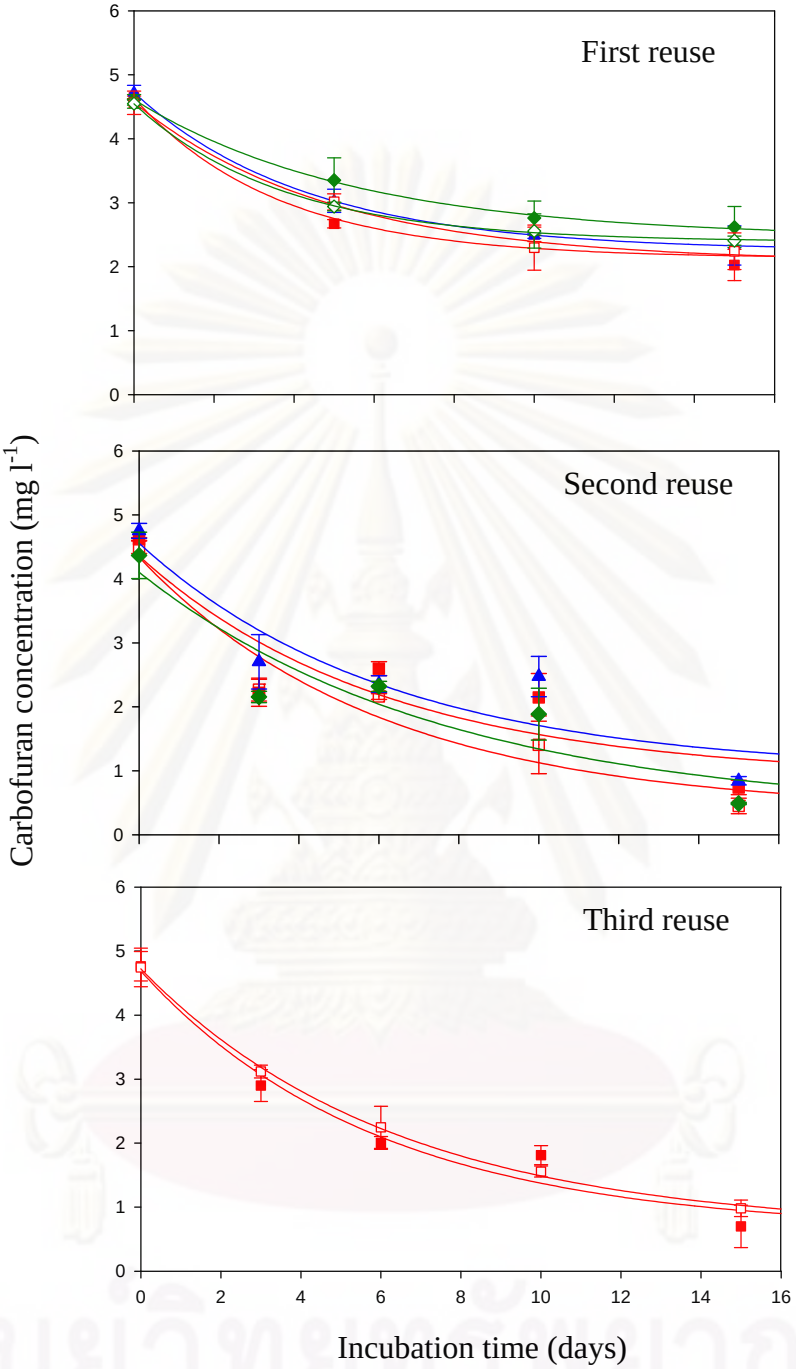

Figure 4.10 Degradation of carbofuran in synthetic wastewater by *B. cepacia* PCL3 immobilized on undelignified support materials (■ = coir, ▲ = bulrush, ◆ = banana stem, and delignified support materials (□ = coir, ◇ = banana stem); lines indicated the carbofuran degradation in synthetic wastewater fitted to the modified first-order kinetic model)

Table 4.5 Degradation rate constants (k_1) and half-lives ($t_{1/2}$) of carbofuran in synthetic wastewater with reused immobilized cells on each support materials

Immobilized *B. cepacia* PCL3	k_1, day^{-1}	$t_{1/2}$, days*	r^{2**}
Immobilized cells on ;			
Undelignified coir			
First reuse	0.2766±0.0654	2.58±0.61	0.98
Second reuse	0.1582±0.0171	4.41±0.48	0.91
Third reuse	0.1693±0.0275	4.15±0.67	0.98
Delignified coir			
First reuse	0.2113±0.0473	3.36±0.75	0.99
Second reuse	0.1646±0.0074	4.21±0.19	0.96
Third reuse	0.1123±0.0127	4.43±0.52	0.99
Undelignified bulrush			
First reuse	0.2318±0.0029	2.99±0.04	0.98
Second reuse	0.1662±0.0169	4.19±0.43	0.92
Undelignified banana stem			
First reuse	0.2090±0.0515	3.42±0.84	0.99
Second reuse	0.1347±0.0216	5.21±0.84	0.93
Delignified banana stem			
First reuse	0.2701±0.0100	2.57±0.10	0.98

* Comparison between treatment in column are not significantly different (Duncan, $p \geq 0.05$)
** Coefficients of determination for non-linear regressions

decay of the support materials implied that the immobilized cells were environmentally friendly in which they will not cause the disposal problem after finished bioremediation process in the contaminated site.

Number of the leaking cells from support materials to synthetic wastewater decreased with an increase in reuse step. The similar trend of the cell number inside the support materials could be observed. The results suggested that the leaking of the cells to synthetic wastewater would reach the steady state when the equilibrium between cell number inside the support material and synthetic wastewater was achieved.

From the obtained results, it could be concluded that undelignified coir is the most suitable support material for PCL3 immobilization to be used to remediate carbofuran contaminated water. The immobilized PCL3 on undelignified coir

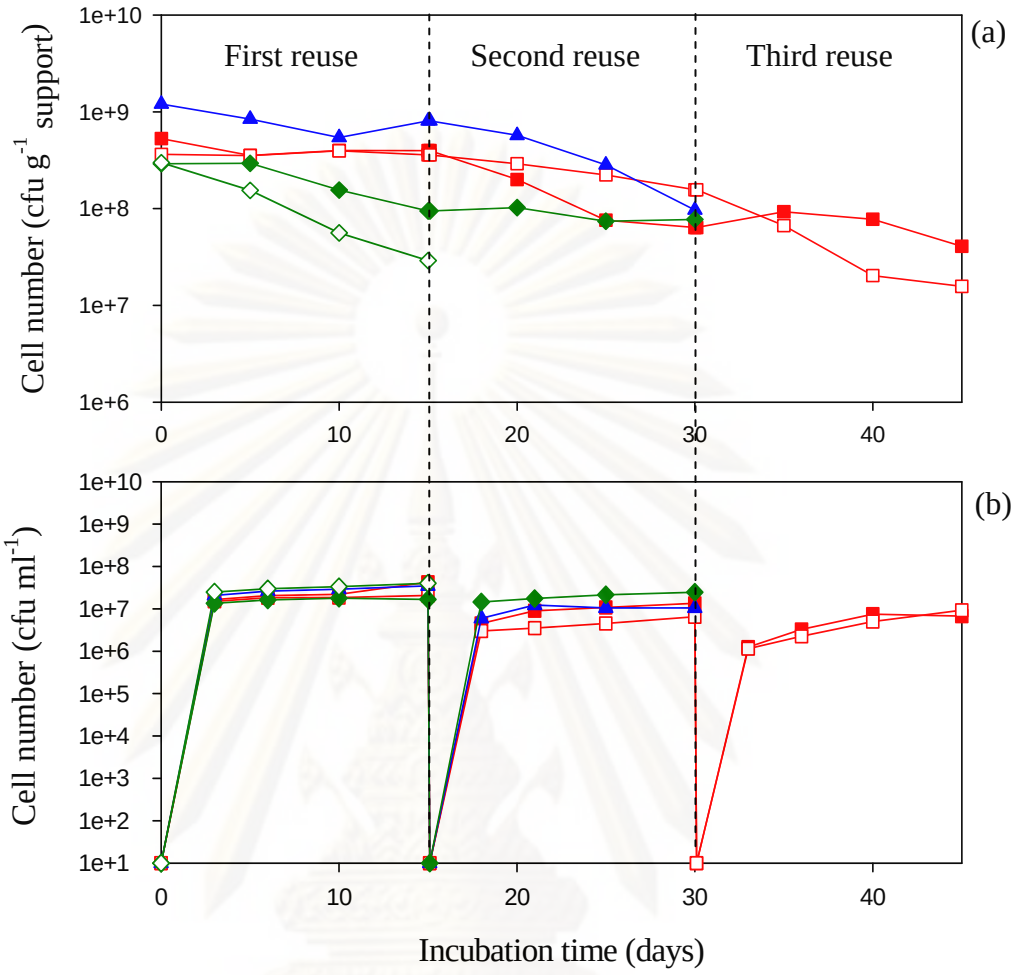

Figure 4.11 Number of *B. cepacia* PCL3 on support materials (a) and number leaking cell (b) from support materials observed in synthetic wastewater during reusability study (undelignified ■ = coir, ▲ = bulrush, ◆ = banana stem, ● = water hyacinth stem and delignified □ = coir, △ = bulrush, ◇ = banana stem)

possessed a high stability of 78.72%, high carbofuran degradation ability with the short $t_{1/2}$ of 3.40 days and could be reused three times with the remaining of carbofuran degradation ability ($t_{1/2}$ of 2.58-4.15 days). The effects of carbofuran concentration on growth patterns and degradation kinetic of immobilized PCL3 on coir were studied in the further experiment.

4.5 Effect of initial carbofuran concentration in synthetic wastewater on the growth and carbofuran degradation kinetic of *B. cepacia* PCL3

4.5.1 Growth kinetic

The growth patterns of free and immobilized PCL3 in synthetic wastewater at the various initial carbofuran concentrations of 5-250 were shown in Fig. 4.12 and 4.13, respectively. It could be seen that growth curves of the free cells have typically exponential and stationary phases. Lag phase increased as the initial concentration of carbofuran increased (Fig. 4.12). It could be seen that the carbofuran concentrations of 5-50 mg l^{-1} did not show any inhibitory effect on the isolate PCL3 indicated by there was almost no lag phase during the growth period. A relatively short lag phase of 3 days was observed during the growth of the isolate PCL3 at initial carbofuran concentration of 100 mg l^{-1}, and the longer lag period of 4 days were evident in the growth patterns of PCL3 at the initial carbofuran concentrations of 150 and 200 mg l^{-1} (Fig. 4.12). At the carbofuran concentrations of greater than 200 mg l^{-1}, the growth of the isolate PCL3 was almost completely inhibited.

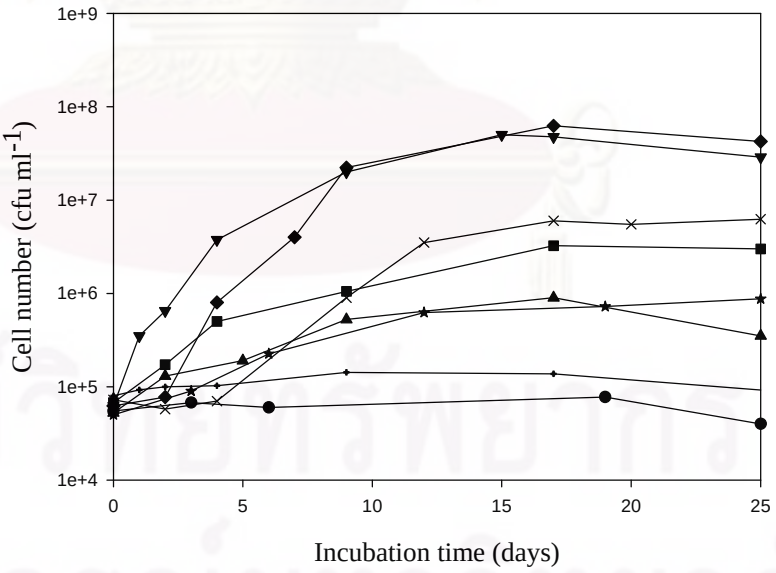

Figure 4.12 Time profile of the growth of *B. cepacia* PCL3 in free cell form at different initial carbofuran concentration; (+ = 0 mg l^{-1}, ▲ = 5 mg l^{-1}, ■ = 10 mg l^{-1}, ▼ = 50 mg l^{-1}, ◆ = 100 mg l^{-1}, x = 150 mg l^{-1}, ★ = 200 mg l^{-1} and ● = 250 mg l^{-1})

Growth and survival of the immobilized PCL3 were determined by the numbers of carbofuran degraders in undelignified coir as a support material. Number of cells leaked from undelignified coir was observed in culture media during incubation. The numbers of the isolate PCL3 on coir and in synthetic wastewater were depicted Fig. 4.13a and 4.13b, respectively. Results indicated that cell numbers on coir increased suddenly after inoculation without any lag period of growth and were stable in the range of 10^8-10^9 cfu g^{-1} dry support material after 3 days of incubation in

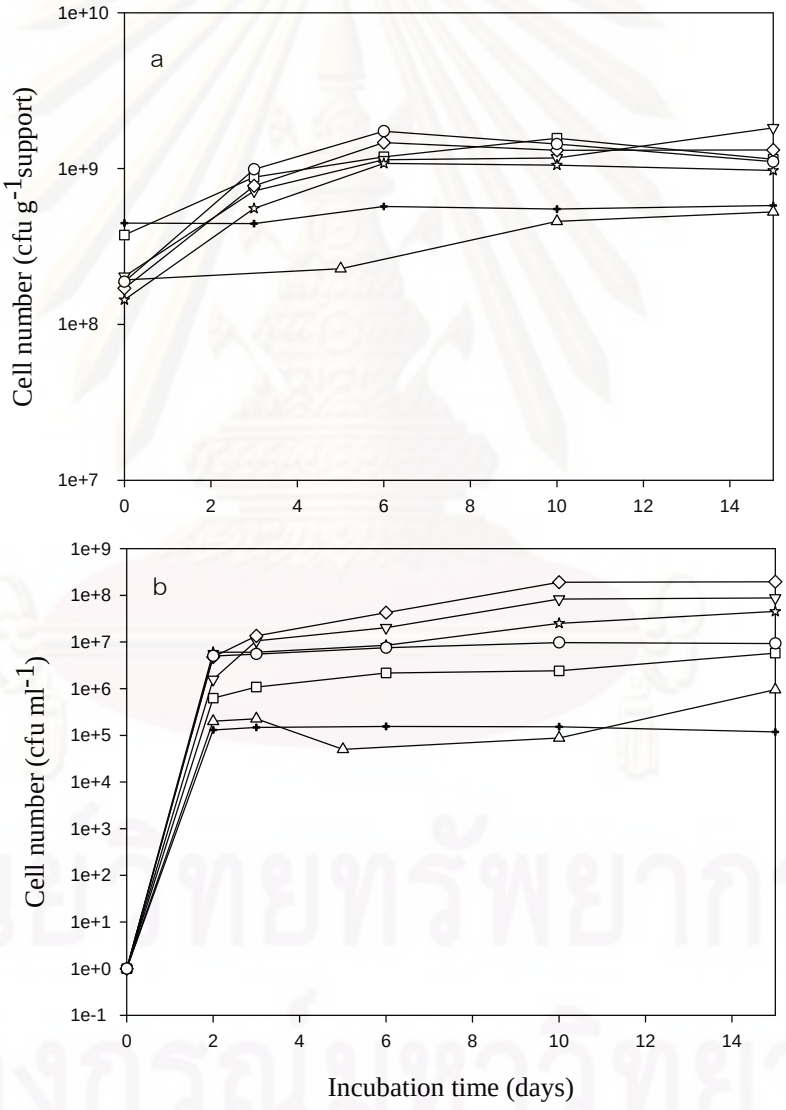

Figure 4.13 Time profile of the number of *B. cepacia* PCL3 (a) immobilized and (b) leaked from undelignified coir at different initial carbofuran concentration; (+ = 0 mg l^{-1}, △ = 5 mg l^{-1}, □ = 10 mg l^{-1}, ▽ = 50 mg l^{-1}, ◇ = 120 mg l^{-1}, ☆ = 200 mg l^{-1} and ○ = 280 mg l^{-1})

all treatments (Fig. 4.13). The number of leaking cells in culture media was negligible at day 0 and increased to be greater than 10^6 to 10^7 cfu ml^{-1} after 3 days of incubation.

