รายงานวิจัยฉบับสมบูรณ์

โครงการ

การประยุกต์ใช้จุลินทรีย์ที่มีความสามารถในการย่อยสลายคาร์โบฟูราน *Burkholderia cepacia* PCL3 ในการกู้ฟื้นฟูดินและน้ำที่มีการปนเปื้อนคาร์โบฟูราน

(Application of carbofuran degrader in remediation of carbofuran contaminated water and soil)

โดย

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กรกฎาคม 2553

สัญญาเลขที่ DBG5180001

รายงานวิจัยฉบับสมบูรณ์

โครงการ การประยุกต์ใช้จุลินทรีย์ที่มีความสามารถในการย่อยสลายคาร์โบฟูราน *Burkholderia cepacia* PCL3 ในการกู้ฟื้นฟูดินและน้ำที่มีการปนเปื้อนคาร์โบฟูราน

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

งานวิจัยนี้ได้รับทุนอุดหนุนการวิจัยสำนักงานกองทุนสนับสนุนการวิจัย ประจำปีงบประมาณ 2551 ชุดโครงการวิจัยแบบมุ่งเป้า “การพัฒนาเกษตรยั่งยืน” คณะผู้วิจัยใคร่ขอขอบคุณภาควิชาเทคโนโลยีชีวภาพ คณะเทคโนโลยี มหาวิทยาลัยขอนแก่น ที่สนับสนุนสถานที่เครื่องมือและอุปกรณ์ที่ใช้ในการวิจัย

บทคัดย่อ

งานวิจัยมีวัตถุประสงค์หลักคือเพื่อประยุกต์ใช้จุลินทรีย์ที่มีความสามารถในการย่อยสลายคาร์โบฟูราน *Burkholderia cepadia* PCL3 ในการกู้ฟื้นฟูดินและน้ำที่มีการปนเปื้อนคาร์โบฟูราน โดยแบ่งการทดลองออกเป็น 4 ชุด ในชุดการทดลองที่ 1 ได้ศึกษาย่อยสลายคาร์โบฟูรานโดยเซลล์ตรึงของ PCL3 บนซังข้าวโพด ในอาหารเลี้ยงเชื้อ Basal Salt Medium (BSM) โดยใช้ถังปฏิกรณ์ชีวภาพแบบสลับเป็นกะ (Sequencing batch reactor, SBR) ที่เป็นขวดแก้วขนาด 2 ลิตร ความเข้มข้นของคาร์โบฟูรานเริ่มต้นเท่ากับ 20 มก./ลิตร โดยศึกษาผลของระยะเวลากักเก็บ (14-6 วัน) ผลการทดลองพบว่า ที่ HRT 14-8 วัน คาร์โบฟูรานจะถูกย่อยสลายได้อย่างสมบูรณ์ (100%) เมื่อลด HRT เป็น 6 วัน ประสิทธิภาพการย่อยสลายคาร์โบฟูรานลดลงเหลือเพียง 73.5% ดังนั้นจึงสามารถสรุปได้ว่า HRT ที่เหมาะสมสำหรับการบำบัดคาร์โบฟูรานใน SBR เท่ากับ 8 วัน จากนั้นได้ศึกษาผลของการกระตุ้นจุลินทรีย์โดยการเติมแหล่งอาหาร (โมลาส กากมัน รำข้าว และกากยีสต์จากอุตสาหกรรมผลิตเบียร์) แต่ละชนิดที่ HRT เท่ากับ ¾ เท่าของ HRT ที่เหมาะสม (6 วัน) ผลการทดลอง พบว่า รำข้าวช่วยเพิ่มประสิทธิภาพการย่อยสลายคาร์โบฟูรานได้ดีที่สุด จากนั้นได้ศึกษาผลความเข้มข้นของคาร์โบฟูราน (20-80 มก./ลิตร) ต่อประสิทธิภาพการย่อยสลายคาร์โบฟูรานใน SBR ที่มีการเติมรำข้าว ที่ HRT เท่ากับ 6 วัน ผลการทดลองพบว่า ความเข้มข้นคาร์โบฟูรานสูงสุดที่สามารถบำบัดได้ 100% คือ 40 มก./ล. และให้ค่าคงที่อัตราการสลาย (k_1) และค่าครึ่งชีวิต ($t_{1/2}$) ของคาร์โบฟูรานใน BSM เท่ากับ 0.044 ต่อชม. และ 16 ชม.ตามลำดับ

ในชุดกการทดลองที่ 2 ได้ศึกษาประสิทธิภาพการย่อยสลายคาร์โบฟูรานในถังปฏิกรณ์ชีวภาพแบบกึ่งแข็งกึ่งเหลว (soil slurry phase reactor) ที่ดำเนินการแบบสลับเป็นกะ ทำการทดลองใน reactor ที่เป็นขวดแก้วขนาด 2 ลิตร ปริมาตรทำการ 1.5 ลิตร ที่ระดับความเข้มข้นคาร์โบฟูรานเริ่มต้นเท่ากับ 20 มก./กก. ดินแห้ง แบ่งการทดลองออกเป็น 8 ชุด เพื่อศึกษาผลของจุลินทรีย์ประจำถิ่น การเติมจุลินทรีย์ PCL3 และการกระตุ้นจุลินทรีย์โดยใช้โมลาส ผลการทดลองพบว่า ถังปฏิกรณ์ที่มีการเติมเซลล์ตรึง PCL3 บนซังข้าวโพดให้ค่าประสิทธิภาพการย่อยสลายคาร์โบฟูรานสูงที่สุด 96.97% รองลงมาคือถังปฏิกรณ์ที่มีการเติมเซลล์ตรึง PCL3 และโมลาส (82.23%) ส่วนการย่อยสลายคาร์โบฟูรานโดยจุลินทรีย์ประจำถิ่นเพียงอย่างเดียวให้ค่าการย่อยสลายเพียง 67.69% แสดงให้เห็นว่าวิธีการบำบัดทางชีวภาพเป็นวิธีที่มีประสิทธิภาพในการบำบัดคาร์โบฟูรานในดิน ใน

ในชุดการทดลองที่ 3 ได้ศึกษาการบำบัดดินที่ปนเปื้อนคาร์โบฟูรานโดยใช้เทคนิคการเติมจุลินทรีย์ (bioaugmentation) ในแปลงทดสอบขนาด 1x1.2x0.2 ม. โดยเปรียบเทียบระหว่างเซลล์ตรึงบนซังข้าวโพดกับเซลล์อิสระของ PCL3 ผลการทดลองพบว่า ในดินที่มีจุลินทรีย์ประจำถิ่นเพียงอย่างเดียว การย่อยสลายคาร์โบฟูรานเกิดขึ้นอย่างช้าๆ มีค่า $t_{1/2}$ เท่ากับ 127 วัน การเติมเซลล์ตรึงของ PCL3 ช่วยให้ค่า $t_{1/2}$ ของคาร์โบฟูรานในดินสั้นลง (16 วัน) ค่า $t_{1/2}$ ของ คาร์โบฟูรานในดินที่เติมเซลล์อิสระของ PCL3 มีค่าเท่ากับ 28 วันซึ่งยาวนานกว่า เมื่อเปรียบเทียบกับการใช้เซลล์ตรึง ผลการทดลองแสดงให้เห็นว่า PCL3 ในรูปเซลล์อิสระอาจไม่สามารถนำมาใช้ในการบำบัดคาร์โบฟูรานที่ปนเปื้อนในสิ่งแวดล้อมจริงได้ นอกจากนี้ในการทดลองยังพบว่าประสิทธิภาพการย่อยสลายคาร์โบฟูรานในดินมีความสัมพันธ์โดยตรงกับจำนวนจุลินทรีย์ที่มีความสามารถในการย่อยสลายคาร์โบฟูรานที่เติมลองไปในดินที่อยู่รอดและเจริญในระบบ

การทดลองที่ 4 ได้ทำการส่งถ่ายยีน *luxAB* เข้าสู่ PCL3 ทำให้ได้จุลินทรีย์สายพันธุ์ใหม่ ให้ชื่อว่า PCL3:*luxAB*1 ซึ่งมีความสามารถในการปลดปล่อยแสง luminescence เมื่อสัมผัสกับ n-decanal โดยให้ค่าความเข้มแสง $1.6x10^{-3}$ RLU/cfu PCL3:*luxAB*1 มีรูปแบบการเจริญและประสิทธิภาพการย่อยสลายคาร์โบฟูรานไม่แตกต่างจาก PCL3 ความสามารถในการปลดปล่อยแสง luminescence ของ PCL3:*luxAB*1มีความสัมพันธ์กับช่วงของการเจริญ กล่าวคือ การปลดปล่อยแสงระหว่างการเจริญในช่วง log phase มีค่าสูงกว่าช่วง stationary phase ประมาณ 10 เท่า ค่าความเป็นกรดด่าง อุณหภูมิ และความเข้มขนของ n-decanal ที่เหมาะสำหรับการปลดปล่อยแสงของ PCL3:*luxAB*1 คือ 7.0 35 ^{o}C และ 0.01% ตามลำดับ โดยการใช้ระบบ PCL3:*luxAB*1 ในการบำบัดดินหรือน้ำที่ปนเปื้อนคาร์โบฟูราน จะทำให้สามารถตรวจสอบการอยู่รอดของ PCL3:*luxAB*1 ที่เติมลงไปในระบบ ระหว่างการทดลองซึ่งเป็นสัดส่วนโดยตรงกับประสิทธิภาพการย่อยสลายคาร์โบฟูรานได้ เนื่องจาก PCL3:*luxAB*1 มีความสามารถในการปลดปล่อยแสง luminescence ซึ่งแตกต่างจากจุลินทรีย์ประจำถิ่น

Abstract

The main objective of this study is to demonstrate the application of carbofuran degrader *Burkholderia cepacia* PCL3 in remediation of carbofuran contaminated water and soil. The experiments were divided into 4 parts. In part (1), the performance of Sequencing Batch Reactors (SBRs) augmented with immobilized PCL3 on corncob for biodegradation of carbofuran in Basal Salt Medium (BSM) was studied. The effect of Hydraulic Retention Time (HRT) (14-6 d) on carbofuran degradation efficiency was investigated at a carbofuran concentration in the feed medium of 20 mg l^{-1}. The shortest HRT of 8 d resulted in complete degradation of carbofuran. At 75% of the optimum HRT (6 d), the effects of biostimulation using carbon sources, i.e. molasses and cassava pulp and nitrogen sources, i.e. rice bran and spent yeast as well as the effect of carbofuran concentration in the feed medium (20-80 mg l^{-1}) were investigated. The optimum conditions for SBRs were achieved with an initial carbofuran concentration of 40 mg l^{-1} by using 0.1 g l^{-1} of rice bran as a biostimulated amendment. The carbofuran degradation efficiency of SBR at the optimum conditions was 100% with a k_1 value and $t_{1/2}$ of 0.044 h^{-1} and 15.57 h, respectively.