The relation between specific growth rate of PCL3 in free and immobilized cells forms and initial concentration of carbofuran was depicted in Fig. 4.14 and 4.15, respectively. The decrease in specific growth rate with the increase in initial carbofuran concentration in free cell experiment (Fig. 4.14) implied that carbofuran acts as an inhibitor on PCL3. Therefore, the substrate inhibition model (Luong model) was used to explain the growth kinetic of PCL3 in free cell form. The Monod model was used to explain the growth kinetic of the immobilized PCL3. The results indicated that there was no inhibitory effect of carbofuran at the concentration up to 280 mg l^{-1} (Fig. 4.15) on the immobilized PCL3 which confirmed that the immobilized technique could protect the PCL3 cell from substrate inhibition effect. The growth kinetic parameters of PCL3 in free and immobilized cell forms estimated from nonlinear regression with the kinetic models were tabulated in Table 4.6. The regressions coefficients, r^2, ranged between 0.98-0.99 indicating a good fit of the

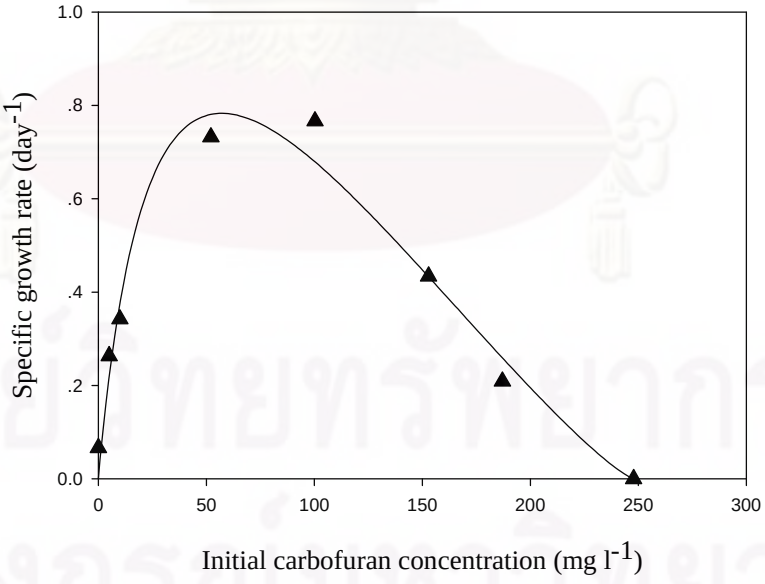

Figure 4.14 Specific growth rate of PCL3 in free cell form at different initial carbofuran concentration; lines indicated the specific growth rate of PCL3 in synthetic wastewater fitted to the Luong model

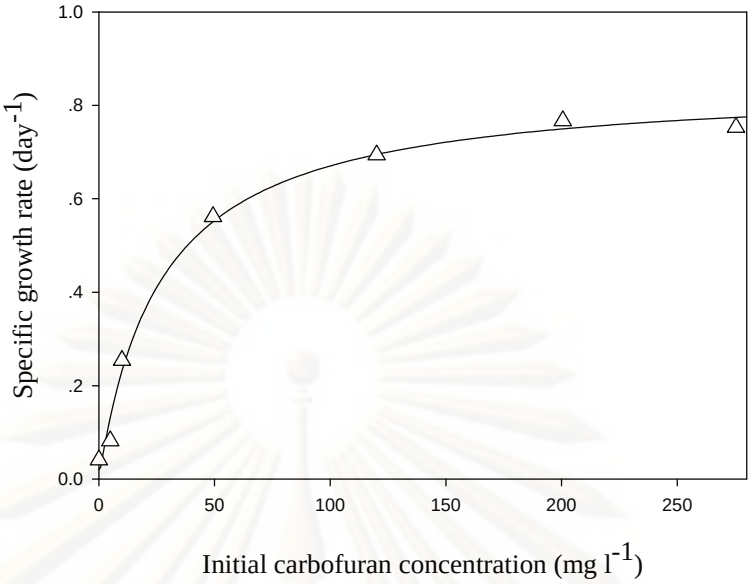

Figure 4.15 Specific growth rate of immobilized PCL3 on undelignified coir at different initial carbofuran concentration; lines indicated the specific growth rate of PCL3 in synthetic wastewater fitted to the Luong model

Table 4.6 Kinetic parameters of PCL3 in free and immobilized cell forms estimated from nonlinear regression with growth kinetic models

Kinetic parameters	Free cells of PCL3	Immobilized PCL3
Model	Luong	Monod
μ_{max} (day^{-1})	1.705	0.850
K_s (mg l^{-1})	33.12	26.77
S_m (mg l^{-1})	247.83	-
n	1.22	-
r^2	0.98	0.99

data to the models. The estimated kinetic parameters for free cells of PCL3 from the Luong model were μ_{max} = 1.70 day^{-1} and K_s = 33.12 mg l^{-1}. The constants n (1.22) estimated by Luong model were greater than 1 suggesting that the non-linear relationship between μ and S existed during inhibition process. The maximum substrate concentration above which carbofuran degradation was completely inhibited (S_m) was 247.83 mg l^{-1}. The estimated growth kinetic parameters for the immobilized PCL3 obtained from the Monod model were μ_{max} = 0.85 day^{-1} and K_s = 26.77 mg l^{-1}. The lower μ_{max} value was obtained when the immobilized cells were used as compared to free cells which might be because the immobilized cells could not grow

freely with the limited space inside the support material. The lower K_s value obtained indicated that at the low carbofuran concentration the immobilized PCL3 was able to access carbofuran in synthetic wastewater better than free cells. This result confirmed that the sorption of carbofuran to support material could enhance the mass transferring between carbofuran and cells inside the support.

4.5.2 Degradation kinetic

The degradation profiles of carbofuran in synthetic wastewater with various concentration of carbofuran inoculated with free cells of PCL3 was depicted in Fig. 4.16. Results revealed that carbofuran could be degraded immediately at the initial concentration up to 100 mg l^{-1}. The lag period could be observed at initial carbofuran concentration of greater than 100 mg l^{-1}. At the initial carbofuran concentration of 250 mg l^{-1}, the microbial growth was not observed (Fig. 4.12), but a slightly decrease of carbofuran concentration was observed (Fig. 4.16,). The result indicated that at a high initial carbofuran concentration, PCL3 tended to use carbofuran for maintaining the cells. In addition, there might be abiotic processes responsible for carbofuran

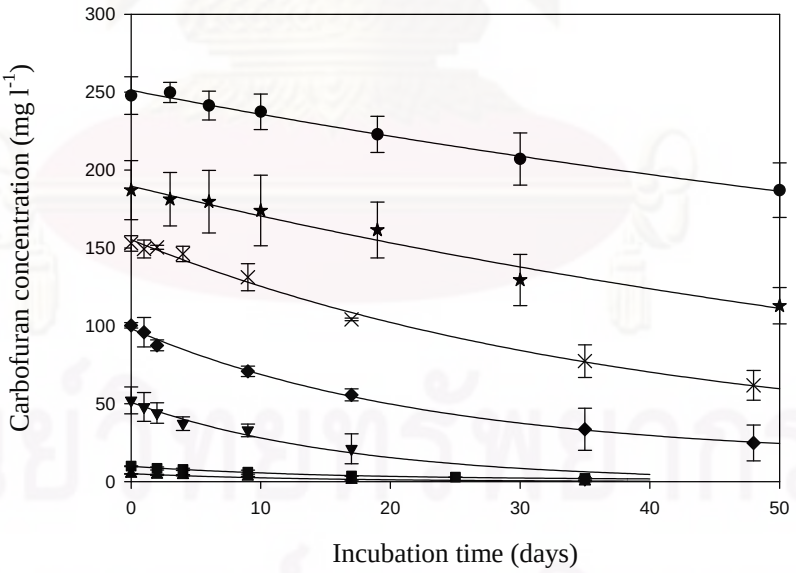

Figure 4.16 Degradation of carbofuran in synthetic wastewater by free cells of *B. cepacia* PCL3 at different initial carbofuran concentration (▲ = 5 mg l^{-1}, ■ = 10 mg l^{-1}, ▼= 50 mg l^{-1}, ◆ = 100 mg l^{-1}, x = 150 mg l^{-1}, ★= 200 mg l^{-1} and ● = 250 mg l^{-1}); lines indicated the carbofuran degradation in synthetic wastewater fitted to the modified first-order kinetic model)

degradation. This might be resulted from the hydrolysis and volatization 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 process which could be found in the abiotic control as reported in previous studies (Plangklang and Reungsang, 2009; Evert, 2002; Laha et al., 1996).

The carbofuran degradation in synthetic wastewater by the immobilized PCL3 on undelignified coir was depicted in Fig. 4.17. It could be seen from the results that the immobilized cells could degrade carbofuran at carbofuran concentrations up to 280 mg l^{-1} without an obvious inhibitory effect. The results were in correlation with the growth patterns in which the growth of immobilized PCL3 was not inhibited by any range of carbofuran concentration used in this study (Fig. 4.13). The degradation rate coefficient of carbofuran was calculated by fitting to a modified first-order kinetic model in order to examine $t_{1/2}$ of carbofuran in synthetic wastewater. The regressions coefficient, r^2, ranged between 0.97-0.99 indicating a good fit of the data to the model

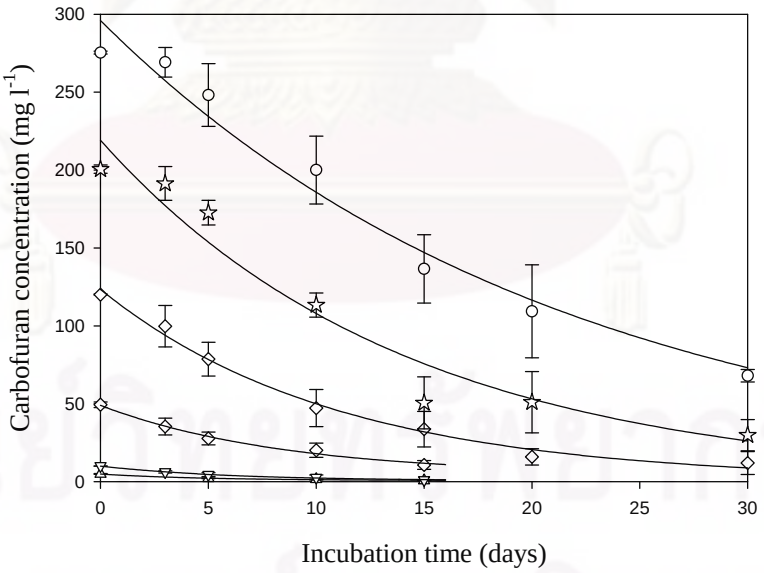

Figure 4.17 Degradation of carbofuran in synthetic wastewater by immobilized *B. cepacia* PCL3 on undelignified coir at different initial carbofuran concentration (△ = 5 mg l^{-1}, □ = 10 mg l^{-1}, ▽ = 50 mg l^{-1}, ◇= 120 mg l^{-1}, ☆= 200 mg l^{-1} and ○ = 280 mg l^{-1}); lines indicated the carbofuran degradation in synthetic wastewater fitted to the modified first-order kinetic model)

(Table 4.7). Results indicated that immobilized cells were more efficient to degrade carbofuran than free cells at any range of carbofuran concentration used in this study. In free cells experiment, the significant longer $t_{1/2}$ of carbofuran ($p<0.05$) was obtained when the initial carbofuran concentration was increased (Table 4.7). The $t_{1/2}$ of carbofuran at the initial carbofuran concentration at 250 mg l^{-1} was 8.2 times longer than the carbofuran $t_{1/2}$ at the initial carbofuran concentration of 5 mg l^{-1}. In the immobilized cells experiments, $t_{1/2}$ of carbofuran (3.4-6.3 days) were not significantly different at the initial concentration from 5 to 50 mg l^{-1} (Table 4.7). The slightly longer $t_{1/2}$ of carbofuran $t_{1/2}$ of 15 days could be found when the concentration of carbofuran was increased from 50 mg l^{-1} to 250 mg l^{-1}. These results indicated that the inhibitory effect on carbofuran degradation by PCL3 at high carbofuran concentration could be reduced by immobilization technique.

The specific carbofuran degradation rate, q (day^{-1})**,** of free and immobilized PCL3 at various initial carbofuran concentration were calculated and depicted in Fig. 4.18a and 4.18b, respectively. The Luong model was used to explain the biodegradation kinetic of carbofuran by PCL3 in free cells experiment which carbofuran acted as inhibitor on PCL3. The correlation coefficients, r^2 of 0.94 (Table 4.8) could be obtained. The carbofuran degradation kinetic parameters

Table 4.7 Degradation rate constants (k_1) and half-lives ($t_{1/2}$) of carbofuran in PCL3 inoculated synthetic wastewater with various initial carbofuran concentrations

Initial carbofuran conc. (mg l^{-1})	Free cells of PCL3			Initial carbofuran conc. (mg l^{-1})	Immobilized PCL3		
	k_1, day^{-1}	$t_{1/2}$, days (*)	r^2 (**)		k_1, day^{-1}	$t_{1/2}$, days(*)	r^2 (**)
5	0.0746±0.0076	9.34a±0.95	0.98	5	0.2102±0.0523	3.40a±0.85	0.99
10	0.0698±0.0280	10.80ab±4.33	0.99	10	0.1625±0.0203	4.30a±0.54	0.99
50	0.0602±0.0052	11.56b±0.99	0.98	50	0.1188±0.0451	6.29ab±2.39	0.99
100	0.0457±0.0117	15.68b±4.03	0.99	120	0.0929±0.0229	7.69b±1.90	0.99
150	0.0200±0.0033	35.23c±5.87	0.99	200	0.0711±0.0124	9.90b±1.73	0.97
200	0.0117±0.0017	59.86d±8.68	0.98	280	0.0469±0.0081	15.00c±2.58	0.98
250	0.0091±0.0006	76.30e±4.74	0.99				

* Comparison between treatment in column are significantly different (Duncan, $p\leq0.05$) if mark in different small letters

** Coefficients of determination for non-linear regression

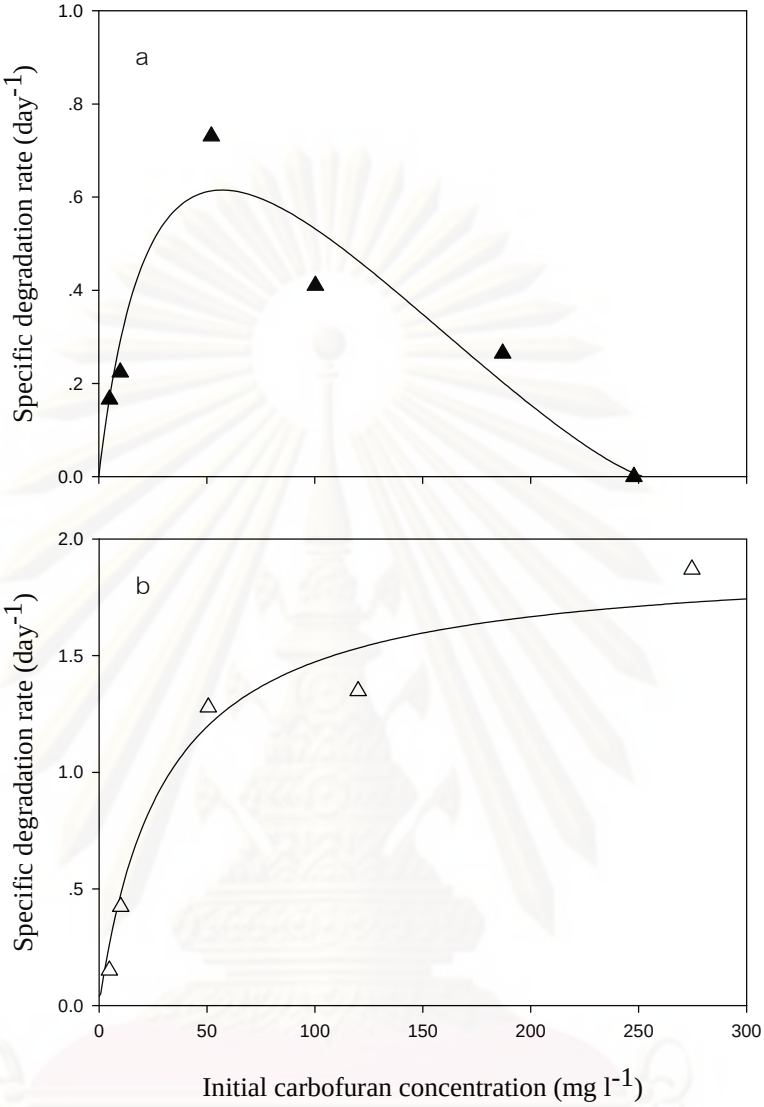

Figure 4.18 The specific degradation rate of carbofuran by PCL3 in free cell form (a) and immobilized cell form (b) at various initial carbofuran concentrations.

Table 4.8 Kinetic parameters of free and immobilized cells PCL3 on coir estimated from nonlinear regression with degradation kinetic models

Kinetic parameters	Free cells of PCL3	Immobilized PCL3
Model	Luong	Monod
q_{max} (day^{-1})	1.380	1.920
K_s (mg l^{-1})	34.78	30.32
S_m (mg l^{-1})	252.80	-
n	1.30	-
r^2	0.94	0.98

were q_{max} = 1.38 day^{-1} K_s = 34.78 mg l^{-1}, and n = 1.3 (Table 4.8). The Monod model was used to explain the biodegradation kinetic of carbofuran by PCL3 in the immobilized cell form. The estimated kinetic parameters q_{max} and K_s were 1.92 day^{-1} and 30.32 mg l^{-1}, respectively (Table 4.8). The inhibitory effect of carbofuran was observed in free cells experiment with the maximum substrate concentration above which carbofuran degradation was completely inhibited (S_m) of 252.80 mg l^{-1} (Table 4.8), while, the inhibitory effect of carbofuran to immobilized cells was not observed. The results confirmed that the immobilization technique could protect the PCL3 cell from substrate inhibition hence enhancing carbofuran degradation efficiency. The enhancement of contaminant degradation activity by immobilization technique has also been reported in different mechanisms. Chung et al. (2003) suggested that immobilization of viable cells could alter their physiological features of metabolism such as enhanced the induction of enzyme responsible for contaminant degradation. Wang et al. (2007) and Plangklang and Reungsang (2008) indicated that the support materials could act as the protective shelter against the toxicity of the contaminant and adverse environmental condition hence the survival of the cells could be improved resulting in the increase in degradation efficiency.

4.6 Selection of support materials for immobilization of *B. cepacia* PCL3 to remediate carbofuran in soil

4.6.1 Degradation of carbofuran by free and immobilized cells of PCL3

Degradation of carbofuran in soil by free and immobilized PCL3 was investigated at the initial carbofuran concentration of 5 mg kg^{-1} dry soil. The profiles of carbofuran dissipation in soil were shown in Fig. 4.19, 4.20 and 4.21. The corresponding kinetic data fitted to the modified first-order kinetic model with the r^2 of greater than 0.90 were tabulated in Table 4.9. In the treatment with the present of only indigenous microorganisms (Treatment A), carbofuran could be slowly degraded with the half-lives of 54 days (Table 4.9). The addition of PCL3 in free cell (Treatment B) or immobilized cell (Treatment C) forms could improve carbofuran degradation efficiency in which $t_{1/2}$ of carbofuran in PCL3 inoculated soil were approximately 2.1-3.5 times shorter than in soil without inoculation (Table 4.9).

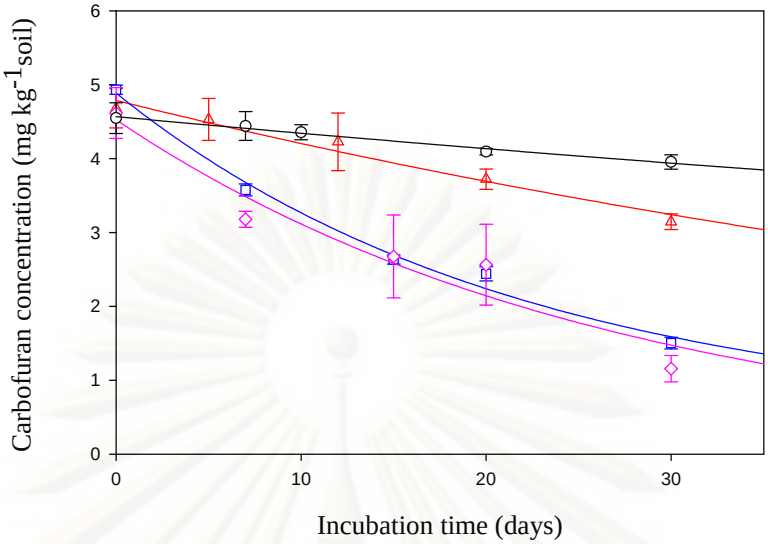

Figure 4.19 Degradation of carbofuran in soils inoculated with free cells of *B. cepacia* PCL3 (◇ = autoclaved bulk soil, □ = bulk soil) and without *B. cepacia* PCL3 (○ = autoclaved bulk soil, △ = bulk soil); lines indicated carbofuran concentrations in soil fitted to the first-order kinetic model)

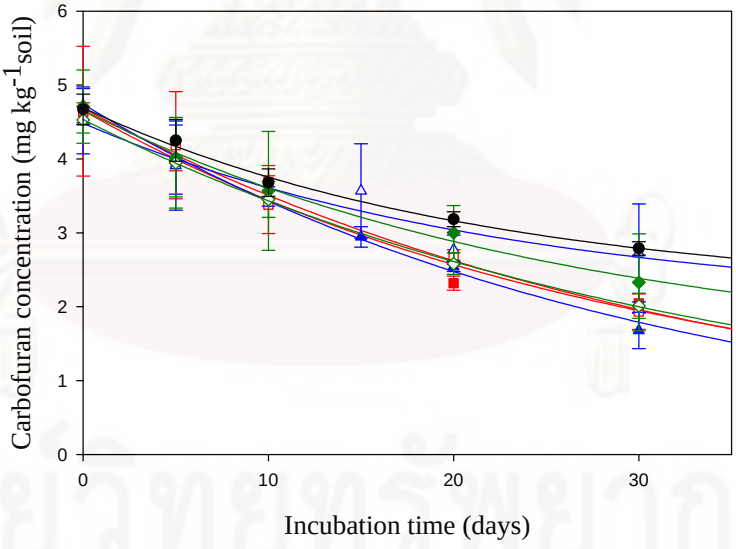

Figure 4.20 Degradation of carbofuran in soil by *B. cepacia* PCL3 immobilized on undelignified support materials (■ = coir, ▲ = bulrush, ◆ = banana stem, ● = water hyacinth stem) and delignified support materials (□ = coir, △ = bulrush, ◇ = banana stem); lines indicated the carbofuran degradation in soil fitted to the modified first-order kinetic model)

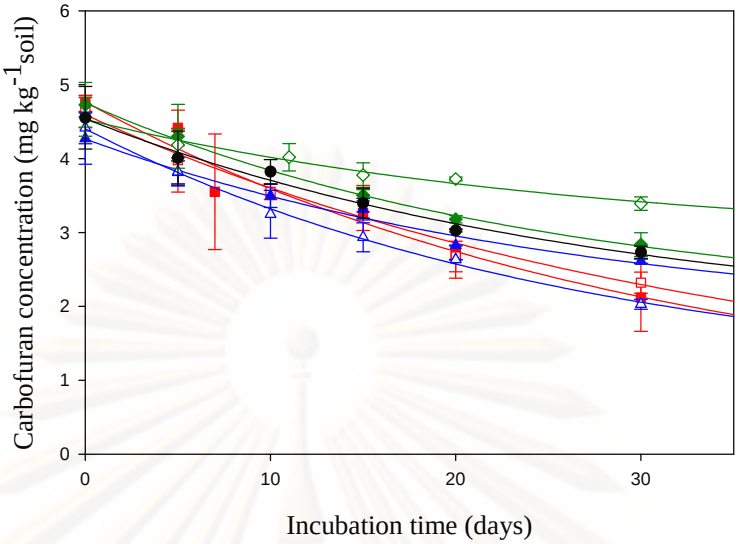

Figure 4.21 Degradation of carbofuran in autoclaved soil by *B. cepacia* PCL3 immobilized on undelignified support materials (■ = coir, ▲ = bulrush, ◆ = banana stem, ● = water hyacinth stem) and delignified support materials (□ = coir, △ = bulrush, ◇ = banana stem); lines indicated the carbofuran degradation in soil fitted to the modified first-order kinetic model)

These results indicated that PCL3 has a potential to be used as an effective tool for carbofuran remediation in soil. The insignificant different ($p \geq 0.05$) between carbofuran degradation in soil inoculated with free and immobilized PCL3 indicated that these support materials did not hinder the transferring of carbofuran in soil to the cells inside the support materials. In addition, the $t_{1/2}$ of carbofuran in soil inoculated with the immobilized PCL3 on different support materials were not significantly different ($p \geq 0.05$) suggesting that all kinds of agricultural residues used in this experiment could be the good support materials for PCL3 immobilization to be used in bioremediation of carbofuran in soil. The slightly decrease in $t_{1/2}$ of carbofuran from 54 days (Treatment A) to approximately 47 days (Treatment D) could be observed in soil by the addition of autoclaved support materials (Table 4.9) implied that these support materials could stimulate the ability of indigenous microorganisms to degrade carbofuran in soil.

Carbofuran degradation ability of free and immobilized PCL3 was evident in the augmented autoclaved soil in which the $t_{1/2}$ of carbofuran could be shorten from 93-104 days (Treatment H and G) to be 17-22 days (Treatment E and F) by inoculated the autoclaved soil with free or immobilized PCL3 (Table 4.9). Other evidence to

Table 4.9 Degradation rate coefficients (k_1) and half-lives ($t_{1/2}$) of carbofuran in soil

Treatment	Experimental setup	k_1, day^{-1}	$t_{1/2}$,days*	r^{2**}
A	Soil	0.0130±0.0008	53.61b±3.22	0.98
B	Soil + free cells of PCL3	0.0456±0.0047	15.28a±1.56	0.99
E	Autoclaved soil + free cells of PCL3	0.0375±0.0023	18.13a±1.74	0.96
H	Autoclaved soil	0.0071±0.0004	97.78c±5.84	0.99
C	Soil + PCL3 immobilized on ;			
	Undelignified coir	0.0381±0.0109	18.96a±5.42	0.98
	Delignified coir	0.0327±0.0037	21.37a±2.45	0.99
	Undelignified bulrush	0.0330±0.009	21.07a±1.22	0.99
	Delignified bulrush	0.0421±0.0077	16.76a±3.07	0.95
	Undelignified banana stem	0.0380±0.0077	18.65a±3.79	0.99
	Delignified banana stem	0.0301±0.0021	23.12a±1.58	0.99
	Undelignified water hyacinth stem	0.0492±0.0160	14.87a±4.83	0.99
D	Soil + autoclaved support material;			
	Undelignified coir	0.0147±0.0009	47.40b±2.97	0.96
	Delignified coir	0.0147±0.0006	47.18b±1.82	0.91
	Undelignified bulrush	0.0153±0.0018	45.62b±5.48	0.94
	Delignified bulrush	0.0148±0.0004	46.84b±1.34	0.98
	Undelignified banana stem	0.0147±0.0018	47.65b±5.75	0.97
	Delignified banana stem	0.0152±0.0021	46.17b±6.25	0.98
	Undelignified water hyacinth stem	0.0144±0.0017	48.46b±5.71	0.99
F	Autoclaved soil + PCL3 immobilized on ;			
	Undelignified coir	0.0323±0.0066	21.94a±4.47	0.96
	Delignified coir	0.0329±0.0089	21.87a±5.92	0.98
	Undelignified bulrush	0.0359±0.0082	19.82a±4.53	0.99
	Delignified bulrush	0.0381±0.0110	19.00a±5.47	0.99
	Undelignified banana stem	0.0409±0.0054	17.09a±2.25	0.99
	Delignified banana stem	0.0362±0.0045	19.32a±2.38	0.98
	Undelignified water hyacinth stem	0.0356±0.0039	19.61a±2.15	0.99
G	Autoclaved soil + autoclaved support material ;			
	Undelignified coir	0.0072±0.0007	96.72c±9.50	0.99
	Delignified coir	0.0075±0.0008	93.53c±9.76	0.98
	Undelignified bulrush	0.0068±0.0014	104.16c±21.66	0.97
	Delignified bulrush	0.0073±0.0004	95.09c±5.23	0.99
	Undelignified banana stem	0.0072±0.0006	96.55c±7.59	0.99
	Delignified banana stem	0.0068±0.0003	102.00c±4.24	0.99
	Undelignified water hyacinth stem	0.0070±0.0013	101.61c±19.64	0.97

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

** Coefficients of determination for non-linear regressions

support this finding is that carbofuran degradation in the augmented soil ($t_{1/2}$ of 15-23 days) (Treatment B and C) were not significantly different ($p \geq 0.05$) from carbofuran degradation in augmented autoclaved soil ($t_{1/2}$ of 17-22 days). The addition of autoclaved support materials into autoclaved soil (Treatment G) did not affect the carbofuran degradation efficiency which indicated that sorption of carbofuran to support material was not significant in carbofuran dissipation in soil.

Carbofuran degradation in soil by *B. cepecia* PCL3 in free cell form and immobilized cell form on corncob and sugarcane bagasse was previously investigated in the study of Plangklang and Reungsang (2009). The similar trend to the present study could be observed in which the immobilization did not worsen or improve carbofuran degradation ability of PCL3, however, the survival of the cells had been improved. The $t_{1/2}$ of carbofuran in PCL3 augmented soil (13-19 days) (Plangklang and Reungsang, 2009) were not markedly different from the results obtained in the present study (15-23 days) (Table 4.9).

Survival and leakage of the immobilized cells in soil at day 30 of incubation were investigated. Results indicated that immobilized cells on all support materials augmented to soil and autoclaved soil (treatment C and F) could survive in the range of 10^8 to 10^9 cfu g^{-1} dry support materials. The free cells augmented to soil and autoclaved soil (treatment B and E) increased from 10^5 at day 0 to 10^6 cfu g^{-1}dry soil at day 30 (Fig. 4.22 and 4.23). The number of indigenous carbofuran degraders in treatment A (soil without inoculation) and D (soil amended with autoclaved support materials) were stable at approximately 10^3 cfu g^{-1} dry soil throughout the experiment (Fig. 4.23).

Leaking of cells from support materials was represented by a cell number observed in soil inoculated with the immobilized cells (treatments C and F) (Fig. 4.23). At day 0, the numbers of carbofuran degrader in the soil were approximately 10^3 cfu g^{-1} dry soil (treatment C) and the numbers of carbofuran degrader in the autoclaved soil were negligible (treatment F). After 30 days of incubation, number of carbofuran degrader in both treatments increased to be 10^7 cfu g^{-1} dry support (Fig. 4.23), while the number of carbofuran degrader inside the support material were not decreased (Fig. 4.22). The results implied that the immobilized cells could act as the inoculum source during bioremediation operation in which the cells could grow and

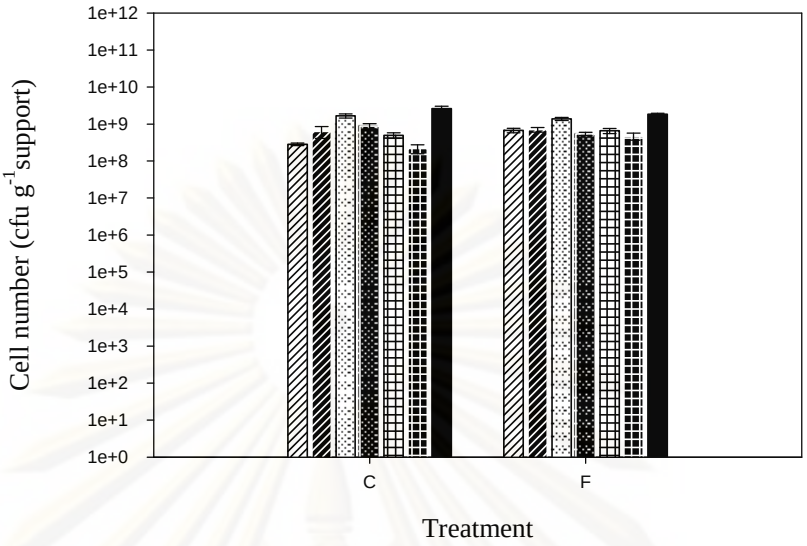

Figure 4.22 Total numbers of carbofuran degraders in undelignified support materials (▨ = coir, ⬚ = bulrush, ▦ = banana stem, ■ = water hyacinth stem) and delignified support materials (▨ = coir, ▩ = bulrush, ▦ = banana stem) at day 30 of incubation. Treatment C = soil + immobilized PCL3; F = Autoclaved soil + immobilized PCL3.