In part (2), the effectiveness of bioremediation technology in the removal of carbofuran from contaminated soil using a bioslurry phase sequencing batch reactor (SBR) was investigated. A 2-L laboratory glass bottle was used as a bioreactor with a working volume of 1.5 L. The carbofuran concentration in the soil was 20 mg kg^{-1} soil. One total cycle period of the SBR was comprised of 1 h of fill phase, 82 h of react phase, and 1 h of decant phase. Immobilized PCL3 on corncob was used as the inoculum. The results revealed that bioaugmentation treatment (addition of PCL3) gave the highest percentage of carbofuran removal (96.97%), followed by bioaugmentation together with biostimulation (addition of molasses) treatment (88.23%). Abiotic experiments, i.e. autoclaved soil slurry with corncob and no PCL3 treatment and autoclaved soil slurry with no PCL3 treatment, could adsorb 31.86% and 7.70% of carbofuran, respectively, which implied that soil and corncob could act as sorbents for the removal of carbofuran.

In part (3), the small-scale field studies of *in situ* bioaugmentation by using free and immobilized PCL3 on corncob were conducted in the plots with a dimension of 1 m x 1.25 m x 0.20 m. In the soil with the presence of only indigenous microorganisms, the degradation of carbofuran was slow with the long $t_{1/2}$ of 127 d. Bioaugmented the soil with immobilized PCL3 could shorten the $t_{1/2}$ of carbofuran in soil to be 16 d. The significant longer $t_{1/2}$ of 28 d in soil was observed when the free cells of PCL3 were used in comparison to immobilized cells which suggested that bioaugmentation of carbofuran by using PCL3 in free cell form might not applicable. Growth and survival of carbofuran degraders in soil and in support materials were examined and the results indicated that the efficiency of carbofuran degradation directly correlated with the number of introduced carbofuran degrader surviving in the system.

In part (4), the *luxAB*-mutant of PCL3*:luxAB*1 was constructed with the capability to emit the luminescence signal of 1.6×10^{-3} RLU cfu^{-1}. The mutant has the growth pattern and carbofuran degradation ability similar to PCL3 wild-type. The luminescent emission by PCL3*:luxAB*1 is directly in correlation with the metabolic activity of the cells in which the exponential growth cells gave 100-fold higher value of light output in comparison to the cell in stationary phase. The optimal pH, temperature and n-decanal concentration on luminescence emission are 7.0, 35 ^{o}C and 0.01%, respectively. With the luciferase system, the degradative fraction of the augmented PCL3*:luxAB*1 and the difference between the active augmented PCL3*:luxAB*1 and indigenous microorganisms at the contaminated site could be indicated.

สารบัญเรื่อง

สารบัญเรื่อง (ต่อ)

สารบัญภาพ

หน้า

สารบัญภาพ (ต่อ)

สารบัญตาราง

หน้า

สารบัญตาราง (ต่อ)

บทสรุปผู้บริหาร
(Executive Summary)

The main objective of this study is to demonstrate the application of carbofuran degrader *Burkholderia cepacia* PCL3 in remediation of carbofuran contaminated water and soil. The experiments were divided into 4 parts as follows:

Part I: The performance of Sequencing Batch Reactors (SBRs) augmented with immobilized *B. cepacia* PCL3 for biodegradation of carbofuran in Basal Salt Medium (BSM) was studied. The effect of Hydraulic Retention Time (HRT) (14-6 d) on carbofuran degradation efficiency was investigated at a carbofuran concentration in the feed medium of 20 mg l^{-1}. The shortest HRT of 8 d resulted in complete degradation of carbofuran. At 75% of the optimum HRT (6 d), the effects of biostimulation using carbon sources, i.e. molasses and cassava pulp and nitrogen sources, i.e. rice bran and spent yeast as well as the effect of carbofuran concentration in the feed medium (20-80 mg l^{-1}) were investigated. The optimum conditions for SBRs were achieved with an initial carbofuran concentration of 40 mg l^{-1} by using 0.1 g l^{-1} of rice bran as a biostimulated amendment. The carbofuran degradation efficiency of SBR at the optimum conditions was 100% with a k_1 value and $t_{1/2}$ of 0.044 h^{-1} and 15.57 h, respectively.

Part II: The effectiveness of bioremediation technology in the removal of carbofuran from contaminated soil using a bioslurry phase sequencing batch reactor (SBR) was investigated. A 2-L laboratory glass bottle was used as a bioreactor with a working volume of 1.5 L at room temperature (27±2 ^{o}C). One total cycle period of the SBR was comprised of 1 h of fill phase, 82 h of react phase, and 1 h of decant phase. The carbofuran concentration in the soil was 20 mg kg^{-1} soil. Immobilized PCL3 on corncob was used as the inoculum. The results revealed that bioaugmentation treatment (addition of PCL3) gave the highest percentage of carbofuran removal (96.97%), followed by bioaugmentation together with biostimulation (addition of molasses) treatment (88.23%). Abiotic experiments, i.e. autoclaved soil slurry with corncob and no PCL3 treatment and autoclaved soil slurry with no PCL3 treatment, could adsorb 31.86% and 7.70% of

carbofuran, respectively, which implied that soil and corncob could act as sorbents for the removal of carbofuran.

Part III: The small-scale field studies were conducted in the plots with the dimension of 1 m x 1.25 m x 0.20 m in order to validate the *in situ* bioaugmentation by using free and immobilized PCL3 on corncob before implemented this technology on a large scale. In the soil with the presence of only indigenous microorganisms, the degradation of carbofuran was slow with the long $t_{1/2}$ of 127 d. Bioaugmented the soil with immobilized PCL3 could shorten the $t_{1/2}$ of carbofuran in soil to be 16 d. The significant longer $t_{1/2}$ of 28 d in soil was observed when the free cells of PCL3 were used in comparison to immobilized cells which suggested that bioaugmentation of carbofuran by using PCL3 in free cell form might not applicable. Growth and survival of carbofuran degraders in soil and in support materials were examined and the results indicated that the efficiency of carbofuran degradation directly correlated with the number of introduced carbofuran degrader surviving in the system.

Part IV: The *luxAB*-mutant of PCL3*:luxAB*1 was constructed with the capability to emit the luminescence signal of 1.6×10^{-3} RLU cfu^{-1}. The mutant has the growth pattern and carbofuran degradation ability similar to PCL3 wild-type. The capability of tetracycline resistance was stable in PCL3:*luxAB*1 during repeated subculture over 120 generations. The luminescent emission by PCL3*:luxAB*1 is directly in correlation with the metabolic activity of the cells in which the exponential growth cells gave 100-fold higher value of light output in comparison to the cell in stationary phase. The optimal pH, temperature and n-decanal concentration on luminescence emission are 7.0, 35 ^{o}C and 0.01%, respectively. PCL3*:luxAB*1 could be used to assess the toxicity of carbofuran and carbofuran phenol in Basal salt medium (BSM) in which the difference sensitivity of the cells is depending on the biomass concentration. PCL3*:luxAB*1 was used to biodegrade carbofuran in BSM and soil. With the luciferase system, the degradative fraction of the augmented PCL3*:luxAB*1 and the difference between the active augmented PCL3*:luxAB*1 and indigenous microorganisms at the contaminated site could be indicated.

เนื้อหางานวิจัย

งานวิจัยนี้ได้แบ่งการทดลองออกเป็น 4 ส่วน ซึ่งในแต่ละส่วนได้นำเสนอรายละเอียดงานวิจัย ได้แก่ บทคัดย่อ (Abstract) บทนำ (introduction) แสดงหลักการ เหตุผล และที่มาของหัวข้อวิจัย วัสดุและวิธีการทดลอง (Materials and Methods) ผลการทดลองและวิจารณ์ (Results and Discussions) สรุปผลการทดลอง (Conclusion) กิตติกรรมประกาศ (Acknowledgements) และเอกสารอ้างอิงของงานวิจัย (References) ทั้งนี้ ได้จัดทำรายงานในรูปแบบ manuscript ภาษาอังกฤษเพื่อเตรียมตีพิมพ์ในวารสารวิชาการต่อไป โดยหัวข้อวิจัยทั้ง 4 การทดลอง มีดังนี้

ส่วนที่ 1 Biodegradation of carbofuran in sequencing batch reactor augmented with the immobilized *Burkholderia cepacia* PCL3 on corncob

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ส่วนที่ 2 Bioaugmentation of carbofuran by *Burkholderia cepacia* PCL3 in a bioslurry phase sequencing batch reactor

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ส่วนที่ 3 Bioaugmentation of carbofuran residues in soil by *Burkholderia cepacia* PCL3: A small-scale field studies

ได้จัดทำบทความวิจัยต้นฉบับแบบ Short Communication เพื่อพิจารณาตีพิมพ์ในวารสาร New Biotechnology

ส่วนที่ 4 *lux*-Marking and application of carbofuran degrader *Burkholderia* cepacia PCL3

ได้จัดทำบทความวิจัยต้นฉบับแบบ Short Communication เพื่อพิจารณาตีพิมพ์ในวารสาร New Biotechnology

PART I

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

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ABSTRACT

The performance of Sequencing Batch Reactors (SBRs) augmented with immobilized *Burkholderia cepacia* PCL3 for biodegradation of carbofuran in Basal Salt Medium (BSM) was studied. The effect of Hydraulic Retention Time (HRT) (14-6 d) on carbofuran degradation efficiency was investigated at a carbofuran concentration in the feed medium of 20 mg l^{-1}. The shortest HRT of 8 d resulted in complete degradation of carbofuran. At 75% of the optimum HRT (6 d), the effects of biostimulation using carbon sources, i.e. molasses and cassava pulp and nitrogen sources, i.e. rice bran and spent yeast as well as the effect of carbofuran concentration in the feed medium (20-80 mg l^{-1}) were investigated. The optimum conditions for SBRs were achieved with an initial carbofuran concentration of 40 mg l^{-1} by using 0.1 g l^{-1} of rice bran as a biostimulated amendment. The carbofuran degradation efficiency of SBR at the optimum conditions was 100% with a k_1 value and $t_{1/2}$ of 0.044 h^{-1} and 15.57 h, respectively.

Key words: Burkholderia cepacia PCL3; bioaugmentation; biostimulation; carbofuran; immobilization; sequencing batch reactor

1. Introduction

Carbofuran (2,3-dihydro-2,2 dimethylbenzofuran-7-yl methylcarbamate) is a broad-spectrum insecticide widely used in agriculture to control insects and nematodes on contact or after ingestion. Carbofuran is of environmental concern because it is soluble in water and highly mobile in soil resulting in a high potential for groundwater contamination and can cause acute toxicity to mammals through cholinesterase inhibition (Gapta, 1994). In 2003, Thailand imported carbofuran in both solid and liquid forms up to 826.6 and 45.5 m, respectively, for use in agriculture, especially in rice fields (Department of Agriculture, 2003). Contamination of carbofuran in the aqueous environment can occur as a result of discharges from manufacturing plants and rinsate, storage sites, accidental spills, and surface runoff through agricultural areas where carbofuran has been applied.

An effective route for pesticide removal is microbial degradation by specific degrader and/or indigenous microorganisms. Previous research reported the discovery of microorganisms capable of degrading carbofuran (Qiu-Xiang et al., 2007; Ryeol et al., 2006) and other pesticides such as atrazine (Lima et al., 2009), dichloroaniline (Tongarun et al., 2008) and fenitrothion (Hong et al., 2007) from contaminated natural matrices. These degraders could use the pesticide as their energy sources, i.e. C- or N- or C and N-sources. The addition of microbial cultures capable of degrading pesticides, or the so-called bioaugmentation technique, is reported to be an effective bioremediation approach to improve pesticide degradation in contaminated soil and water that lack microbial activities (Ventaka-Mohan et al., 2006; Plangklang and Reungsang, 2008). Not only bioaugmentation but also biostimulation, the addition of organic and/or inorganic amendments to stimulate the activity of indigenous microorganisms, has been reported to enhance the degradation rate of contaminants of interest (Robles-Gonzalez et al., 2006).

Bioaugmentation and biostimulation can be applied both *in situ* by directly adding degraders and/or amendments to the contaminated areas and *ex situ* through biodegradation in the bioreactor. Although *in situ* treatment has the advantages of simplicity and cost-effectiveness, it requires a long time to complete the degradation process and can be restrained by some limiting conditions such as low permeability and heterogeneity of the contaminated matrices (Prasanna et al., 2008). Therefore, bioreactor technology which can be specially designed in a variety of configurations

to maximize microbial activity has drawn our attention to the bioremediation of carbofuran.

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

Evidence of the biodegradation of carbofuran in synthetic media and soil have been published by many researchers (Ogram et al., 2000; Bano et al., 2004; Plangklang and Reungsang, 2009; 2010). However, to the best of our knowledge, there is very limited information available on its biodegradation potential in bioreactors. Therefore, the main objective of this study was to explore the performance of SBRs in the bioremediation of carbofuran. The effects of HRT, biostimulation technique, and carbofuran concentration on carbofuran degradation efficiency in SBRs were investigated. The kinetic aspects of carbofuran degradation in the SBRs were further studied to explain the degradation behavior in SBR treatments.

2. Materials and Methods

2.1 Chemicals

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

purchased from Merck, Germany. Dichloromethane (analytical grade) was purchased from BDH, England. All other chemicals were analytical grade and purchased from BDH, England.

2.2 Feed medium

The culture medium used in this study was C-limited Basal Salt Medium (pH 7) containing (in g l^{-1}): 5.57, $NaHPO_4$; 2.44, KH_2PO_2; 2.00, NH_4Cl; 0.20, $MgCl_2.6H_2O$; 0.0004, $MnCl_2.4H_2O$; 0.001, $FeCl_3.6H_2O$; and 0.001, $CaCl_2$. Carbofuran stock solution in methanol, at the given concentrations was added to BSM after sterilization by autoclaving at 121^oC for 15 min before using as the feed medium.

2.3. Immobilization of B. cepacia PCL3

2.3.1 Microorganism preparation

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

2.3.2 Supporting material preparation

Corncob was used as a support material to immobilize *B. cepacia* PCL3. This material has high matrix porosity and a pore size that could enhance the cell adsorption capability during immobilization. Corncob was cut into 0.7x0.7x0.7 cm pieces using a knife. Three hundred g of cut corncob was boiled in 3 l of 1% NaOH for 3 h to remove lignin and fibers inside the materials which might react with the cells (Bardi and Koutnas, 1994). The alkaline-boiled corncob was washed three times with 3 l of distilled water, soaked in distilled water overnight, and then sterilized by autoclaving at 121^oC for 15 min and kept at 4^oC until use.

2.3.3 Cell immobilization

Adsorption was used as the immobilization method in this study. This method is typically performed when porous media are used as support materials with the advantage of ease of operation. The immobilization technique was conducted by adding 75 g of sterile corncob to 250 ml of sterile NB containing 5 mg l^{-1} carbofuran before inoculating with PCL3 (10^6 CFU ml^{-1}). The flask was then incubated at 150 rpm, at room temperature, for 48 h. After incubation, the support material was transferred to fresh NB containing 5 mg l^{-1} of carbofuran and incubated, as previously

described, before harvesting by filtration through a 0.45 µm filter using a Buchner filter funnel and washing with 0.85% NaCl using an aseptic technique. This process was repeated twice. Immobilized cells were kept at 4^{o}C until use in subsequent experiments. The internal cell density on the corncob after immobilization was approximately 5.3x10^{8} CFU g^{-1} dry corncob.

2.4 Reactor configuration

A 2-L laboratory glass bottle was used as a reactor in this study with an operating liquid volume of 1.5 l and suitable inlet and outlet arrangements as shown in Fig. 1. Four ports were installed in the reactor for filling, decanting and sample collection, air supplying and gas venting. Air was supplied from an air compressor through an air diffuser at the bottom of the bottle. The contents of the reactor were continuously mixed by a magnetic stirrer while feeding and decanting were carried out by appropriately calibrated peristaltic pumps.

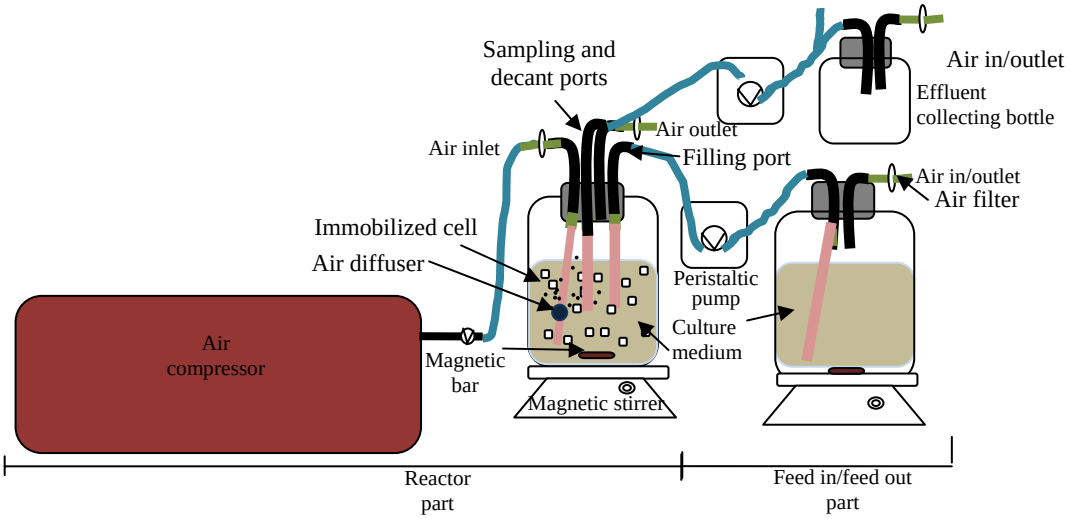

Fig. 1. Schematic diagram of the bioreactor used (not subject to scale)

2.4 Reactor operation

2.4.1 Carbofuran degradation in batch system

Carbofuran degradation in the bioaugmented reactor was first studied in the batch system. Wet corncob (200 g) immobilized *B. cepacia* PCL3 (10^{8} CFU g^{-1} dry corncob, 40% moisture content) was added to the sterilized reactor. A 1.5-l volume of BSM with an initial carbofuran concentration of 20 mg l^{-1} was fed into the reactor at a flow rate of 25 ml min^{-1} using a peristaltic pump. The reactor was then operated at room temperature (28-30^{o}C) with continuous mixing using a magnetic stirrer. Dissolved Oxygen (DO) level in the medium was maintained at above 2.5 mg l^{-1} by

pumping air through an oxygen diffuser at an air flow rate of 600 ml min^{-1}. The reactor was operated until the carbofuran in the culture medium was completely degraded. During the reactor operation, the culture medium in the reactor was sampled every 6 h to determine the concentrations of carbofuran and its metabolites. An abiotic control reactor was inoculated with autoclaved corncob which was operated in a similar manner. Results from the batch experiment were used as the basic information for SBR operation.

2.4.2 SBR experiments

Carbofuran degradation in the SBRs was started in a similar manner to that in the batch system. The reactors were aerobically operated in the sequencing batch mode with a fixed total cycle period of 48 h without a settling step in all treatments. The cycle period consisted of a fill phase of 1.0 h, a react phase of 46 h and a 1.0 h decant phase. The conditions for SBR operation were set based on the results of the batch experiment. During the react phase, the reactor was continuously aerated and stirred to homogenize the contents and air diffusion. The culture medium was sampled after the filling phase was finished and at the end of each cycle period (effluent), *via* the sampling port using the peristaltic pump, to determine the concentrations of carbofuran and its metabolites. The number of PCL3 on the supporting material was determined at the initial and at the end of the reactor operation in all SBR treatments.

2.4.2.1 Effect of HRT

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

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

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

2.4.2.2 Biostimulation of SBRs

In order to stimulate the carbofuran degradation ability of immobilized PCL3, various types of C-sources (10 g l^{-1}), i.e. molasses and cassava pulp and N-sources (0.1 g l^{-1}), i.e. brewery spent yeast powder and rice bran were used as organic amendments. These organic amendments were chosen because they are common agricultural wastes in Thailand. Each amended material was sterilized by autoclaving and then added to the feed medium before feeding to the reactor. As the experimental results indicated that the optimum HRT was 8 d, the SBRs biostimulation experiments were thus operated with a total cycle period of 48 h as described in similar earlier procedures. However, a shorter HRT (75% of the optimum HRT) was used in this experiment based on the assumption that biostimulation would provide a greater efficiency of carbofuran removal than the experiment without biostimulation. Therefore, the reactor was operated at a HRT of 6 d. In each treatment, the reactor was operated until a steady state was achieved. The optimal amendment for carbofuran degradation in SBRs was used in subsequent experiments.

2.4.2.3 Effect of carbofuran concentration

This experiment was conducted to determine the maximum concentration of carbofuran needed for high carbofuran removal efficiency in the SBRs. The SBRs were operated separately at high carbofuran concentrations of 40 and 80 mg l^{-1} at the HRT of 6 d (75% of the optimum HRT). The reactors were amended with the nitrogen source that yielded the highest carbofuran degradation from the previous experiment (2.4.2.2). The conditions for the SBR experiments are summarized in Table 1.