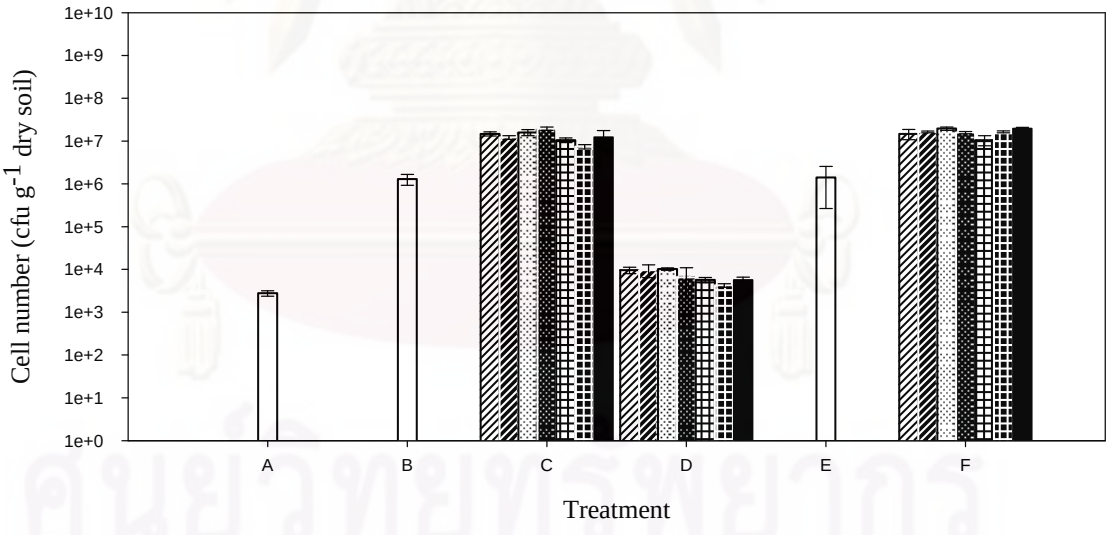

Figure 4.23 Total numbers of carbofuran degraders in soil and autoclaved soil at day 30 of incubation. Treatment A = soil; treatment B = soil + free cells of PCL3; treatment C = soil + immobilized PCL3; treatment D = soil + autoclaved support material; treatment E = autoclaved soil + free cells of PCL3; treatment F = Autoclaved soil + immobilized PCL3. Leaking of cells from undelignified support materials (▨ = coir, ⬚ = bulrush, ▦ = banana stem, ■= water hyacinth stem) and delignified support materials (▨ = coir, ▩= bulrush, ▦ = banana stem) at day 30 of incubation.

further leaked out to the soil due to the space limit inside the support. With this behavior, the high cell density and cell load in the bioremediation system would be maintained which might prevent the loss of degradation efficiency in long term operation.

4.6.2 Reusability of the immobilized *B. cepacia* PCL3 on each support material

The reusability of immobilized PCL3 for carbofuran remediation in soil (treatment C) and autoclaved soil (treatment F) was investigated. The immobilized cells on undelignified water hyacinth stem and bulrush and delignified bulrush and banana stem were broken after 30 days of incubation in the first time usage, hence they were not reused in this study. The results clearly demonstrated that the efficiency of reused immobilized cells on undelignified coir, delignified coir and undelignified banana stem in soil and autoclaved soil ($t_{1/2}$ of 19-21 days) was not significantly different from the first usage ($t_{1/2}$ of 17-22 days) (Table 4.9, 4.10). Numbers of the immobilized cells on undelignified and delignified coir were observed to be stable at 10^8 cfu g^{-1} dry support material (Fig. 4.24) and the leaking cells to the soil were increased to be 10^8 cfu g^{-1} dry soil (Fig. 4.25). The structures of delignified and undelignified coir were not ruined after reuse. These results indicated the potential of further reusing the immobilized cells on delignified coir and undelignified coir in bioremediation of carbofuran in soil. In the case of undelignified banana stem,

Table 4.10 Degradation rate coefficients (k_1) and half-lives ($t_{1/2}$) of carbofuran in soil on cycle 2

Treatment	Experimental setup	k_1, day^{-1}	$t_{1/2,}$ days*	r^{2**}
C	Soil + PCL3 immobilized on ;			
	Undelignified coir	0.0357±0.0088	20.06±4.97	0.97
	Delignified coir	0.0337±0.0048	20.78±2.96	0.99
	Undelignified banana stem	0.0377±0.0061	18.62±3.00	0.99
F	Autoclaved soil + PCL3 immobilized on ;			
	Undelignified coir	0.0355±0.0056	19.79±3.12	0.98
	Delignified coir	0.0343±0.0081	20.78±4.88	0.99
	Undelignified banana stem	0.0356±0.0077	19.96±4.33	0.97

* Comparison between treatment in column are not significantly different (Duncan, $p \geq 0.05$)
** Coefficients of determination for non-linear regressions

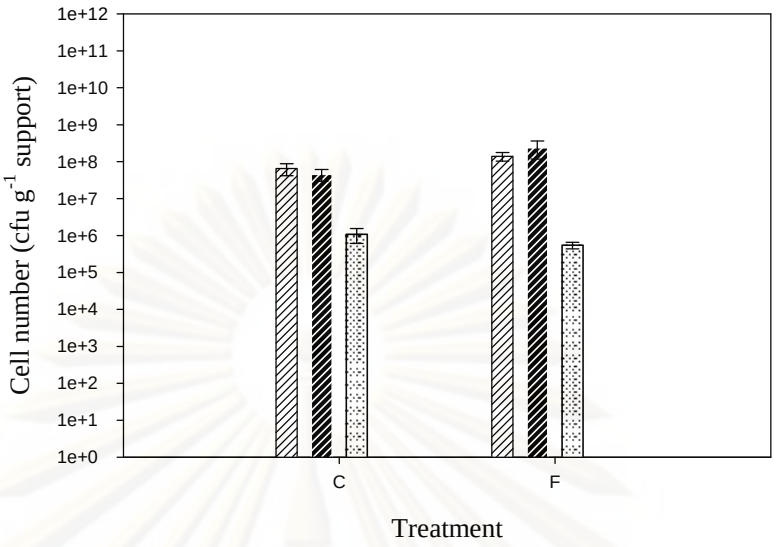

Figure 4.24 Total numbers of carbofuran degraders in undelignified support materials (▨ = coir and ⬚ = banana stem) and delignified support materials (▩ = coir) at day 30 of incubation in reuse experiment. Treatment C = soil + immobilized PCL3; F = Autoclaved soil + immobilized PCL3.

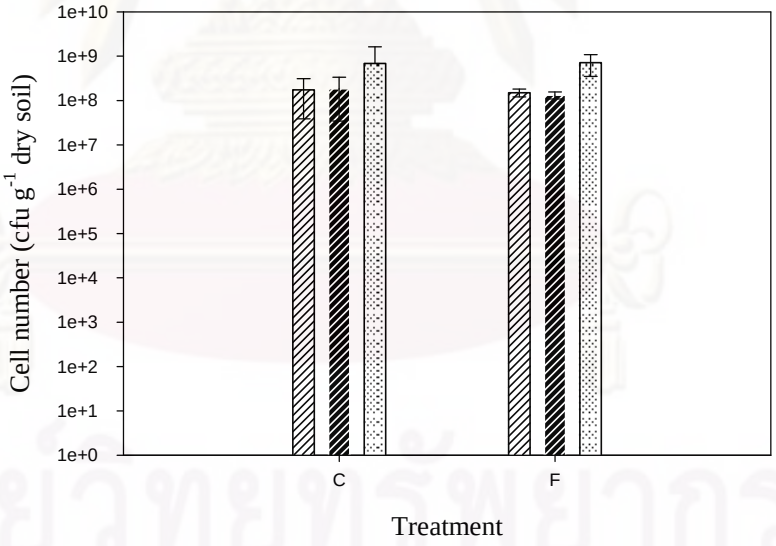

Figure 4.25 Total numbers of carbofuran degraders in soil and autoclaved soil at day 30 of incubation. Treatment C = soil + immobilized PCL3; treatment F = Autoclaved soil + immobilized PCL3. Leakage of cells from undelignified support materials (▨= coir, ⬚ = banana stem) and delignified support materials (▩ = coir) at day 30 of incubation in reuse experiment.

number of immobilized cells decreased to be 10^6 cfu g^{-1} support (Fig. 4.24) after reuse and the number of leaking cells in soil and autoclaved soil increased to be approximately 10^9 cfu g^{-1} dry soil (Fig. 4.25). This might be due to the structure of undelignified banana stem was broken (Fig. 4.26c) after reuse which preventing us from the further reuse.

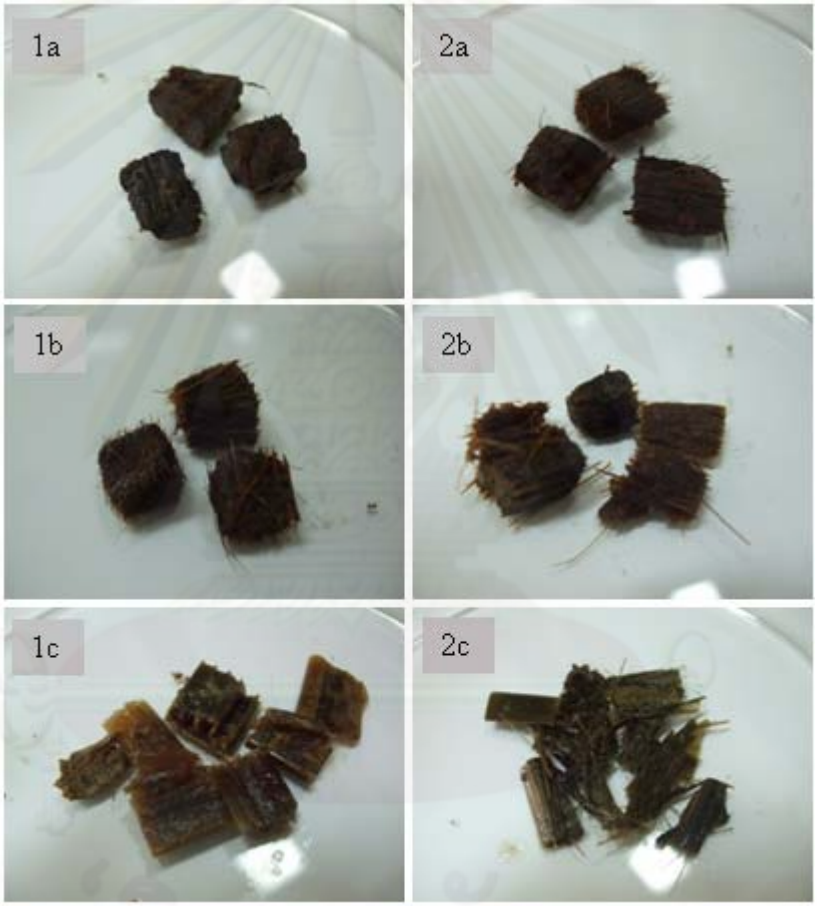

Figure 4.26 Structure of support materials with PCL3 at the end of (1) cycle 1 (2) cycle 2 in undelignified (subscript a = coir, c = banana stem) and delignified (subscript b = coir) support materials when amended in soil.

4.7 Effect of carbofuran concentrations on growth and carbofuran degradation ability of free and the immobilized PCL3 in soil

4.7.1 Growth kinetic

The specific growth rate of carbofuran degraders in soil and autoclaved soil at the various initial carbofuran concentrations of 5-250 mg kg^{-1} dry soil were depicted in Fig. 4.27. In the treatment of soil without inoculation (treatment A) and soil and autoclaved soil inoculated with free cells of PCL3 (treatments B and E), the specific growth rate of carbofuran degraders increased with increase in substrate concentration up to 100 mg kg^{-1} soil (Fig. 4.27a-c). The decline trend of the plot beyond 100 mg kg^{-1} soil indicates that carbofuran is inhibitory type substrate and the inhibition effect of carbofuran becomes predominant above 100 mg kg^{-1}soil. The Luong model was used to model the growth kinetics of the carbofuran degrader in these treatments. The obtained kinetic parameters were shown in Table 4.11. The higher μ_{max} (0.444-0.550 day^{-1}) and S_m (278.90-284.10 mg kg^{-1} soil) could be obtained in the treatments augmented with free cells of PCL3 in comparison to soil without inoculation (μ_{max} = 0.130 day^{-1}, S_m = 248.32 mg kg^{-1} soil) (Table 4.11). The results suggested that PCL3 could grow more rapid and better torelance to carbofuran concentration than indigenous microorganisms in the soil.

The Monod model was used to predict the growth kinetic parameters of carbofuran degraders in the treatment with immobilized PCL3 (treatments C and F) because the inhibitory effect of carbofuran on their growth was not observed. The μ_{max} value of 0.39 day^{-1} (Table 4.11) could be obtained. The smaller K_s values of 16.16-20.08 mg kg^{-1} (Table 4.11) soil could be obtained which indicated that at the low concentration of carbofuran, PCL3 in immobilized form could access and degrade carbofuran in soil more efficient than in free cell form.

4.7.2 Degradation efficiency

The effect of carbofuran concentration on carbofuran degradation efficiency by free cells in comparison to immobilized cells in soil was evaluated. The example of carbofuran degradation profile at the initial carbofuran concentration of 50 mg kg^{-1} soil was shown in Fig. 4.28. 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 soil. In the treatments with only indigenous

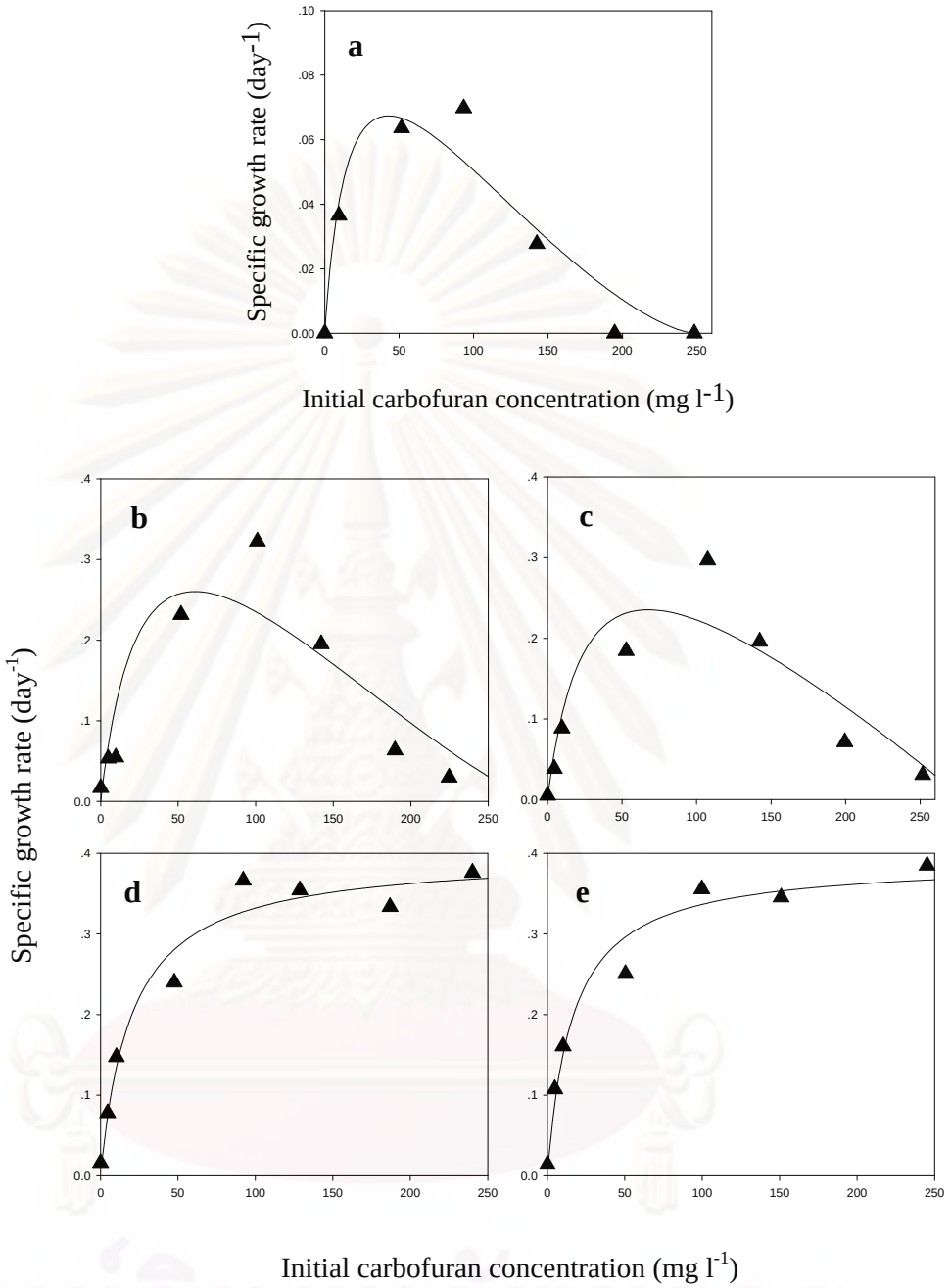

Figure 4.27 Specific growth rate of (a) carbofuran degrader in soil (b) free cells of PCL3 inoculated in soil and (c) autoclaved soil and immobilized PCL3 inoculated in (d) soil and (e) autoclaved soil at different initial carbofuran concentration; lines indicated the specific growth rate in soil fitted to the Luong model and Monod model)

microorganisms (treatments A and D), carbofuran could be effectively degraded at the initial carbofuran concentration up to 50 mg kg^{-1} soil with the $t_{1/2}$ of 47-67 days. The carbofuran degradation efficiency of the indigenous microorganisms was