2.5 Analytical methods

2.8.1 Analysis of the concentrations of carbofuran and its metabolites

Extraction of carbofuran from culture media using liquid-liquid partitioning and the analysis of carbofuran concentration by HPLC followed the method of Plangklang and Reungsang (2008).

2.8.2 SBRs performance

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

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

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

2.8.3 Kinetics of carbofuran degradation

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

2.8.4 Enumeration of carbofuran degraders

The number of *B. cepacia* PCL3 in suspended form, as cells leaked from the support materials, was determined by the plate count technique which was modified from Zilli et al. (2004). To observe the number of PCL3 on corncob, 10 g of wet immobilized cells were taken from culture media and washed with sterile 0.85% NaCl solution three times. The washed immobilized cells were blended to small particles using a blender and then added to 50 ml sterile 0.85% NaCl solution and shaken at 250 rpm for 5 min in order to dislodge cells from the corncob. The number of PCL3 in the liquid phase was determined by plate count on the carbofuran-coated BSM agar.

Table 1 SBR experiments

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

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

3. Results and Discussion

3.1 Degradation of carbofuran in batch system

This experiment investigated the ability of immobilized *B. cepacia* PCL3 on corncob to degrade carbofuran in BSM in a batch bioreactor. Carbofuran was rapidly degraded by immobilized PCL3 from 0 to 160 h of reactor operation followed by gradual degradation (Fig. 2). The degradation profile of carbofuran in BSM was well described by a modified first-order kinetic model with a regression coefficient, r^2 of 0.96. The half-life of carbofuran in the augmented degrader was 52 h. The complete degradation of carbofuran was achieved at 336 h (14 d) of reactor operation (Fig. 2). Carbofuran phenol was the major metabolite observed during carbofuran degradation. The accumulation of carbofuran phenol was observed up to 72 h of reactor operation and then started to decrease and could not be detected in BSM after 258 h of reactor operation (Fig. 2). Results indicated that PCL3 could metabolize carbofuran and carbofuran phenol as its energy sources.

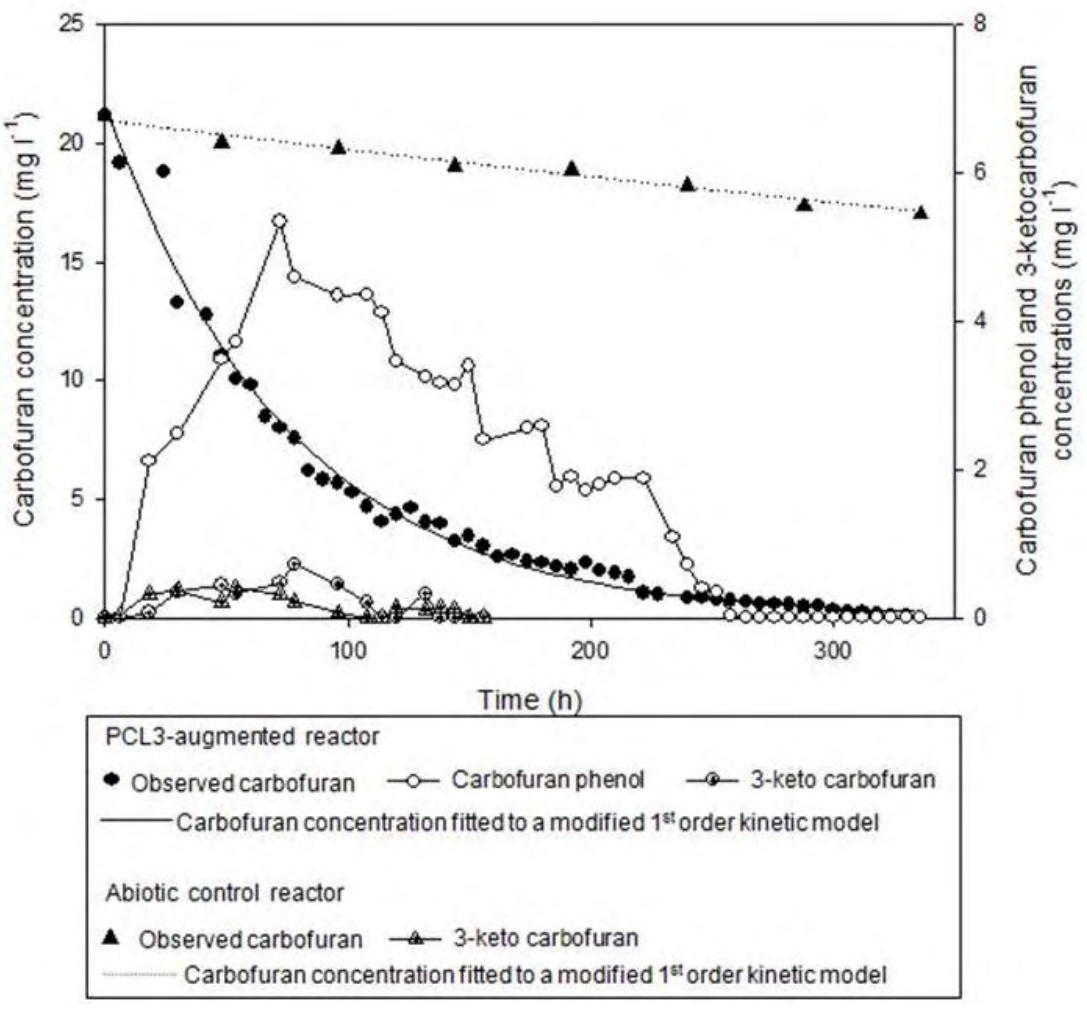

Fig. 2. Carbofuran degradation in batch reactors

Carbofuran was slowly degraded in the abiotic control reactor with removal of approximately 15% at 14 d of reactor operation (Fig. 2), suggesting that degradation of carbofuran mainly resulted from biological activity of the immobilized PCL3. Carbofuran phenol and 3-keto carbofuran were the metabolites detected in this study (Fig. 2). The metabolite 3-keto carbofuran was detected in both the PCL3-augmented reactor and the abiotic control. This metabolite is normally detected when the oxidation of carbofuran has taken place.

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

At the end of the reactor operation, the number of immobilized PCL3 on corncob increased approximately two-fold (from $4.6x10^8$ to $9.1x10^8$ CFU g^{-1} dry corncob). The number of PCL3 in suspended form increased continuously during the first 3 days of reactor operation, and was then stable at approximately $8.4x10^7$ CFU ml^{-1} (data not shown). These results indicated that cells were leaking out from the porous corncob due to limited space and they continued to grow in the medium causing an increase in cell concentration. Cell detachment might have resulted due to the fact that there were no barriers between the cells and the media which led to the possibility of cell relocation with potential establishment of cell-equilibrium inside the supports and culture media (Bekatorou et al., 2004).

3.2 Degradation of carbofuran in SBRs

3.2.1 Effect of HRT

The effect of HRT, by means of feed volume variation, on the performance of SBRs in the removal of carbofuran was investigated. Results indicated that carbofuran was completely

degraded when the SBRs were operated at the HRT of 14 to 8 d (Fig. 3). When the HRT was further decreased to 6 d, the carbofuran degradation efficiency decreased from 100% to approximately 73.50% (Fig. 3). This may have been due to the fact that a decrease in HRT led to a higher accumulation of carbofuran concentration in the culture medium (Fig. 3), which generally needs a longer time for complete degradation. The numbers of PCL3 in the immobilized form at the start and at the steady state of each HRT were not markedly different (Fig. 4). However, the numbers of PCL3 in suspended form which accumulated in the culture medium decreased with reduced HRT (Fig. 4), and the lowest number of PCL3 in suspended form were found at an HRT of 6 d. Therefore, a decrease in the number of suspended PCL3 might have resulted in a decrease in carbofuran degradation efficiency at an HRT of 6 d. From these results, it can be concluded that the optimal HRT for carbofuran remediation in SBR was 8 d.

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

3.2.2 Biostimulation effects

The effect of organic amendments on the carbofuran degradation efficiency of SBRs was examined at an HRT of 6 d. The carbofuran degradation efficiencies of stimulated SBRs are shown in Fig. 5. Results indicated that the addition of nitrogen sources, i.e. rice bran (RB) or spent yeast powder (SY) led to the complete degradation of carbofuran in SBRs (Fig. 5). However, the addition of supplementary carbon sources, i.e. cassava pulp (CP) or molasses (ML) resulted in a decrease in carbofuran degradation efficiency from 74% for the non - stimulated reactor to 37.5 and 15.0%, respectively (Fig. 5). These results implied that by adding supplementary nitrogen sources to the culture medium, the degradation of carbofuran could be enhanced, however, supplementary carbon sources are not required. This might be because PCL3 utilized carbofuran as its carbon source, thus the added supplementary carbon sources may be in competition with carbofuran resulting in a decrease in carbofuran degradation efficiency.

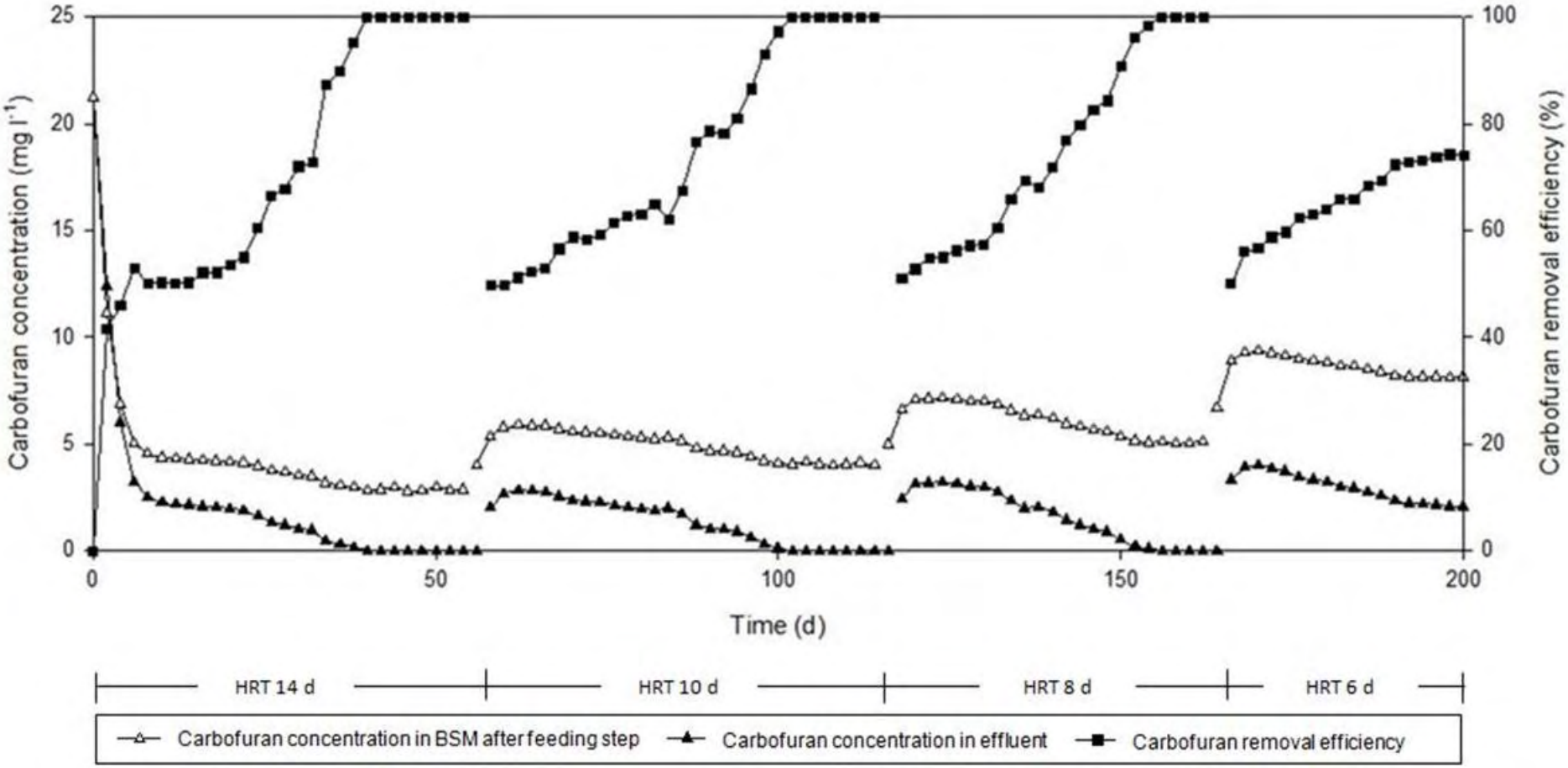

Fig. 3. Effect of HRT on carbofuran degradation efficiency in SBR

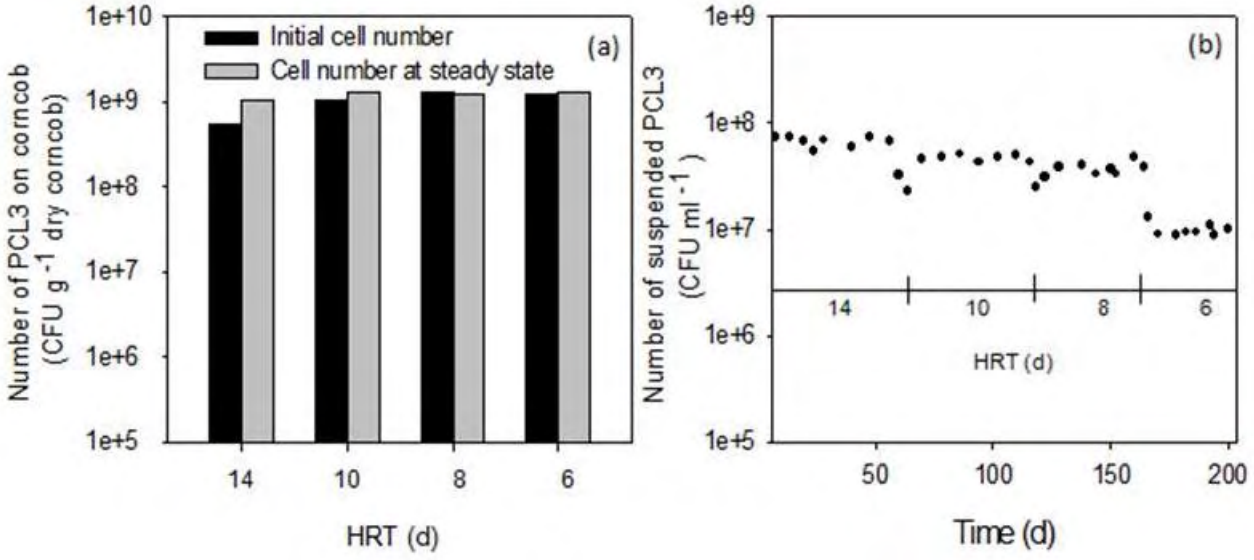

Fig. 4. Number of PCL3 in immobilized (a) and suspended (b) forms in SBRs operated at the varying of HRT.

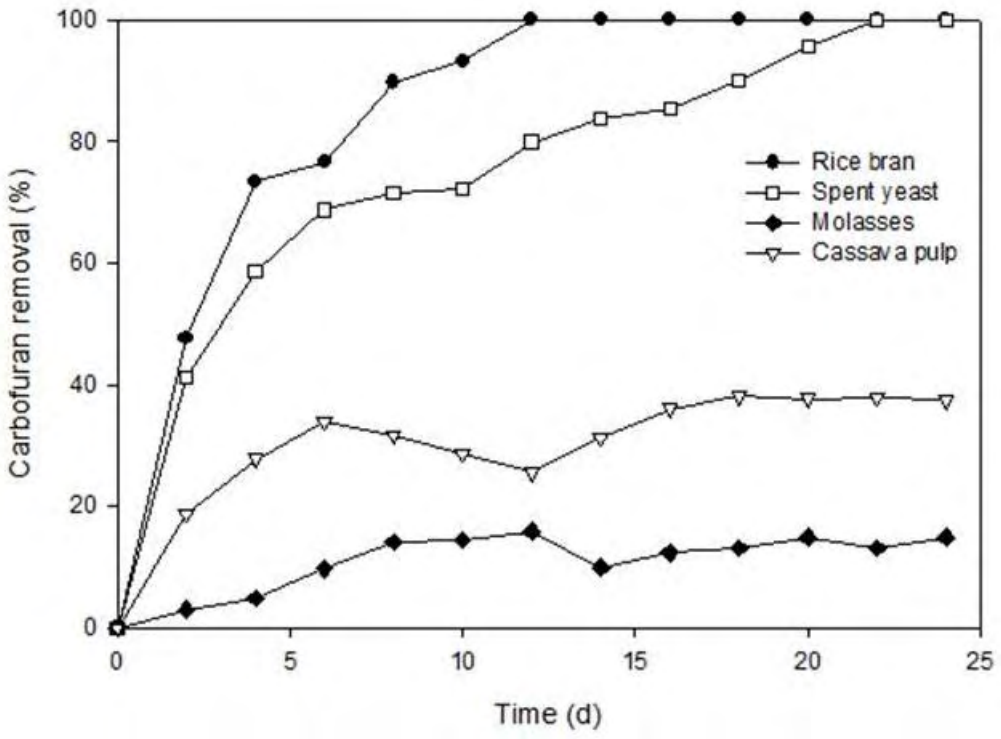

Fig. 5. Effect of organic amendments on carbofuran degradation efficiency of SBRs

The numbers of PCL3 in immobilized and suspended forms in the stimulated SBR experiments are shown in Fig. 6. Results revealed that in the SBRs stimulated with ML, RB or SY, the number of immobilized PCL3 on corncob at the steady state increased approximately two-fold compared to the initial number of immobilized cells. The addition of CP to SBR resulted in an approximate 5-fold decrease in the number of immobilized PCL3 (Fig. 6), which might have resulted from the cyanide that is generally contained in CP at 40-200 mg kg-^{1} (Oboh, 2006).

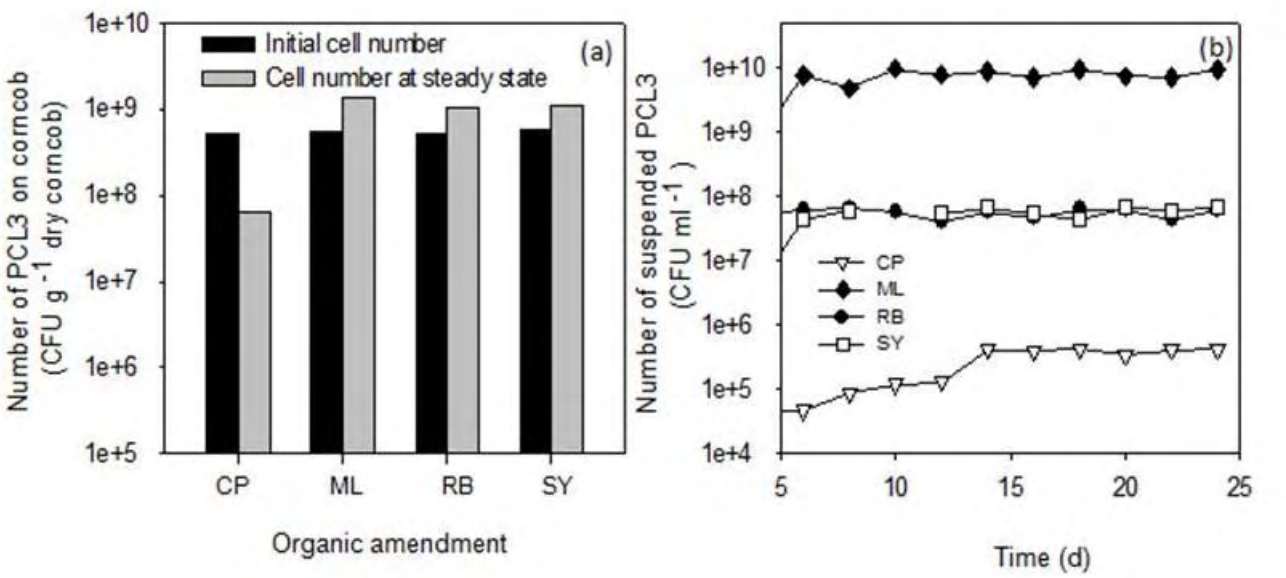

Fig. 6. Number of PCL3 in immobilized (a) and suspended (b) forms in biostimulated SBRs; CP = cassava pulp (10 g l^{-1}), ML = molasses (10 g l^{-1}), RB = rice bran (0.1 g l^{-1}) and SY = spent yeast from beer fermentation process (0.1 g l^{-1}).

The number of suspended PCL3 in SBRs stimulated with RB and SY increased and was stable at approximately 6.3x10^{7} CFU ml^{-1} (Fig. 6), while a lower number of 4.3x10^{5} CFU ml^{-1} was observed in the SBR stimulated with CP. This might have been due to the toxic effects of CP as described earlier. In the SBR stimulated with ML, the number of suspended PCL3 was approximately 9.4x10^{9} CFU ml^{-1} (Fig. 6) which was markedly higher than that in the other stimulated reactors. This result was in contrast to the carbofuran degradation efficiency in the ML-stimulated SBR (Fig. 5). ML may have contained sugar which is a preferred substrate and has fewer complex structures than carbofuran, thus PCL3 could metabolize ML for its growth without the degradation of carbofuran.