Table 4.11 Kinetic parameters of carbofuran degrader and PCL3 estimated from nonlinear regression with growth kinetic models

Treatment	Experimental setup	μ_{max} (day^{-1})	K_s (mg kg^{-1})	S_m (mg kg^{-1})	n	r^2
A	Soil	0.130	19.35	248.32	1.48	0.96
B	Soil + free cells of PCL3	0.550	33.24	284.10	1.30	0.90
C	Soil + immobilized PCL3	0.399	20.08	-	-	0.98
E	Autoclaved soil + free cells of PCL3	0.444	30.11	278.90	1.96	0.92
F	Autoclaved soil + immobilized PCL3	0.391	16.16	-	-	0.99

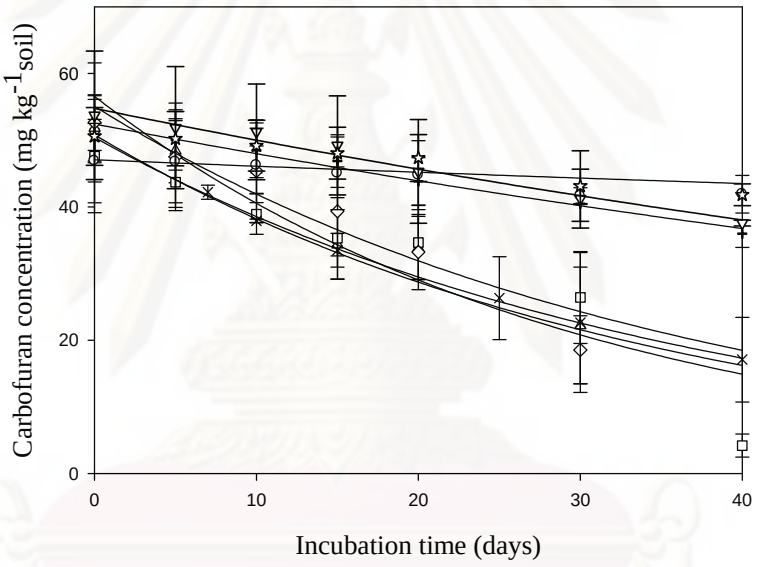

Figure 4.28 Degradation of carbofuran in soil by indigenous microorganisms and PCL3 at carbofuran concentration of 50 mg l^{-1} (+ = treatment A , Δ = treatment B, □ = treatment C, ▽ = treatment D, ◇ = treatment E, x = treatment F, ☆ = treatment G and ○ = treatment H); lines indicated the carbofuran degradation in synthetic wastewater fitted to the modified first-order kinetic model

inhibited at the initial carbofuran concentrations of greater than 50 mg kg^{-1} soil indicated by a longer half-life of carbofuran of 82-543 days (treatments A and D; Table 4.12). A significant shorter $t_{1/2}$ of carbofuran could be found in the treatments augmented with PCL3 in free and immobilized cell forms (treatment B, C, E and F) as compared to the treatments with only indigenous microorganisms in any range of initial carbofuran concentration (Table 4.12). This indicated the effectiveness of PCL3 to improve carbofuran degradation efficiency in soil.

In the treatments with bioaugmentation (treatment B, C, E, F), carbofuran could be effectively degraded by free and immobilized cells at the initial carbofuran concentration of 5-50 mg kg^{-1} soil with the short haft-lives ranged between 15-27 days (Table 4.12). The significant longer $t_{1/2}$ of carbofuran (36-154 days) were obtained when the initial carbofuran concentrations in soil were increased to 100-250 mg kg^{-1} soil (Table 4.12). The results indicated that the activity of PCL3 could be inhibited at the initial carbofuran concentration of 100-250 mg kg^{-1} soil which was in correlation with the growth of carbofuran degrader in soil.

At the initial carbofuran concentrations of 5-150 mg kg^{-1} soil, $t_{1/2}$ of carbofuran obtained from the treatment inoculated with immobilized PCL3 is not significantly different from the treatment with free cells ($p>0.05$) (Table 4.12). The results demonstrated that immobilization did not worsen or improve carbofuran degradation efficiency of PCL3 in soil at the initial carbofuran concentration ranged between 5-100 mg kg^{-1} soil. The enhancement of carbofuran degradation efficiency by immobilization technique was evidenced in the treatments with high concentrations of carbofuran of 200-250 mg kg^{-1} soil in which the $t_{1/2}$ of carbofuran in soil augmented with immobilized PCL3 were significant shorter than in the treatment with free cells (Table 4.12). These results indicated potential of using immobilized PCL3 for bioremediation of soil contaminated with carbofuran at the high concentration

Table 4.12 Half-lives ($t_{1/2}$) of carbofuran in soil

Initial carbofuran Concentration (mg kg^{-1})	Treatment					
	Soil	Soil+free cells	Soil+immobilized cells	Soil+autoclaved support	Autoclaved soil+free cells	Autoclaved soil+immobilized cells
5(*)	53.61aB±3.22	15.26aA±1.58	18.96aA±5.42	47.40aB±2.97	18.08aA±1.66	21.92aA±4.51
10	58.83aB±3.53	17.78aA±2.86	20.47aA±2.17	57.84aB±5.72	19.31aA±1.52	23.71aA±3.74
50	67.22aB±5.97	20.76aA±2.56	24.20abA±1.67	65.94aB±5.75	25.41aA±1.91	26.74abA±3.54
100	86.49abB±8.36	38.98bA±6.13	36.13bcA±6.55	82.61aB±4.17	40.35bA±3.80	36.00bcA±2.64
150	109.47bB±8.53	46.41bA±4.38	39.63cA±1.60	99.84aB±5.08	50.26bA±2.06	40.97cA±2.56
200	396.32cC±16.01	92.81cB±8.75	58.15dA±6.85	413.36bC±30.25	99.08cB±4.00	62.00dA±3.91
250	543.03dC±42.00	137.56dB±9.63	95.38eA±9.24	534.41cC±54.59	154dB±9.70	111.23eA±8.81

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

CHAPTER V

CONCLUSIONS

Four types of agricultural residues i.e., coir, bulrush, banana stem and water hyacinth stem in delignified and undelignified forms were used as support materials for the immobilization of *B. cepacia* PCL3 in carbofuran remediation. Among these support materials, undelignified coir is the most suitable for PCL3 immobilization to be used to remediate carbofuran contaminated in both synthetic wastewater and soil. In synthetic wastewater, the immobilized PCL3 on undelignified coir possessed a high stability of 78.72% and high carbofuran degradation ability with the short $t_{1/2}$ of 3.40 days. In addition, it could be reused at least three times with the remaining of carbofuran degradation ability ($t_{1/2}$ of 2.58-4.41 days) and the structure of undelignified coir was not ruined. The other support materials were degraded and broken overtime, as a result, the immobilized cells on these support materials could be reused for a shorter time as compared to the immobilized cells on undelignified coir. In soil, immobilized PCL3 on undelignified coir gave the high carbofuran degradation efficiency with the short $t_{1/2}$ of 18.96 days. It exhibited a potential to be reused for a longer time, as compared to the immobilized cells on other support materials, with the remaining of the carbofuran degradation, growth and cell survival abilities and good appearance structure.

The effect of carbofuran concentration on growth and degradation ability of free cells and immobilized cells of PCL3 on undelignified coir during the remediation of carbofuran in synthetic wastewater and soil was investigated. In synthetic wastewater, the growth and degradation ability of PCL3 in free cell forms was inhibited by carbofuran concentration of greater than 100 mg l^{-1}. Immobilization technique was able to reduce the inhibitory effect of carbofuran on PCL3 in which the growth and carbofuran degradation ability of the immobilized PCL3 on coir were not worsen at the concentration up to 280 mg l^{-1}.

The growth of free cells in soil was inhibited at the concentration of greater than 100 mg kg^{-1} soil, while the growth of the immobilized cells was not inhibited in any range of carbofuran concentrations from 5 to 250 mg kg^{-1} soil. At the initial carbofuran concentration of 1-150 mg kg^{-1} soil, the carbofuran degradation efficiency of PCL3 in free and immobilized cell forms was not significant different. However, at the high carbofuran concentration of 200-250 mg kg^{-1} soil, the $t_{1/2}$ of carbofuran in soil augmented with immobilized PCL3 were significantly (approximately 1.5 times) shorter than in the treatments with free cells. These results indicated that the immobilized PCL3 on undelignified coir has a great potential to be used for bioremediation of water and soil contaminated with carbofuran at the high concentrations.

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APPENDICES

APPENDIX A

RAW DATA

Table A-1 Equilibrium solution concentration (C_e, mg l^{-1}) and amount of carbofuran sorbed per unit weight of support materials (q_e, mg kg^{-1})

carbofuran	UC*		DC		UB		DB	
conc.	C_e	q_e	C_e	q_e	C_e	q_e	C_e	q_e
0.1	0.0772	0.0005	0.0669	0.0009	0.0865	0.0002	0.084	0.0007
1	1.0305	0.0011	0.8387	0.0017	0.8972	0.0011	1.0057	0.0013
5	4.5219	0.0138	5.1158	0.0069	4.0742	0.0091	4.9394	0.0072
10	9.3933	0.0149	9.9131	0.0146	8.2581	0.0065	11.184	0.0096
20	19.4382	0.0259	20.1422	0.0153	19.1847	0.0174	18.9578	0.0094
carbofuran	UBA		DB		UW			
conc.	C_e	q_e	C_e	q_e	C_e	q_e		
0.1	0.0687	0.0008	0.1102	0.0006	0.0842	0.0002		
1	0.8614	0.0012	1.0165	0.0013	0.914	0.0007		
5	5.1999	0.0048	4.9699	0.0022	4.5095	0.0034		
10	10.0722	0.0064	9.674	0.0029	8.863	0.0039		
20	20.4023	0.0088	19.0751	0.0073	19.6647	0.0112		

* UC = undelignified coir, DC = delignified coir, UB = undelignified bulrush, DB = delignified bulrush, UBA = undelignified banana stem, DBA = delignified banana stem, UW = undelignified water hyacinth stem

Table A-2 Number of PCL3 immobilized on support materials

Support type	Cell conc. (cfu g^{-1})
Undelignified coir	1.45E+08
Delignified coir	2.91E+08
Undelignified bulrush	6.87E+08
Delignified bulrush	1.73E+08
Undelignified banana stem	2.17E+08
Delignified banana stem	1.97E+08
Undelignified water hyacinth stem	6.00E+08

Table A-3 Number of cell observed in synthetic wastewater (WC) and number of cell observed in support material (IMC) for stability test

support type	WC cfu g^{-1}	IMC cfu g^{-1}	stability (%)
Undelignified coir	3.68E+07	1.36E+08	78.69
Delignified coir	7.24E+07	2.52E+08	77.69
Undelignified bulrush	1.20E+08	5.65E+08	82.47
Delignified bulrush	4.96E+07	1.13E+08	69.57
Undelignified banana stem	4.42E+07	1.49E+08	77.14
Delignified banana stem	5.07E+07	1.59E+08	75.86
Undelignified water hyacinth stem	2.78E+08	3.74E+08	57.41

Table A-4 Degradation of carbofuran by PCL3 immobilized on each support material cultured in synthetic wastewater

Time of used	Incubation time (day)	Type of support materials*						
		UC	DC	UB	DB	UBA	DBA	UW
1	0	4.96	4.93	4.93	4.81	4.87	4.36	4.61
	5	2.11	1.79	1.79	2.93	2.36	2.17	2.25
	10	1.47	0.97	0.97	1.76	1.37	1.28	1.48
	15	0.79	0.40	0.40	1.03	0.71	1.33	0.77
2	0	4.65	4.56	4.72		4.61	4.55	
	5	2.67	3.01	3.03		3.26	2.94	
	10	2.51	2.30	2.48	–	2.76	2.56	–
	15	2.02	2.24	2.33		2.62	2.41	
3	0	4.62	4.50	4.75		4.37		
	3	2.22	2.27	2.70		2.15		
	6	2.60	2.16	2.36	–	2.32	–	–
	10	2.15	1.41	2.47		1.88		
	15	0.74	0.45	0.84		0.49		
4	0	4.76	4.75					
	3	2.90	3.12					
	6	2.00	2.25	–	–	–	–	–
	10	1.81	1.56					
	15	0.70	0.98					

* UC = undelignified coir, DC = delignified coir, UB = undelignified bulrush, DB = delignified bulrush, UBA = undelignified banana stem, DBA = delignified banana stem, UW = undelignified water hyacinth stem

Table A-5 Concentrations of carbofuran phenol in synthetic wastewater (mg l^{-1}) cultured with PCL3 immobilized on each support material

Time of used	Incubation time (day)	Type of support materials*						
		UC	DC	UB	DB	UBA	DBA	UW
1	0	0	0	0	0	0	0	0
	5	1.75	0.45	0	0.07	0.76	0.02	0.97
	10	0	0.08	0.84	0	0.42	0.21	0.41
	15	0.11	0	0.09	0	0	0.64	0.20
2	0	0	0	0		0	0	
	5	0.67	0	0.77		0.35	0	
	10	1.12	1.02	0.24	–	0	0.52	–
	15	0	0.3	0.04		0.05	0.34	
3	0	0	0	0		0		
	3	0.42	0.89	0.81		0.92		
	6	0.97	0	0.53	–	0	–	–
	10	0	0.02	0.64		0.59		
	15	0.01	0	0.01		0		
4	0	0	0					
	3	0.98	1.01					
	6	0.42	0	–	–	–	–	–
	10	0.04	0.73					
	15	0	0.04					

* UC = undelignified coir, DC = delignified coir, UB = undelignified bulrush, DB = delignified bulrush, UBA = undelignified banana stem, DBA = delignified banana stem, UW = undelignified water hyacinth stem

Table A-6 Concentrations of 3-keto carbofuran in synthetic wastewater (mg l^{-1}) cultured with PCL3 immobilized on each support material

Time of used	Incubation time (day)	Type of support materials*						
		UC	DC	UB	DB	UBA	DBA	UW
1	0	0	0	0	0	0	0	0
	5	0.02	0.01	0	0.16	0	0.06	0.02
	10	0.08	0	0	0.05	0.12	0	0.70
	15	0	0.02	0.03		0.06	0	0.03
2	0	0	0	0	–	0	0	–
	5	0.01	0.03	0.04		0.02	0.12	
	10	0.03	0	0.06		0	0.04	
	15	0.01	0.02	0.02		0.07	0.01	
3	0	0	0	0	–	0	–	–
	3	0.07	0.07	0.09		0.04		
	6	0	0.01	0.27		0.06		
	10	0.08	0.02	0.03		0.06		
	15	0.04	0	0.07		0.03		
4	0	0	0	–	–	–	–	–
	3	0.05	0.07					
	6	0.03	0					
	10	0.03	0.8					
	15	0.05	0.06					