As the SBRs stimulated with RB achieved complete degradation of carbofuran within the shortest time (Fig. 5), RB was considered to be the best organic amendment and was used in subsequent experiments.

3.2.3 Effect of carbofuran concentration

The experiments in SBRs stimulated by RB were conducted at a HRT of 6 d with an increase in the carbofuran concentration in the feed medium from 20 to 40 and 80 mg l^{-1}. The carbofuran degradation patterns after 24 d of reactor operation are shown in Fig. 7. At a carbofuran concentration in the feed medium of 20 mg l^{-1}, the reactor displayed a rapid adaptation behavior about 12 d before achieving complete degradation of carbofuran. When the carbofuran concentration in the feed medium was at 40 mg l^{-1}, PCL3 needed a longer time (20 d) to achieve complete degradation (Fig. 7). A further increase in carbofuran concentration in the feed medium to 80 mg l^{-1} resulted in a partial degradation of carbofuran in the SBR of 84% (Fig. 7). The numbers of PCL3 in immobilized and suspended forms are shown in Fig. 8. Results indicated that the number of PCL3 in the immobilized form increased approximately 1.7-fold at a carbofuran concentration in the feed medium of 40 mg l^{-1}, and the number of suspended PCL3 was stable at approximately $1.6x10^6$ CFU ml^{-1} (Fig. 8). At a carbofuran concentration in the feed medium of 80 mg l^{-1}, the number of immobilized PCL3 was reduced from $5.4x10^8$ to $1.2x10^7$ CFU g^{-1} dry corncob (Fig. 8). In addition, the number of suspended PCL3 was stable at only $1.3x10^3$ CFU ml^{-1}. These results indicated that the growth of PCL3 in SBRs could be inhibited when the carbofuran concentration in the feed medium was 80 mg l^{-1} resulting in a decrease in the carbofuran degradation efficiency in the SBRs.

3.3 Kinetics of carbofuran degradation

The kinetic rate constants associated with carbofuran degradation in batch reactors and SBRs at the steady state were calculated and the results are summarized in Table 2. Overall, the biodegradation of carbofuran was well described by the modified first-order kinetic model indicated by the regression coefficient, r^2, which ranged from 0.97 to 0.99 (Table 2). The microbial degradation of carbofuran has also been reported to follow the first order kinetic model (Plangklang and Reungsang, 2008; 2009). It was evident that the rates of carbofuran degradation remained unchanged with an average value of 0.036 h^{-1} when the SBRs were operated at a HRT of 14 to 8

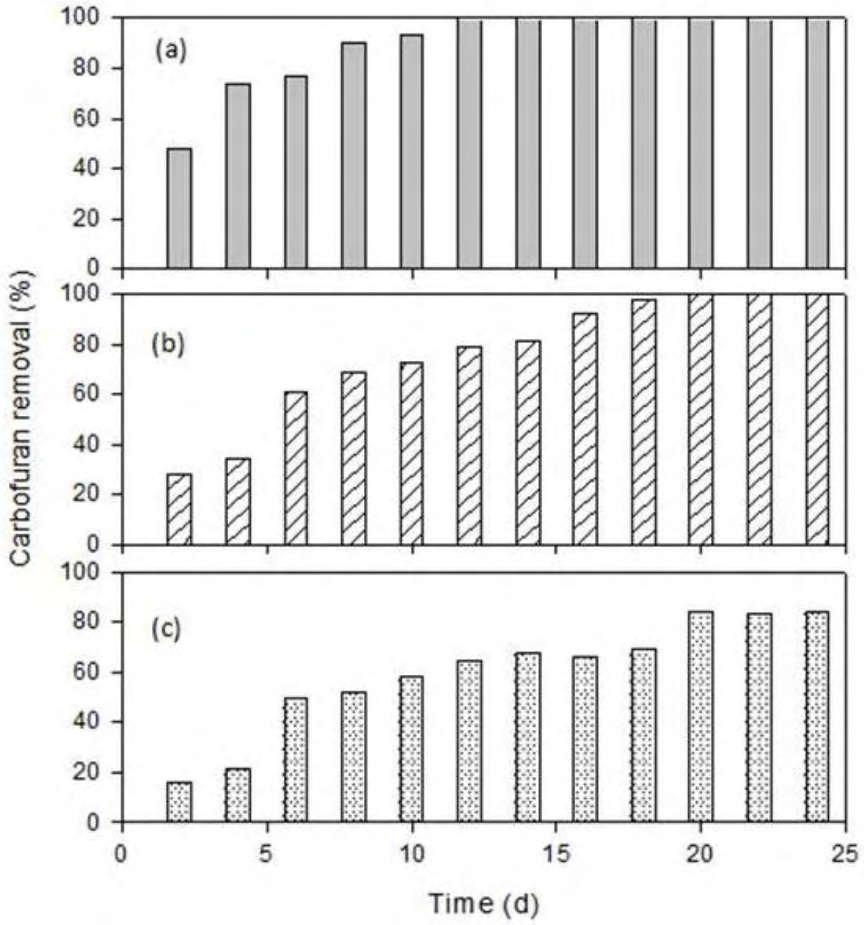

Fig. 7. Effect of carbofuran concentrations on the performance of SBRs; (a) = 20 mg l^{-1}, (b) = 40 mg l^{-1} and (c) = 80 mg l^{-1}.

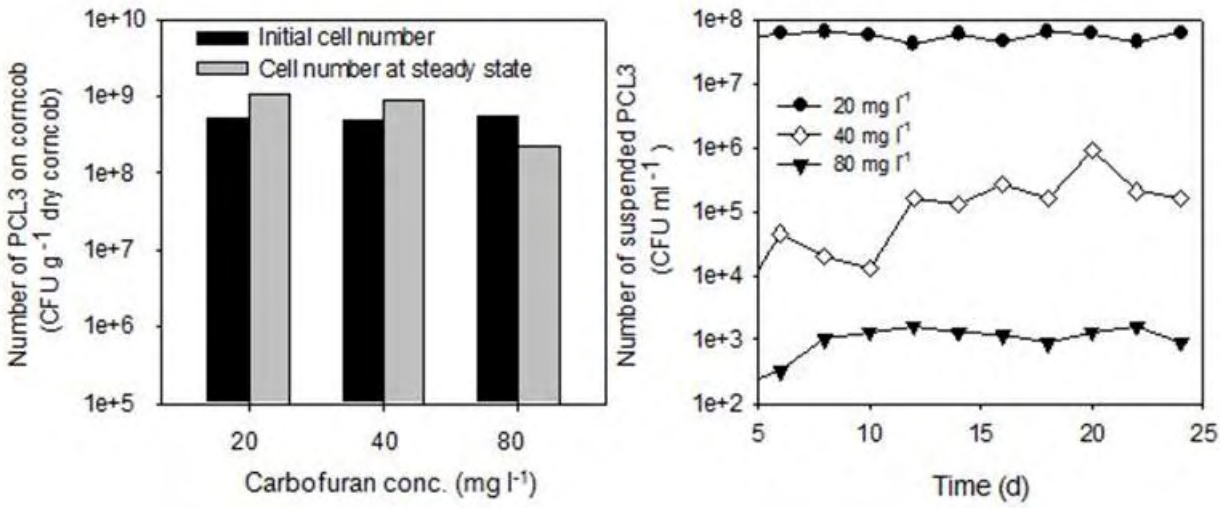

Fig. 8. Number of PCL3 in immobilized (a) and suspended (b) forms in SBRs Operated with varying carbofuran concentrations.

d. However, a further decrease in HRT to 6 d resulted in a decline in the rate of carbofuran degradation to 0.020 h^{-1} (Table 2). The degradation rates of carbofuran in SBRs were influenced by the addition of organic amendments. The addition of nitrogen sources, i.e. RB and SY resulted in an increase in the carbofuran degradation rate constant to approximately 0.043 h^{-1}. Whereas, the addition of CP and ML led to a marked decrease in the rate constants to 0.007 and 0.002 h^{-1}, respectively (Table 2). The kinetic constants of carbofuran degradation remained almost constant at 0.44 h^{-1} when the carbofuran concentration in the feed medium was increased from 20 mg l^{-1} to 40 mg l^{-1}. When the carbofuran concentration in the feed medium was increased to 80 mg l^{-1}, an obvious decrease in the degradation rate to 0.025 mg l^{-1} (Table 2) was observed. It can be concluded that the trends in the kinetic rate observed in this study reflected the inhibitory effect of carbofuran at high concentrations on the performance of SBRs.

The half-lives of carbofuran with regard to the degradation rate constant values were calculated and are shown in Table 2. These results indicated that the SBR together with bioaugmentation and biostimulation improved carbofuran degradation by approximately 3.5-fold compared to the batch reactor. The shortest half-life of carbofuran which was 15.57 h was obtained in SBRs stimulated with RB, and these SBRs had the highest carbofuran degradation efficiency when the carbofuran concentration was up to 40 mg l^{-1} in the feed medium.

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

Kinetic parameter	Batch		SBR										
	Control reactor	PCL3–augmented reactor	HRT (d)				Stimulated amendment *				Carbofuran conc. (mg l^{-1})		
			14	10	8	6	CP	ML	RB	SY	20	40	80
First-order rate, k_1 (h^{-1})**	0.0006g± 0.0001	0.013d± 0.001	0.037b± 0.002	0.035b± 0.002	0.34b± 0.003	0.020c± 0.002	0.007e± 0.001	0.002f± 0.0007	0.044a± 0.004	0.042a± 0.005	0.044a± 0.003	0.044a± 0.005	0.025c± 0.005
Half-life, $t_{1/2}$ (h)**	1,155.00g± 264.28	52.11d± 4.12	18.63b± 1.02	19.63b± 1.12	20.03b± 1.81	34.65c± 3.51	99.00e± 14.48	346.50f± 80.80	15.57a± 1.59	16.50a± 1.65	15.57a± 1.49	15.57a± 1.81	27.72c± 5.81
r^2	0.97	0.97	0.98	0.99	0.98	0.99	0.97	0.99	0.99	0.99	0.99	0.99	0.98

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

** Comparison between the treatments in row are significant different (Duncan, $p \leq 0.05$) if marked with different small letters.