* UC = undelignified coir, DC = delignified coir, UB = undelignified bulrush, DB = delignified bulrush, UBA = undelignified banana stem, DBA = delignified banana stem, UW = undelignified water hyacinth stem

Table A-7 Number of *B. cepacia* PCL3 leaked from support materials (cfu ml^{-1}) during incubation in synthetic wastewater

Time of used	Incubation time (day)	Type of support materials*						
		UC	DC	UB	DB	UBA	DBA	UW
1	0	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00
	3	2.25E+05	2.25E+05	2.43E+06	5.25E+05	6.50E+05	1.33E+06	3.75E+06
	5	5.00E+04	3.25E+05	4.75E+06	1.20E+07	1.58E+06	1.30E+06	4.50E+06
	10	8.75E+04	5.25E+05	4.25E+05	3.00E+06	1.90E+06	8.00E+05	5.75E+06
	15	9.50E+05	9.50E+05	3.00E+05	3.50E+06	3.50E+06	1.43E+06	1.50E+07
2	0	1.00E+00	1.00E+00	1.00E+00		1.00E+00	1.00E+00	
	3	1.65E+06	1.50E+06	2.08E+06		1.35E+06	2.50E+06	
	5	2.05E+06	1.83E+06	2.60E+06	–	1.63E+06	2.98E+06	–
	10	2.20E+06	1.85E+06	2.90E+06		1.78E+06	3.33E+06	
	15	4.25E+06	2.08E+06	3.50E+06		1.68E+06	4.00E+06	
3	0	1.00E+00	1.00E+00	1.00E+00		1.00E+00		
	3	4.50E+05	3.00E+05	6.00E+05		1.45E+06		
	6	9.00E+05	3.50E+05	1.23E+06	–	1.75E+06	–	–
	10	1.08E+06	4.50E+05	1.05E+06		2.15E+06		
	15	1.35E+06	6.50E+05	1.05E+06		2.48E+06		
4	0	1.00E+00	1.00E+00					
	3	1.25E+05	1.15E+05					
	6	3.25E+05	2.25E+05	–	–	–	–	–
	10	7.50E+05	5.00E+05					
	15	6.75E+05	9.25E+05					

* UC = undelignified coir, DC = delignified coir, UB = undelignified bulrush, DB = delignified bulrush, UBA = undelignified banana stem, DBA = delignified banana stem, UW = undelignified water hyacinth stem

Table A-8 Number of *B. cepacia* PCL3 immobilized on support materials (cfu g^{-1}) during incubation in synthetic wastewater

Time of used	Incubation time (day)	Type of support materials*						
		UC	DC	UB	DB	UBA	DBA	UW
1	0	1.93E+08	2.84E+08	7.12E+08	2.46E+08	2.05E+08	1.83E+08	7.08E+08
	5	2.27E+08	3.65E+08	9.73E+08	7.25E+08	2.86E+08	3.29E+08	8.87E+08
	10	4.58E+08	3.40E+08	1.35E+09	7.83E+08	2.89E+08	2.58E+08	8.85E+09
	15	5.28E+08	3.63E+08	1.20E+09	9.76E+08	2.91E+08	2.97E+08	7.84E+09
2	0	5.28E+08	3.63E+08	1.20E+09	–	2.91E+08	2.97E+08	–
	5	3.55E+08	3.54E+08	8.41E+08		2.93E+08	1.54E+08	
	10	3.98E+08	3.96E+08	5.42E+08		1.55E+08	5.60E+07	
	15	3.97E+08	3.58E+08	8.05E+08		9.44E+07	2.90E+07	
3	0	3.97E+08	3.58E+08	8.05E+08	–	9.44E+07	–	–
	6	2.00E+08	2.90E+08	5.70E+08		1.02E+08		
	10	7.59E+07	2.23E+08	2.83E+08		7.41E+07		
	15	6.37E+07	1.56E+08	9.69E+07		7.73E+07		
4	0	6.37E+07	1.56E+08	–	–	–	–	–
	6	9.29E+07	6.66E+07					
	10	7.75E+07	2.03E+07					
	15	4.08E+07	1.57E+07					

* UC = undelignified coir, DC = delignified coir, UB = undelignified bulrush, DB = delignified bulrush, UBA = undelignified banana stem, DBA = delignified banana stem, UW = undelignified water hyacinth stem

Table A-9 Dissipation of carbofuran in synthetic wastewater with sterile support materials

Incubation time (day)	Carbofuran concentration (mg l^{-1})						
	Type of support materials*						
	UC	DC	UB	DB	UBA	DBA	UW
0	4.85	4.81	4.63	4.53	4.35	4.34	4.60
6	4.75	4.41	4.46	4.09	4.07	4.18	4.48
10	4.42	3.70	4.02	3.48	3.87	3.73	4.19
15	3.94	3.45	3.76	3.19	3.63	3.52	3.76

* UC = undelignified coir, DC = delignified coir, UB = undelignified bulrush, DB = delignified bulrush, UBA = undelignified banana stem, DBA = delignified banana stem, UW = undelignified water hyacinth stem

Table A-10 Growth of *B. cepacia* PCL3 in free cell form during incubation in synthetic wastewater at different initial carbofuran concentration

carbofuran Conc. (mg l^{-1})	Time (day)	cell number (cfu ml^{-1})	x/x_0	ln (x/x_0)	carbofuran Conc. (mg l^{-1})	day	cell number (cfu ml^{-1})	x/x_0	ln (x/x_0)
0	0	8.00E+04	1	0	100	0	6.25E+04	1	0
	1	9.25E+04	1.16	0.15		2	7.75E+04	1.24	0.22
	2	1.00E+05	1.25	0.22		4	8.00E+05	12.8	2.55
	4	1.03E+05	1.28	0.25		7	4.00E+06	64	4.16
	9	1.43E+05	1.78	0.58		9	2.23E+07	356	5.87
	17	1.38E+05	1.72	0.54		17	6.25E+07	1000	6.91
	26	8.75E+04	1.09	0.09		25	4.25E+07	680	6.52
	35	7.00E+04	0.88	-0.13		30	1.33E+06	21.2	3.05
5	0	5.25E+04	1	0		35	5.75E+05	9.2	2.22
	2	1.30E+05	2.48	0.91	150	0	7.25E+04	1	0
	5	1.90E+05	3.62	1.29		2	5.75E+04	0.79	-0.23
	9	5.25E+05	10	2.3		4	7.00E+04	0.97	-0.04
	17	9.00E+05	17.14	2.84		9	9.00E+05	12.41	2.52
	25	3.50E+05	6.67	1.9		12	3.50E+06	48.28	3.88
	35	2.50E+05	4.76	1.56		17	6.00E+06	82.76	4.42
10	0	6.75E+04	1	0		20	5.50E+06	75.86	4.33
	2	1.73E+05	2.56	0.94		25	6.25E+06	86.21	4.46
	4	5.00E+05	7.41	2		35	4.75E+06	65.52	4.18
	9	1.05E+06	15.56	2.74		48	4.75E+06	65.52	4.18
	17	3.25E+06	48.15	3.87	200	0	5.00E+04	1	0
	25	3.00E+06	44.44	3.79		3	9.00E+04	1.8	0.59
	35	3.25E+06	48.15	3.87		6	2.25E+05	4.5	1.5
50	0	6.25E+04	1	0		12	6.25E+05	12.5	2.53
	1	3.50E+05	5.6	1.72		19	7.25E+05	14.5	2.67
	2	6.50E+05	10.4	2.34		25	8.75E+05	17.5	2.86
	4	3.75E+06	60	4.09		30	6.00E+05	12	2.48
	9	2.00E+07	320	5.77		35	4.75E+05	9.5	2.25
	15	5.00E+07	800	6.68		50	1.75E+05	3.5	1.25
	17	4.75E+07	760	6.63	250	0	5.50E+04	1	0
	25	2.88E+07	460	6.13		3	6.75E+04	1.23	0.2
	35	2.30E+07	368	5.91		6	6.00E+04	1.09	0.09
						19	7.75E+04	1.41	0.34
						25	4.00E+04	0.73	-0.32
						30	3.25E+04	0.59	-0.53
						35	2.25E+04	0.41	-0.89
						50	8.75E+03	0.16	-1.84

Table A-11 Growth of immobilized *B. cepacia* PCL3 during incubation in synthetic wastewater at different initial carbofuran concentration

carbofuran concentration ($mg\ l^{-1}$)	day	leaked cell ($cfu\ ml^{-1}$)	immobilized cell ($cfu\ g^{-1}$)	total cell ($cfu\ ml^{-1}$)	x/x_0	$\ln (x/x_0)$
0	0	1.00E+00	3.04E+06	3.04E+06	1.00	0.00
	3	1.48E+05	3.02E+06	3.16E+06	1.04	0.04
	6	1.55E+05	3.88E+06	4.04E+06	1.33	0.29
	10	1.53E+05	3.73E+06	3.89E+06	1.28	0.25
	15	1.18E+05	3.94E+06	4.05E+06	1.34	0.29
5	0	1.00E+00	1.31E+06	1.31E+06	1.00	0.00
	5	5.00E+04	1.55E+06	1.60E+06	1.22	0.20
	10	8.75E+04	3.12E+06	3.20E+06	2.44	0.89
	15	9.50E+05	3.59E+06	4.54E+06	3.46	1.24
10	0	1.00E+00	2.55E+06	2.55E+06	1.00	0.00
	3	1.08E+06	5.99E+06	7.06E+06	2.77	1.02
	6	2.18E+06	8.09E+06	1.03E+07	4.03	1.39
	10	2.40E+06	1.07E+07	1.31E+07	5.12	1.63
	15	5.75E+06	7.80E+06	1.36E+07	5.32	1.67
50	0	1.00E+00	1.38E+06	1.38E+06	1.00	0.00
	3	1.05E+07	4.88E+06	1.54E+07	11.13	2.41
	6	2.03E+07	7.74E+06	2.80E+07	20.25	3.01
	10	8.25E+07	7.95E+06	9.04E+07	65.44	4.18
	15	8.75E+07	1.24E+07	9.99E+07	72.29	4.28
120	0	1.00E+00	1.16E+06	1.16E+06	1.00	0.00
	3	1.35E+07	5.26E+06	1.88E+07	16.17	2.78
	6	4.25E+07	9.98E+06	5.25E+07	45.22	3.81
	10	1.90E+08	8.91E+06	1.99E+08	171.41	5.14
	15	1.95E+08	8.94E+06	2.04E+08	175.74	5.17
	20	1.95E+08	1.15E+07	2.06E+08	177.94	5.18
	30	1.48E+08	5.97E+06	1.53E+08	132.25	4.88
200	0	1.00E+00	9.79E+05	9.79E+05	1.00	0.00
	3	6.00E+06	3.77E+06	9.77E+06	9.99	2.30
	6	8.50E+06	7.33E+06	1.58E+07	16.17	2.78
	10	2.50E+07	7.16E+06	3.22E+07	32.86	3.49
	15	4.50E+07	6.61E+06	5.16E+07	52.74	3.97
	20	5.00E+07	6.44E+06	5.64E+07	57.68	4.05
	30	3.50E+07	4.29E+06	3.93E+07	40.16	3.69
280	0	1.00E+00	1.28E+06	1.28E+06	1.00	0.00
	3	5.50E+06	6.72E+06	1.22E+07	9.57	2.26
	6	7.50E+06	1.18E+07	1.93E+07	15.14	2.72
	10	9.75E+06	9.77E+06	1.95E+07	15.29	2.73
	15	9.25E+06	7.52E+06	1.68E+07	13.14	2.58
	20	7.75E+06	5.36E+06	1.31E+07	10.27	2.33
	30	9.75E+06	6.13E+06	1.59E+07	12.44	2.52

Table A-12 Degradation of carbofuran in soils (mg kg^{-1})

Time (day)	Treatment*			
	A	B	E	H
0	4.68	4.93	4.62	4.55
5	4.53	-	-	-
7	-	3.58	3.18	4.44
12	4.23	2.63	2.68	4.36
20	3.72	2.44	2.56	4.09
30	3.15	1.5	1.16	3.95

*Treatment A = soil; treatment B = soil + free cells of PCL3; treatment E = autoclaved soil + free cells of PCL3; treatment H = autoclaved soil

Table A-13 Concentrations of 3-keto carbofuran and carbofuran phenol in soil

Time (day)	Treatment*					
	3-keto carbofuran (mg l^{-1})			carbofuran phenol (mg l^{-1})		
	A	B	E	A	B	E
0	0	0	0	0	0	0
5	0.01	-	-	0	-	-
7	-	0	0.01	-	0.21	0.13
12	0	0.02	0.02	0.18	1.79	2.05
20	0.03	0.01	0.04	0.07	1.07	0.96
30	0	0	0	0.32	0	0

*Treatment A = soil; treatment B = soil + free cells of PCL3 and treatment E = autoclaved soil + free cells of PCL3

Table A-14 Degradation of carbofuran in soils

Treatment*	day	Support materials**						
		UC	DC	UB	DB	UBA	DBA	UW
C	0	4.63	4.65	4.72	4.51	4.71	4.53	4.67
	5	3.94	4.19	3.99	3.91	4.01	3.95	4.25
	10	3.69	3.38	3.49	3.57	3.57	3.45	3.68
	15	-	-	2.95	-	-	-	-
	20	2.32	2.69	2.54	2.77	3.00	2.58	3.18
	30	2.05	1.93	1.68	2.73	2.33	2.01	2.79
D	0	4.77	4.69	4.80	4.96	4.77	4.56	4.69
	10	4.53	4.57	4.64	4.51	4.45	4.14	4.21
	15	3.85	4.28	4.26	4.07	3.97	3.63	3.80
	20	3.71	3.35	3.64	3.81	3.60	3.31	3.52
	30	3.05	3.07	2.97	3.31	3.08	2.95	3.07
F	0	4.75	4.63	4.27	4.42	4.73	4.56	4.55
	5	4.42	3.98	3.81	3.82	4.30	4.19	4.01
	10	3.55	3.55	3.50	3.25	-	4.02	3.82
	15	3.27	3.46	3.32	2.93	3.50	3.78	3.40
	20	2.71	2.68	2.82	2.63	3.18	3.72	3.03
	30	2.12	2.32	2.61	2.03	2.84	3.39	2.73
G	0	4.21	4.41	4.27	4.39	4.37	4.41	4.35
	5	4.07	4.30	4.12	4.19	4.27	4.30	4.15
	15	3.80	4.09	3.96	3.97	3.94	4.05	3.99
	20	3.67	3.84	3.66	3.83	3.82	3.90	3.83
	30	3.38	3.51	3.49	3.48	3.53	3.59	3.47

*Treatment C = soil + immobilized PCL3; treatment D = soil + autoclaved support material; treatment F = Autoclaved soil + immobilized PCL3 and treatment G = autoclaved soil + autoclaved support material

** UC = undelignified coir, DC = delignified coir, UB = undelignified bulrush, DB = delignified bulrush, UBA = undelignified banana stem, DBA = delignified banana stem, UW = undelignified water hyacinth stem

Table A-15 Concentrations of carbofuran phenol in soil

Treatment*	day	Support materials**						
		UC	DC	UB	DB	UBA	DBA	UW
C	0	0	0	0	0	0	0	0
	5	0.02	0.72	0	0.52	0	0.54	0
	10	0.2	0	0.42	0.03	0.82	0.98	1.24
	15	-	-		-	-	-	-
	20	1.43	1.34	0.73	1.45	1.00	1.37	0
	30	1.27	1.01	0	1.21	1.13	0.97	1.08
D	0	0	0	0	0	0	0	0
	10	0.18	0.04	0.08	0	0.02	0.10	0
	15	0.07	0	0.12	0.28	0	0	0.21
	20	0.34	0	0.38	0.15	0	0.21	0
	30	0.25	0.27	0	0.11	0.24	0.10	0.08
F	0	0	0	0	0	0	0	0
	5	0	0.15	0	0.05	0.01	0.04	0.03
	10	0.42	0.34	1.26	0.26	-	0	0.02
	15	0.97	0	0.43	1.13	0.28	0	1.03
	20	1.05	0.27	1.52	1.36	0.33	0.37	1.01
	30	1.18	0.18	0.64	0.31	0.11	0.26	1.05

*Treatment C = soil + immobilized PCL3; treatment D = soil + autoclaved support material and treatment F = Autoclaved soil + immobilized PCL3