Acknowledgements

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PART II

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

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ABSTRACT

The effectiveness of bioremediation technology in the removal of carbofuran from contaminated soil using a bioslurry phase sequencing batch reactor (SBR) was investigated. A 2-L laboratory glass bottle was used as a bioreactor with a working volume of 1.5 L at room temperature (27±2 ^{o}C). One total cycle period of the SBR was comprised of 1 h of fill phase, 82 h of react phase, and 1 h of decant phase. The carbofuran concentration in the soil was 20 mg/kg soil. A carbofuran degrader isolated from carbofuran phytoremediated soil, namely *Burkholderia cepacia* PCL3 (PCL3) immobilized on corncob, was used as the inoculum. The results revealed that bioaugmentation treatment (addition of PCL3) gave the highest percentage of carbofuran removal (96.97%), followed by bioaugmentation together with biostimulation (addition of molasses) treatment (88.23%), suggesting that bioremediation was an effective technology for removing carbofuran in contaminated soil. Abiotic experiments, i.e. autoclaved soil slurry with corncob and no PCL3 treatment and autoclaved soil slurry with no PCL3 treatment, could adsorb 31.86% and 7.70% of carbofuran, respectively, which implied that soil and corncob could act as sorbents for the removal of carbofuran.

Key words: Bio-slurry phase reactor; *Burkholderia cepacia* PCL3; Bioremediation; Carbofuran; Immobilization; Soil slurry

1. Introduction

Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate) is a broad-spectrum insecticide widely used in agriculture to control insects and nematodes on contact or after ingestion. Carbofuran is of environmental concern because it is soluble in water and highly mobile in soil, resulting in a high potential for groundwater contamination (Gapta, 1994). In 2003, Thailand imported carbofuran in solid and liquid forms up to 826.6 and 45.5 tons, respectively, for using in agriculture especially in rice fields (Department of Agriculture, 2003). Continuous use of carbofuran in the rice fields may subsequently exacerbate the risk of contamination of the soil and groundwater; thus, the removal of carbofuran is necessary.

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

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

contaminated matrices (Prasanna et al., 2008). Therefore, bioreactor technology which can be specially designed in a variety of configurations to maximize microbial activity has drawn our attention as a way to bioremediate carbofuran.

Soil-slurry phase reactors comprised of 10-40% soil in liquid (water) are a relatively new application for soil and sediment bioremediation. It is an alternative technology for decontamination of soil and sediment which minimizes the limiting effect of mass transfer on biodegradation (Venkata-Mohan et al., 2004; Valentin et al., 2007). Contaminant in a soil-slurry treatment system can partition itself to the soil and liquid phases and can be utilized by both indigenous microorganisms in the soil and inoculated specific degraders. Therefore, the degradation process can take place in the soil, water, and/or soil-water phases, resulting in an enhancement of contaminant degradation (Venkata-Mohan et al., 2006). Published data have indicated successful bioremediation of hazardous substances, especially pesticides such as pendimethalin (Ramakrishna et al., 2008) and hexachlorocyclohexane isomers (Quintero et al., 2005; 2006; Robles-Gonzalez et al., 2006), using a soil-slurry phase reactor. In addition, it has been reported that the efficiency of remediation of contaminated soil could be effectively improved by using a bioslurry reactor augmented with specific microorganisms capable of degrading the pesticide of interest. However, to the best of our knowledge, there has been no report on carbofuran remediation in a bioaugmented soil-slurry phase reactor.

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

2. Materials and Methods

2.1. Immobilization of B. cepacia PCL3

2.1.1. Microorganism preparation

B. cepacia PCL3 (accession number of EF990634) was used as the carbofuran degrader (Plangklang, 2004). It was grown in 100 mL nutrient broth (NB) containing 5

mg/L of carbofuran at 30°C and 150 rpm for 36 h and was used as seed inoculum for immobilization.

2.1.2. Support material preparation

Corncob was used as a support material to immobilize *B. cepacia* PCL3. This material has high matrix porosity and a pore size that could enhance the cell adsorption capability during immobilization. Corncob was cut into 0.7x0.7x0.7 cm pieces using a knife and then 300 g of cut corncob was boiled in 3 L of 1% NaOH for 3 h to remove lignin and fibers inside the materials which might react with the cells (Bradi and Koutinas, 1994). The alkaline-boiled corncob was washed three times with 3 L of distilled water, soaked in distilled water overnight, and then sterilized by autoclaving at 121^{o}C for 15 min and kept at 4 ^{o}C prior to usage.

2.1.3. Cell immobilization

Adsorption was used as the immobilization method in this study. This method is typically performed when porous media are used as support materials with the advantage of ease of operation. The immobilization technique was conducted by adding 75 g of sterile corncob to 250 mL of sterile NB containing 5 mg/L of carbofuran before inoculating with PCL3 (10^6 CFU/mL). The flask was then incubated at 150 rpm, at room temperature, for 48 h. After incubation, the support material was transferred to fresh NB containing 5 mg/L of carbofuran and incubated, as previously described, before harvesting by filtration through a Buchner filter funnel and washing with 0.85% NaCl by an aseptic technique. This process was repeated two times. Immobilized cells were kept at 4^{o}C until use in further experiments. The internal cell density on the corncob after immobilization was approximately 10^7 CFU/g dry material. The procedures for cell immobilization followed the method of Plangklang and Reungsang (2009).

2.2. Soil

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

2.3. Soil slurry preparation

Air-dried soil was spiked with carbofuran at a concentration of 20 mg/kg dry soil, well mixed by hand stirring, and kept at 4^{o}C for 24 h to induce the homogenous sorption of carbofuran over the soil particles. For soil slurry preparation, the impregnated soil was added to distilled water at a ratio of 1 g soil:20 mL distilled water before feeding to the reactor. The method of soil slurry preparation was adapted from Venkata-Mohan et al. (2006).

2.4. Soil slurry phase reactor configuration

A 2-L laboratory glass bottle was used as a reactor in this study with a working volume of 1.5 L and a suitable inlet and outlet arrangement as shown in Fig. 1. To prevent sorption of carbofuran on tubing system, the glass tubes were used inside the reactor. Due to the biodegradation of carbofuran was mostly occured by aerobic microorganisms, the reactor was operated with aerobic condition in this study. Oxygen was supplied from an air compressor through an air diffuser at the flow rate of 600 mL/min at the bottom of the bottle. The contents of the reactor were continuously mixed by a magnetic stirrer in which the magnetic bar with 0.4 cm in diameter and 4 cm in length was placed on the center of the bottom of the reactor. This mixing system was set to facilitate the uniform diffusion of air from the bottom of the reactor towards upflow direction and keep the soil slurry phase in a suspension form.

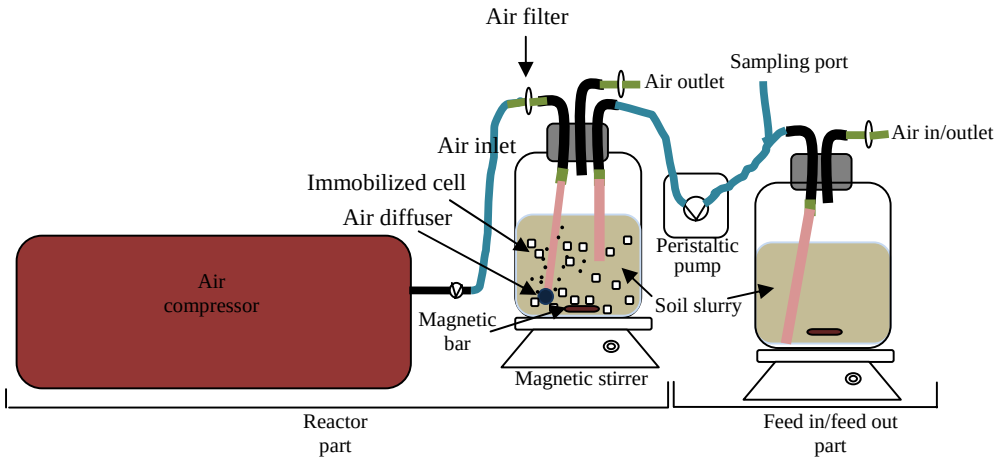

Fig. 1. Schematic diagram of soil slurry phase reactor (not subject to scale)

2.5. Soil slurry phase reactor operation

In this experiment, eight soil slurry phase reactors (A-H) (Table 1) were operated with an initial carbofuran concentration of 20 mg/kg dry soil. The soil slurry was prepared as described above and fed to the reactor at a flow rate of 25 mL/min using a peristaltic pump. For the bioaugmented reactors (A, C, and H), 100 g wet corncob immobilized with *B. cepacia* PCL3 (10^7 CFU/g dry corncob) were added to the reactor before feeding in the soil slurry. For the biostimulated reactors (G and H), molasses (1500 mg-COD/L) was mixed with the soil slurry before feeding to the reactor. Each reactor was aerobically operated in sequencing batch mode with a total cycle period of 84 h (HRT). The cycle period consisted of 1 h of fill phase, 82 h of react phase, and 1 h of

Table 1 Soil slurry phase reactor treatment

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

decant phase. The cycle period was fixed according to the results from our previous study in which the half-life of carbofuran in liquid medium (5 mg/L) degraded by *B. cepacia* PCL3 was 3 d (Plangklang and Reungsang, 2008; 2009). Aerobic conditions in the reactor were maintained by pumping air through an oxygen diffuser at an air flow rate of 600 mL/min. During the react phase, the reactor was continuously stirred using a magnetic stirrer to homogenize the contents and maintain air diffusion in the reactor. The soil slurry was sampled every 6 h via the sampling port using a peristaltic pump. The concentrations of carbofuran and its metabolites, i.e. carbofuran phenol and 3-keto carbofuran, in both the soil and liquid phases were determined by extraction using a liquid-liquid partitioning method followed by HPLC. The pH values of the soil slurry were measured using a digital pH meter (Sartorius, Germany). The number of carbofuran degraders in the soil slurry was enumerated by a drop-plate technique.

2.6. Analysis method

2.6.1. Extraction of carbofuran in soil slurry

In order to extract carbofuran and its metabolites from the soil and liquid phases, the soil slurry was centrifuged at 6000 rpm and 25^{o}C for 15 min to separate the liquid from the soil. The liquid phase was filtered through cellulose acetate paper number 1 (Whatman, England) prior to the liquid-liquid partitioning extraction procedure. Briefly, 2 mL of methanol were added to 2 mL of liquid sample and then the mixture was sonicated twice for 10 min on a 50/60 voltage cycle. After sonication, carbofuran and its metabolites were extracted in a separation funnel with dichloromethane. This extraction was performed 3 times with 4, 2, and 2 mL of dichloromethane. The organic fractions from the extractions were collected, pooled, and evaporated in the fume hood, then re-dissolved in 4 mL of 60% methanol and passed through a 0.45 μm nylon membrane syringe filter before analysis by HPLC.