** UC = undelignified coir, DC = delignified coir, UB = undelignified bulrush, DB = delignified bulrush, UBA = undelignified banana stem, DBA = delignified banana stem, UW = undelignified water hyacinth stem

Table A-16 Concentrations of 3-keto carbofuran in soil (mg kg^{-1})

Treatment*	day	Support materials**						
		UC	DC	UB	DB	UBA	DBA	UW
C	0	0	0	0	0	0	0	0
	5	0	0	0.01	0	0	0	0.01
	10	0.02	0.01	0.01	0	0.02	0.02	0.01
	15	-	-	0.02	-	-	-	-
	20	0.04	0.02	0	0.03	0.02	0.01	0
	30	0.04	0.03	0	0.02	0.01	0.02	0
D	0	0	0	0	0	0	0	0
	10	0	0	0.02	0	0.01	0.02	0.01
	15	0.02	0.01	0.01	0.02	0	0.01	0.02
	20	0	0.02	0	0.02	0	0	0
	30	0.03	0.02	0	0.01	0.02	0	0.01
F	0	0	0	0	0	0	0	0
	5	0	0.01	0	0	0	0.01	0
	10	0.03	0	0.02	0.02	-	0.02	0.01
	15	0.02	0	0.01	0.02	0.01	0	0
	20	0.02	0.02	0.03	0.02	0.03	0	0.02
	30	0.02	0.03	0	0.03	0.01	0.01	0.02

*Treatment C = soil + immobilized PCL3; treatment D = soil + autoclaved support material and treatment F = Autoclaved soil + immobilized PCL3

** UC = undelignified coir, DC = delignified coir, UB = undelignified bulrush, DB = delignified bulrush, UBA = undelignified banana stem, DBA = delignified banana stem, UW = undelignified water hyacinth stem

Table A-17 Degradation of carbofuran in soils in reused experiment

Treatment*	day	Support materials**			
		UC	DC	UB	UBA
C	0	5.03	4.70	4.29	4.54
	5	4.31	4.02	4.01	4.20
	15	4.03	3.42	3.50	3.62
	20	3.51	2.90	3.22	3.28
	30	3.16	2.47	3.03	3.04
D	0	4.41	4.45	4.31	4.10
	5	4.17	4.18	4.11	3.97
	15	3.88	3.84	3.86	3.49
	20	3.60	3.56	3.49	3.28
	30	3.36	3.15	3.34	3.00
F	0	4.68	4.64	4.71	4.64
	5	3.92	4.45	4.47	3.92
	15	3.53	3.94	4.01	3.62
	20	2.95	3.67	3.64	3.30
	30	2.58	3.52	3.52	2.81
G	0	4.62	2.31	4.52	4.53
	5	4.46	4.73	4.37	4.36
	15	4.17	9.59	4.25	4.17
	20	4.13	12.06	4.07	4.01
	30	3.82	16.91	3.93	3.73

*Treatment C = soil + immobilized PCL3; treatment D = soil + autoclaved support material; treatment F = Autoclaved soil + immobilized PCL3 and treatment G = autoclaved soil + autoclaved support material

** UC = undelignified coir, DC = delignified coir, UB = undelignified bulrush UBA = undelignified banana stem

Table A-18 Number of cells leaked into soil during incubation in soil

Treat-ment*	day	Support materials**						
		UC	DC	UB	DB	UBA	DBA	UW
C	0	2.67E+04	1.74E+04	1.16E+04	3.20E+04	1.46E+04	1.76E+04	2.64E+04
	5	3.22E+04	3.70E+04	1.98E+04	3.48E+04	2.59E+04	3.15E+04	3.77E+04
	10	4.22E+05	2.54E+05	5.08E+05	5.41E+05	3.98E+05	5.16E+05	4.85E+05
	15	4.68E+06	9.34E+05	3.21E+06	1.22E+06	1.23E+06	1.55E+06	1.60E+06
	20	1.01E+07	6.63E+06	9.57E+06	1.09E+07	6.05E+06	3.69E+06	1.26E+07
	30	1.48E+07	1.22E+07	1.59E+07	1.95E+07	1.06E+07	7.37E+06	1.23E+07
D	0	2.37E+03	1.45E+03	1.74E+03	1.16E+03	1.75E+03	1.17E+03	1.46E+03
	5	2.63E+03	2.00E+03	1.99E+03	1.74E+03	2.59E+03	1.43E+03	2.03E+03
	10	2.81E+03	4.78E+03	3.68E+03	2.56E+03	2.55E+03	2.01E+03	2.57E+03
	15	3.80E+03	5.84E+03	7.01E+03	5.25E+03	4.38E+03	2.88E+03	4.66E+03
	20	6.05E+03	8.65E+03	8.99E+03	5.75E+03	5.19E+03	3.41E+03	4.86E+03
	30	9.65E+03	9.60E+03	1.02E+04	6.52E+03	5.70E+03	4.25E+03	5.71E+03
F	0	2.37E+04	2.04E+04	2.03E+04	3.49E+04	2.92E+04	3.22E+04	3.52E+04
	5	3.52E+04	3.14E+04	3.41E+04	4.93E+04	4.31E+04	4.01E+04	4.93E+04
	10	3.93E+05	5.91E+05	5.65E+05	6.83E+05	5.38E+05	4.59E+05	6.00E+05
	15	6.43E+06	2.30E+06	5.26E+06	1.57E+06	1.61E+06	7.48E+05	1.75E+06
	20	1.38E+07	1.10E+07	1.13E+07	1.87E+07	6.05E+06	1.53E+07	1.89E+07
	30	1.47E+07	1.64E+07	1.96E+07	1.58E+07	1.05E+07	1.64E+07	1.97E+07

*Treatment C = soil + immobilized PCL3; treatment D = soil + autoclaved support material and treatment F = Autoclaved soil + immobilized PCL3

** UC = undelignified coir, DC = delignified coir, UB = undelignified bulrush, DB = delignified bulrush, UBA = undelignified banana stem, DBA = delignified banana stem, UW = undelignified water hyacinth stem

Table A-19 Number of cells leaked into soil in reused experiment

Treatment*	day	Support materials**		
		UC	DC	UBA
C	0	2.95E+04	3.23E+04	3.52E+04
	5	6.13E+04	5.72E+04	4.67E+04
	10	8.70E+05	3.73E+05	8.67E+05
	15	4.37E+06	1.27E+07	1.38E+08
	20	1.29E+08	7.97E+07	4.28E+07
	30	1.36E+08	1.49E+08	9.45E+08
D	0	2.36E+03	3.83E+03	
	5	2.93E+03	4.01E+03	
	10	4.06E+03	6.65E+03	
	15	5.26E+03	3.75E+03	
	20	3.16E+03	8.83E+03	
	30	5.69E+03	5.75E+03	
F	0	3.53E+04	3.54E+04	3.22E+04
	5	5.84E+04	6.58E+04	2.92E+05
	10	6.09E+05	3.43E+06	8.75E+05
	15	1.52E+07	1.21E+07	1.32E+08
	20	1.17E+08	1.37E+08	8.85E+08
	30	1.51E+08	1.32E+08	7.16E+08

*Treatment C = soil + immobilized PCL3; treatment D = soil + autoclaved support material and treatment F = Autoclaved soil + immobilized PCL3

** UC = undelignified coir, DC = delignified coir and UBA = undelignified banana stem

Table A-20 Number of *B. cepacia* PCL3 immobilized on support materials (cfu g^{-1}) during incubation in soil

	Treat-ment*	day	Support materials**						
			UC	DC	UB	DB	UBA	DBA	UW
1st use	C	0	1.85E+08	2.01E+08	5.29E+08	1.71E+08	2.05E+08	1.72E+08	5.57E+08
		5	3.00E+08	2.20E+08	5.98E+08	2.76E+08	3.15E+08	2.19E+08	6.95E+08
		10	2.18E+08	4.59E+08	8.36E+08	5.62E+08	3.92E+08	2.87E+08	1.31E+09
		15	4.62E+08	6.36E+08	1.82E+09	9.15E+08	3.07E+08	3.21E+08	4.50E+09
		20	5.57E+08	5.18E+08	1.28E+09	7.94E+08	3.77E+08	2.08E+08	5.30E+09
		30	2.84E+08	6.31E+08	1.67E+09	8.79E+08	4.93E+08	2.17E+08	2.62E+09
	F	0	1.68E+08	3.07E+08	5.90E+08	1.80E+08	3.13E+08	2.05E+08	5.36E+08
		5	2.28E+08	3.91E+08	5.99E+08	1.64E+08	2.38E+08	3.00E+08	6.85E+08
		10	2.92E+08	6.62E+08	8.08E+08	2.14E+08	3.38E+08	4.09E+08	8.41E+08
		15	3.27E+08	8.41E+08	6.92E+08	2.02E+08	5.37E+08	4.08E+08	1.43E+09
		20	5.70E+08	7.59E+08	1.17E+09	3.26E+08	7.43E+08	5.43E+08	1.63E+09
		30	6.73E+08	7.02E+08	1.38E+09	5.42E+08	6.56E+08	4.68E+08	1.85E+09
2nd use	C	0	2.84E+08	6.31E+08	-	-	4.93E+08	-	-
		5	1.65E+08	2.16E+08			2.26E+07		
		10	1.79E+08	9.34E+07			8.41E+06		
		15	9.84E+07	6.44E+07			1.34E+07		
		20	1.40E+08	1.23E+08			4.57E+06		
		30	6.49E+07	4.55E+07			1.08E+06		
	F	0	6.73E+08	7.02E+08	-	-	6.32E+08	-	-
		5	1.13E+09	5.15E+08			7.18E+07		
		10	7.50E+07	1.50E+08			1.38E+08		
		15	1.41E+08	1.34E+09			2.43E+06		
		20	5.07E+08	9.46E+07			1.05E+06		
		30	1.40E+08	2.39E+08			5.48E+05		

*Treatment C = soil + immobilized PCL3 and treatment F = Autoclaved soil + immobilized PCL3

** UC = undelignified coir, DC = delignified coir, UB = undelignified bulrush, DB = delignified bulrush, UBA = undelignified banana stem, DBA = delignified banana stem, UW = undelignified water hyacinth stem

Table A-21 Degradation of carbofuran in soils at different initial carbofuran concentration

carbofuran conc. ($mg\ l^{-1}$)	Time (day)	Carbofuran concentration ($mg\ l^{-1}$) Soil treatment*							
		A	B	C	D	E	F	G	H
5	0	4.68	4.93	4.63	4.77	4.62	4.75	4.21	4.55
	5	4.53	3.58	3.94	4.53	3.18	4.42	4.07	4.44
	12	4.23	2.63	3.69	3.85	2.68	3.27	3.80	4.36
	20	3.72	2.44	2.32	3.71	2.56	2.71	3.67	4.09
	30	3.15	1.50	2.05	3.05	1.16	2.12	3.38	3.95
10	0	9.62	9.82	10.19	8.07	9.84	10.11	10.07	10.56
	5	9.22	8.41	8.65	7.94	7.98	8.70	10.04	10.47
	10	8.99	7.17	7.62	7.62	6.95	7.73	9.89	10.33
	15	8.13	6.08	7.29	6.85	6.03	7.04	9.67	10.03
	20	8.04	4.90	5.45	6.20	5.02	6.29	9.58	9.85
	30	6.98	3.04	2.74	5.74	3.88	4.57	8.74	9.69
	40	6.16	2.72	-	5.09	2.34	3.56	8.37	9.32
50	0	51.63	51.83	47.61	53.71	52.65	50.48	50.57	46.86
	5	50.02	48.40	43.70	51.87	47.49	42.20	50.22	46.76
	10	48.47	45.56	38.89	51.25	45.35	37.88	49.17	46.21
	15	46.87	34.34	35.28	49.21	39.32	33.49	48.04	45.03
	20	43.81	33.85	34.64	45.30	33.21	26.30	47.32	44.74
	30	40.46	22.22	26.40	41.24	18.53	22.66	43.11	41.59
	40	35.97	2.28	4.18	37.56	16.84	17.08	41.80	41.89
100	0	93.35	101.16	92.21	103.60	107.39	99.83	98.67	105.73
	5	92.45	94.94	84.98	102.62	102.85	91.59	97.76	104.74
	10	89.53	88.15	75.32	99.29	-	84.95	96.90	103.81
	15	85.88	82.84	70.84	95.25	89.83	76.79	95.36	102.13
	20	82.63	80.31	52.57	89.64	80.56	68.25	95.17	101.94
	30	77.88	67.52	38.43	84.67	65.63	57.39	94.09	100.77
	40	73.13	59.20	18.45	80.53	58.89	48.68	91.61	98.06
150	0	142.51	142.33	128.70	144.21	142.36	150.91	148.56	-
	5	137.50	139.89	122.52	143.48	139.95	144.80	147.98	
	10	128.37	135.62	104.33	137.70	134.11	133.45	147.33	
	15	124.53	125.32	90.96	133.04	121.15	121.55	146.18	
	20	112.45	120.28	69.06	130.84	113.57	114.29	145.80	
	30	104.10	110.23	60.94	120.07	100.95	102.54	144.31	
	40	90.91	101.44	38.34	109.57	89.65	88.75	142.97	
200	0	194.74	189.89	186.98	201.56	199.49	193.49	196.94	212.85
	5	193.04	185.87	181.30	200.44	193.77	188.42	195.44	212.03
	10	188.91	184.20	160.38	198.49	190.47	181.86	194.46	209.55
	15	185.00	176.67	147.45	190.74	188.00	175.15	192.57	207.47
	20	179.27	168.90	130.91	186.22	182.33	161.49	191.46	206.67
	30	172.71	159.53	106.06	177.38	172.61	139.34	188.21	204.39
	40	166.29	149.66	89.54	172.59	163.87	119.83	185.93	200.23
250	0	248.30	224.72	240.16	258.28	251.71	245.18	225.10	255.57
	5	245.82	223.16	238.35	253.46	249.17	240.59	224.68	254.32
	10	244.48	218.50	230.22	252.09	247.09	236.78	224.32	253.67
	15	233.16	213.50	218.87	242.30	244.38	223.31	222.38	251.30
	20	228.08	210.30	206.23	238.94	241.61	220.62	222.17	250.86
	30	224.94	201.64	195.74	234.70	235.26	207.51	220.24	247.71
	35	218.14	-	-	228.83	-	-	-	-
	40	212.41	192.77	167.02	224.51	229.67	187.26	218.97	245.62

*****Treatment A = soil; treatment B = soil + free cells of PCL3; treatment C = soil + immobilized PCL3; treatment D = soil + autoclaved support material; treatment E = autoclaved soil + free cells of PCL3; treatment F = Autoclaved soil + immobilized PCL3; treatment G = autoclaved soil + autoclaved support material and treatment H = autoclaved soil

APPENDIX B

NUMBER OF CARBOFURAN DEGRADER IN SYNTHETIC WASTEWATER AND SOIL

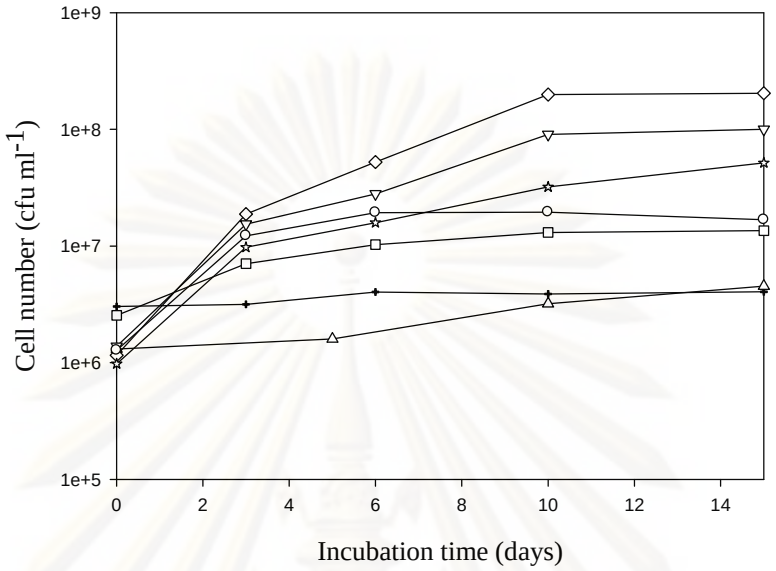

Figure B-1 Number of immobilized *B. cepacia* PCL3 plus leaking cell in synthetic wastewater with different initial carbofuran concentration**;** (+ = 0 mg l^{-1}, △ = 5 mg l^{-1}, □ = 10 mg l^{-1}, ▽ = 50 mg l^{-1}, ◇ = 120 mg l^{-1}, ☆ = 200 mg l^{-1} and ○= 280 mg l^{-1})

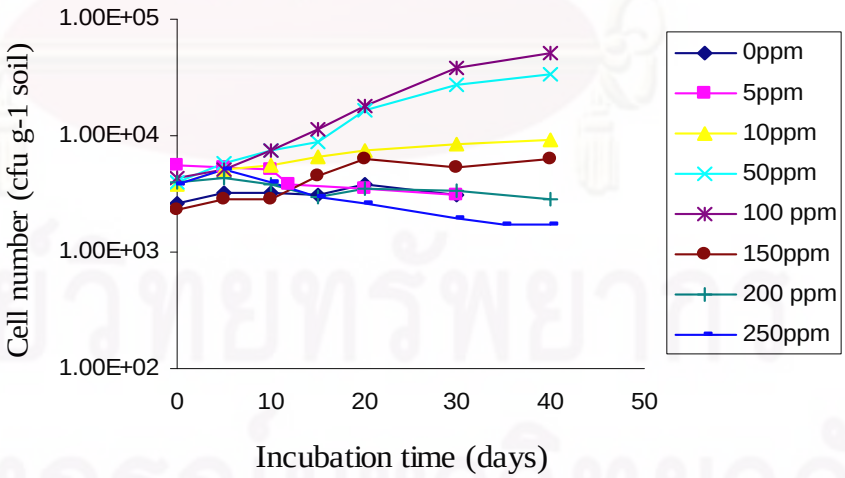

Figure B-2 Growth of indigenous microorganism incubation in soil at different initial carbofuran concentration (treatment A)

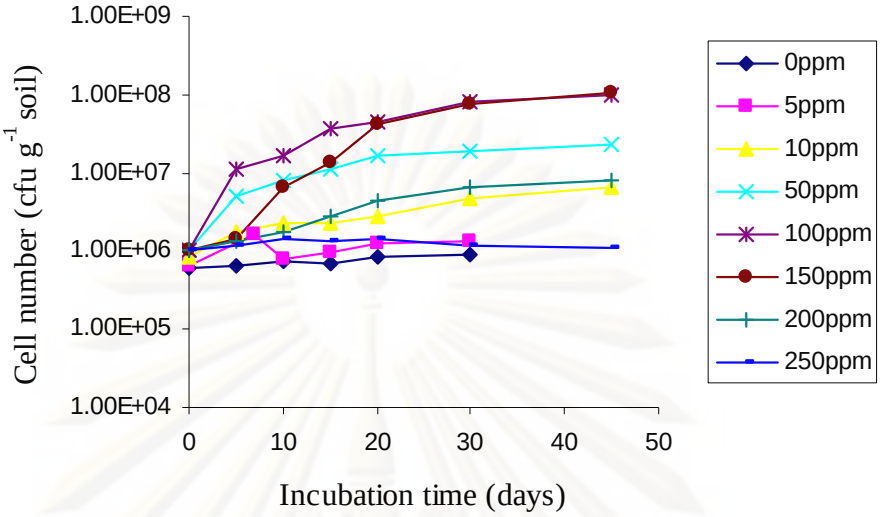

Figure B-3 Growth of carbofuran degrader in soil augmented with PCL3 in free cell form at different initial carbofuran concentration (treatment B)

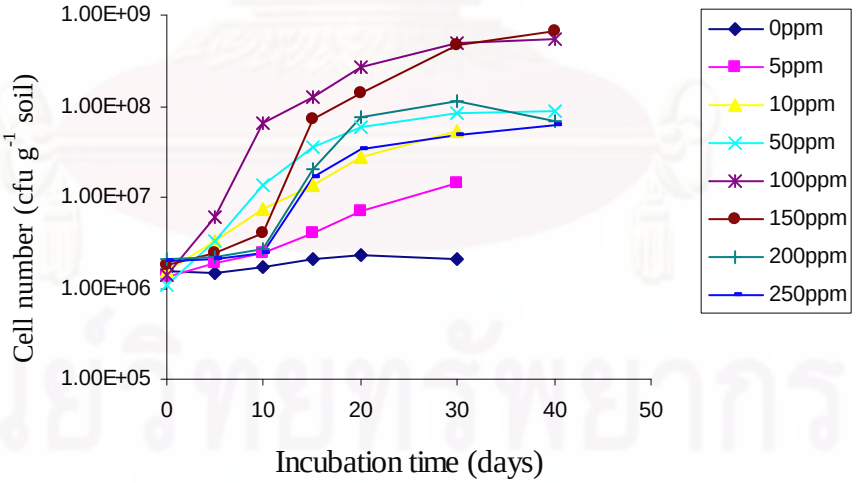

Figure B-4 Growth of carbofuran degrader in soil augmented with immobilized *B. cepacia* PCL3 at different initial carbofuran concentration (treatment C)

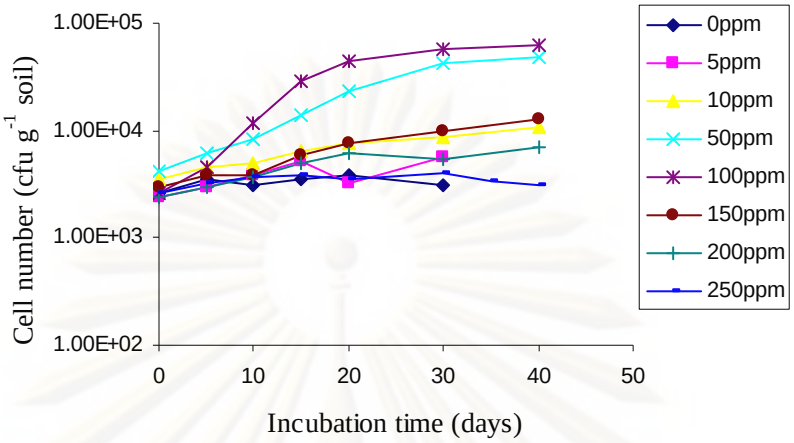

Figure B-5 Growth of carbofuran degrader in soil amended with sterile undelignified coir at different initial carbofuran concentration (treatment D)

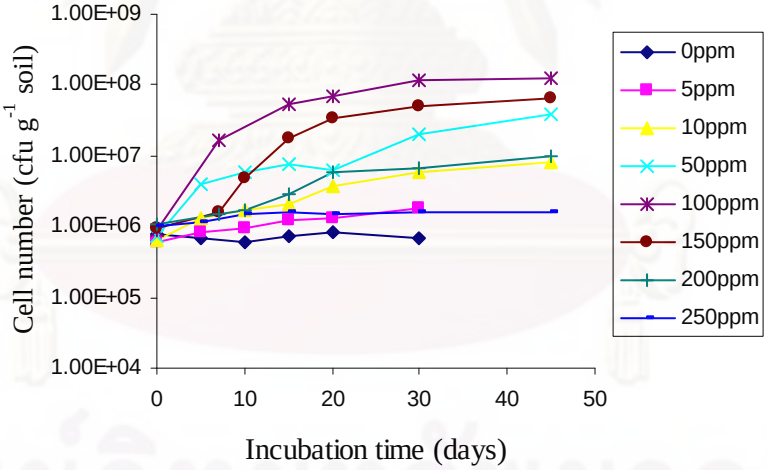

Figure B-6 Growth of PCL3 in autoclaved soil amended with PCL3 in free cell form at different initial carbofuran concentration (treatment E)

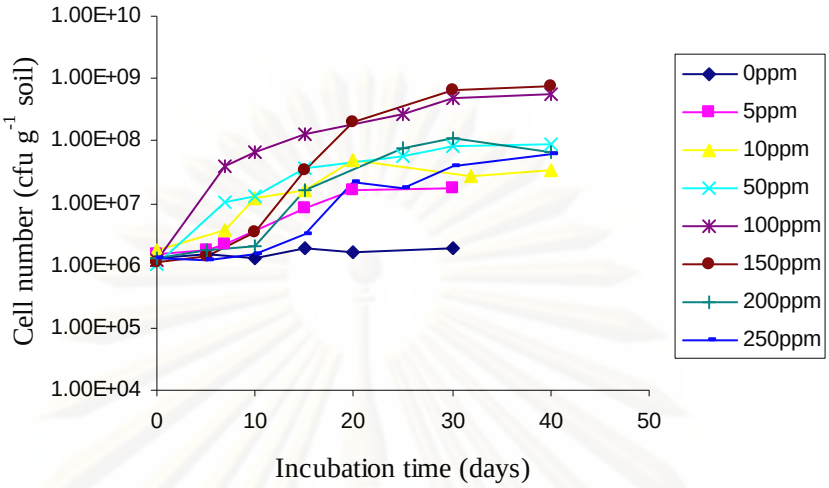

Figure B-7 Growth of PCL3 in autoclaved soil amended with immobilized *B. cepacia* PCL3 at different initial carbofuran concentration (treatment F)

APPENDIX C

SCANNING ELECTRON MICROSCOPEIC OF *B. cepacia* PCL3 IMMOBILIZED ON SUPPORT MATERIALS

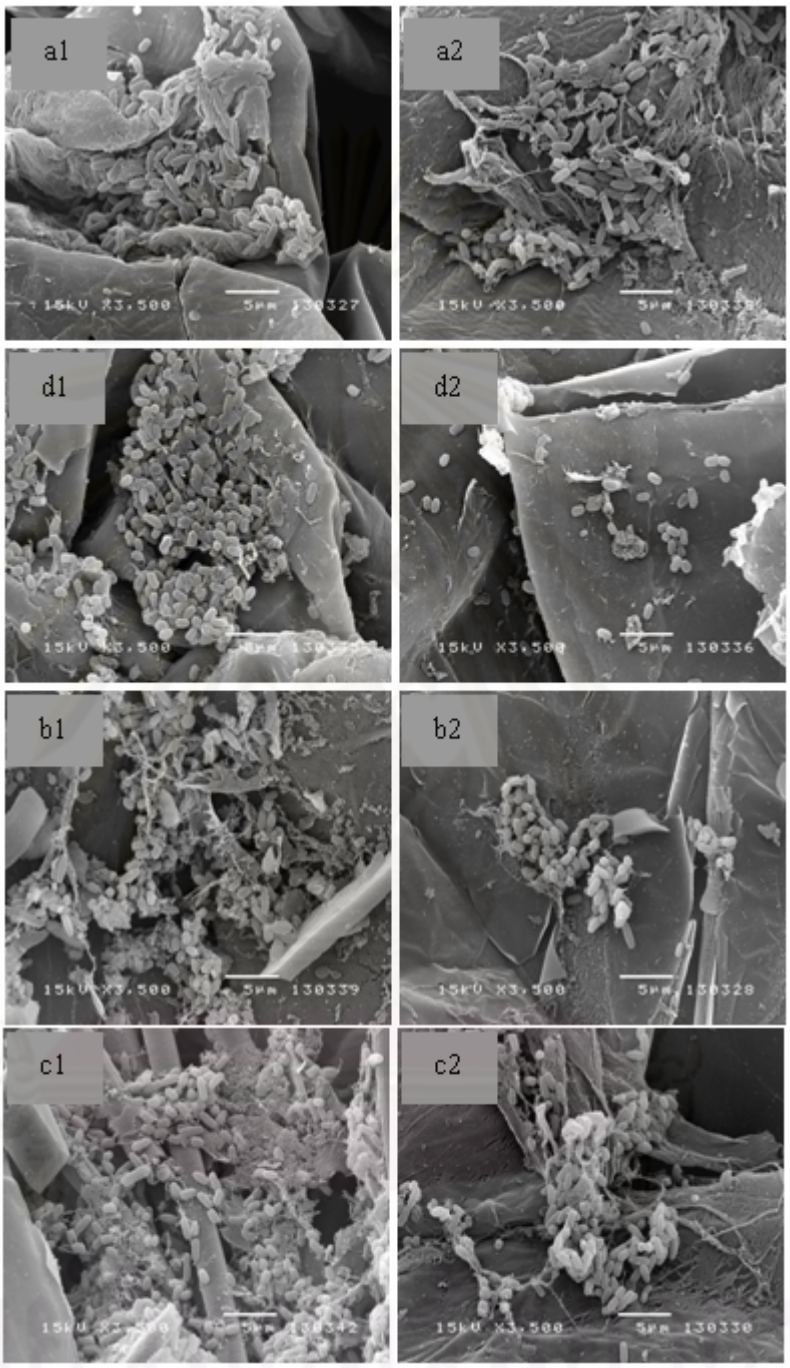

Figure C-1 Scanning electron microscopic image of *B. cepacia* PCL3 immobilized on support materials in undelignified (a = coir, b = bulrush, c = banana stem) and delignified (d = coir) support materials (subscript 1= the end of first use, 2 = the end of first reuse)

APPENDIX D

CALIBRATION CURVE

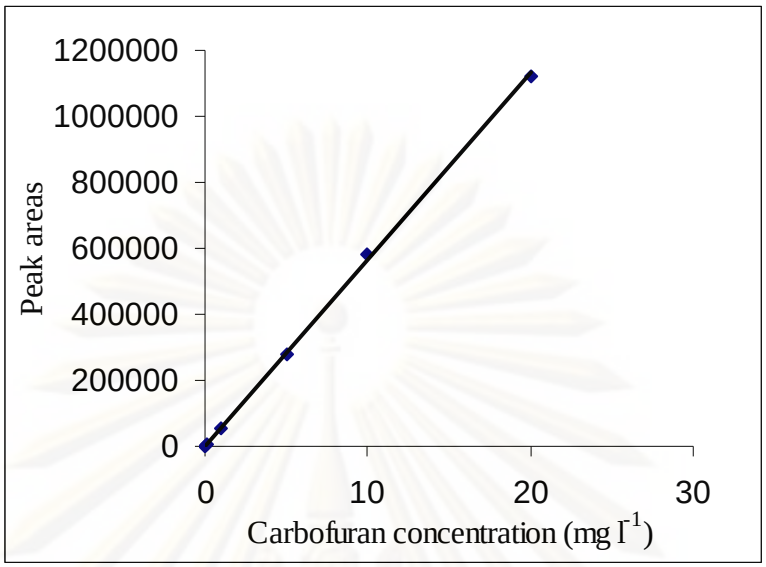

Figure D-1 Standard curve of carbofuran

Linear equation of carbofuran standard

$y = 56523x$ _____(1)

$R^2 = 0.9997$

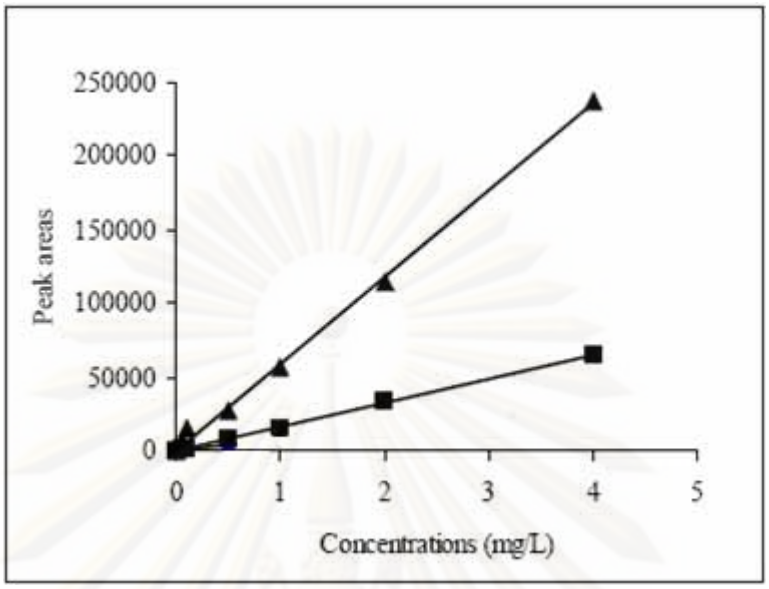

Figure D-2 Standard curve of carbofuran phenol (■) and 3-ketocarbofuran (▲)

Linear equation of carbofuran phenol standard

$y = 16315x$ _____ (2)
$R^2 = 0.9997$

Linear equation of 3-keto carbofuran standard

$y = 58846x$ _____ (3)
$R^2 = 0.9969$

BIOGRAPHY

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