The soil phase was air dried at room temperature (30±2^{o}C) and weighed in an HDPE tube, and 60% methanol was added at a ratio of 1:200 (w/v) soil:methanol. The tube was horizontally shaken at 200 rpm for 30 min and further centrifuged at 6000 rpm and 10^{o}C for 10 min to separate the supernatant. This step was repeated twice and the supernatants were pooled and filtered through cellulose acetate paper number 1 prior to the liquid-

liquid partitioning extraction procedure described above. The final volume of the extract was adjusted to 0.4 mL and then filtered through a nylon membrane filter disc before analysis by HPLC. Percentage recoveries of this procedure were 94.4, 93.2 and 94.0% for carbofuran, carbofuran phenol and 3-ketocarbofuran, respectively.

2.6.2. HPLC analysis of carbofuran and its metabolites

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

2.6.3. Kinetic analysis of carbofuran degradation in soil phase

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

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

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

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

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

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

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

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

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

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

2.6.4. Enumeration of carbofuran degraders in the soil slurry by the drop-plate technique

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

3. Results and Discussions

3.1. Soil slurry phase reactor performance

3.1.1. Non-augmented and abiotic reactors

The degradation profiles and degradation efficiency of carbofuran in the soil slurry for the non-augmented reactors (B and D) and abiotic control reactors (E and F) as a function of operation time are depicted in Fig. 2(a). Reactor B, which had only indigenous microorganisms in the soil, exhibited marked carbofuran degradation (Fig. 2(a)) with a relatively high efficiency of 67.69%. This indicated that there might be some carbofuran degraders presented as native microflora in the soil. The rice field where the soil samples were collected had a history of carbofuran application with a background carbofuran concentration of 0.06 mg/kg dry soil; therefore, microorganisms in the soils would have been able to adapt to use carbofuran as an energy source (Plangklang and Reungsang, 2008).

The addition of autoclaved corncob to the soil slurry (reactor D) increased the carbofuran degradation efficiency to 70.12%. In addition, the abiotic experiments (i.e. autoclaved soil slurry with corncob and no additional PCL3 treatment) demonstrated an adsorbance of 31.86% of carbofuran, which implied that corncob could act as a sorbent

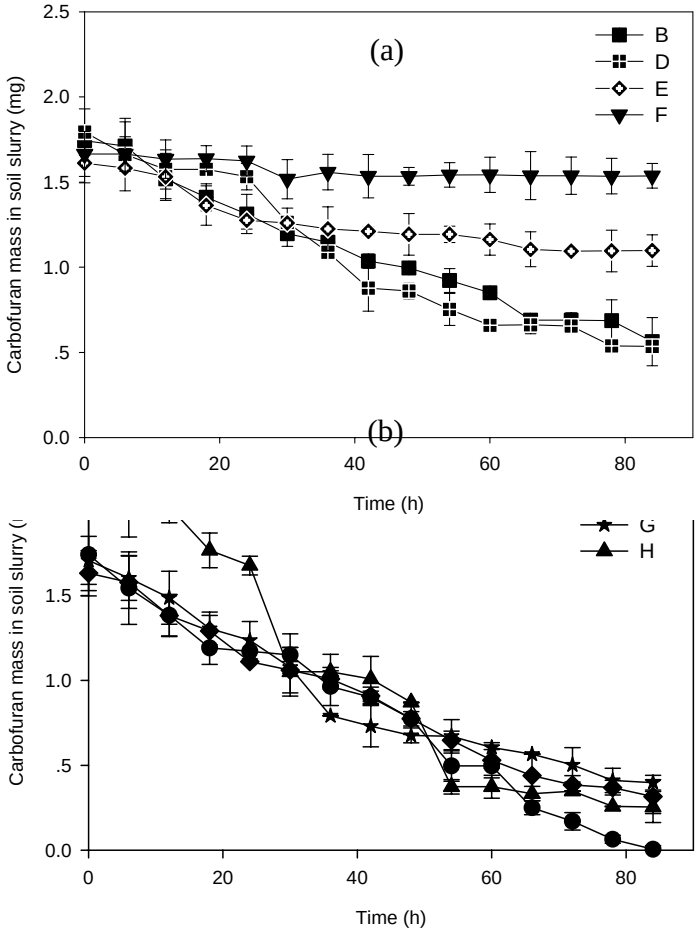

Fig. 2. Degradation profiles of carbofuran in soil slurry phase reactors. (a) Without bioremediation treatment (reactor B: soil slurry; reactor D: soil slurry + autoclaved corncob; reactor E: autoclaved soil slurry + autoclaved corncob; reactor F: autoclaved soil slurry); (b) with bioremediation treatments (reactor A: soil slurry + immobilized PCL3; reactor C: autoclaved soil slurry + immobilized PCL3; reactor G: soil slurry + molasses; reactor H: soil slurry + immobilized PCL3 + molasses)

for the removal of carbofuran. However, it should be noted that the sorption of carbofuran onto the support material could lead to a higher degradation rates when the immobilized PCL3 was used. Abiotic control (reactor F, autoclaved soil slurry) showed an insignificant carbofuran degradation profile (Fig. 2(a)). However, a relatively low carbofuran degradation efficiency of 7.70% was found (Fig 2(a)). This might have

resulted from oxidation and volatilization processes due to the continuous aeration during reactor operation. Although the oxidation and volatilization are not as important as microbial degradation to carbofuran dissipation, they were contributing to dissipation processes which could be found in abiotic control as reported in previous study (Lalah and Wandiga 1996).

3.1.2. Effect of bioremediation treatments

The effects of bioaugmentation, biostimulation, and bioaugmentation together with biostimulation treatments on carbofuran degradation in the soil slurry phase reactor were investigated in this experiment. The carbofuran degradation profiles in soil slurry phase reactors with bioremediation treatments as a function of operation time are depicted in Fig. 2(b). A significant improvement in carbofuran degradation in the soil slurry could be found when these bioremediation techniques were compared to non-augmented experiments. The highest carbofuran degradation of 96.97% could be achieved with the bioaugmentation treatment (addition of immobilized PCL3, reactor A), followed by degradation of 88.23% by bioaugmentation together with biostimulation treatments (addition of PCL3 and molasses, reactor H) and degradation of 76.70% by biostimulation treatment (addition of molasses; reactor G).

Molasses used as the organic amendment in the biostimulation treatment (reactor G) could improve the carbofuran degradation efficiency of the indigenous microorganisms in which the carbofuran degradation was increased from 67.69% to 76.70% as compared to reactor B (Fig. 2). However, the reduction in carbofuran degradation efficiency (8.74%) after the addition of molasses to the augmented reactor could be observed (reactor H compared to reactor A). This might due to the fact that PCL3 prefered to use sugars in molasses than carbofuran since sugars have less complex structures and are easier to be metabolized (Plangklang and Reungsang, 2009). In addition, there might be some adverse effects from substances contained in molasses, such as metal ions (Liu et al., 2008) or by-products of molasses metabolism, on PCL3 augmented to the reactor.

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

Our previous research (Plangklang and Reungsang, 2008) investigated carbofuran degradation in soil and rhizosphere soil microcosms in which soils were collected from the same rice field as presented in this study. The experiments were conducted using soil microcosms with an initial carbofuran concentration of 5 mg/kg dry soil. The bioaugmentation technique, free cell of PCL3 inoculation, was applied to both kinds of soil in order to improve the carbofuran degradation efficiency. When compared to carbofuran degradation in soil microcosms, the soil slurry phase bioreactor showed a better performance in carbofuran degradation. The shortest time to achieve 90% carbofuran degradation in the bioaugmented soil microcosms was 50 d (Plangklang and Reungsang, 2009), which was 14.3 times longer than using the soil slurry phase treatment (3.5 d) (Fig. 2(b)), even though the initial carbofuran concentration in the soil microcosms was 4 times lower than in the soil slurry phase reactor. These findings indicate that the bioslurry phase reactor could be applied for the purpose of significantly enhancing carbofuran degradation efficiency in the soil. The aeration together with mechanical mixing might be responsible for this trend by improving the mass transfer rate and contact among microorganisms, carbofuran, and nutrients, and hence increasing the rates of carbofuran biodegradation. This finding was similar to the results from previous studies (Ventaka-Mohan et al., 2004; 2006; Quintero et al., 2005; 2006).

Carbofuran phenol and 3-keto carbofuran were observed to be the metabolites in both soil and liquid phases in the reactors with biological activity (reactors A, B, C, D, G and H) (Table 2 and 3). In the soil phase, carbofuran phenol and 3-keto carbofuran could be observed in reactors A, B, C, D and H from 6 to 42 h while, in reactors D and G, these metabolites could be found until the end of reactor operation. Higher concentrations of carbofuran metabolites were found in the liquid phase than in the soil phase, and the accumulation of carbofuran phenol and 3-keto carbofuran in the liquid phase could be found in all bioreactors until the end of reactor operation except for reactors A and H. A decrease in carbofuran metabolites during reactor operation implied that the microorganisms in the soil slurry phase reactors might be able to metabolize carbofuran metabolites as their energy sources, as was reported in a study by Yan et al. (2007).

Table 2 Carbofuran metabolites, i.e. carbofuran phenol (CP) and 3-ketocarbofuran (3KC) (mg) observed in soil phase during soil slurry phase reactor operation

Time	Reactor A		Reactor B		Reactor C		Reactor D		Reactor E		Reactor F		Reactor G		Reactor H	
(h)	CP	3KC	CP	3KC	CP	3KC	CP	3KC	CP	3KC	CP	3KC	CP	3KC	CP	3KC
nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
6	nd	nd	nd	0.046	0.051	0.047	0.052	nd	nd	nd	nd	nd	0.058	0.043	nd	nd
12	0.188	0.135	0.077	0.025	0.064	0.066	0.054	0.125	nd	nd	nd	nd	0.078	0.075	0.083	nd
18	0.285	0.057	0.068	0.036	0.095	0.135	0.095	0.060	nd	nd	nd	nd	0.064	0.116	0.058	0.047
24	0.400	nd	0.075	0.030	0.062	0.033	0.051	0.031	nd	nd	nd	nd	0.114	0.085	0.052	0.015
30	nd	nd	0.053	nd	0.054	0.037	nd	0.070	nd	nd	nd	nd	0.134	0.151	nd	0.06
36	0.131	nd	0.060	nd	0.059	0.023	0.059	0.100	nd	nd	nd	nd	0.124	0.147	0.053	nd
42	nd	0.050	0.056	0.034	0.052	0.034	0.059	nd	nd	nd	nd	nd	0.108	0.092	0.050	0.010
48	nd	nd	nd	nd	nd	nd	nd	0.006	nd	nd	nd	nd	nd	0.039	nd	nd
54	nd	nd	nd	nd	nd	nd	nd	0.007	nd	nd	nd	nd	0.055	0.047	nd	nd
60	nd	nd	nd	nd	nd	0.041	nd	nd	nd	nd	nd	nd	0.052	0.056	nd	nd
66	nd	nd	nd	nd	nd	nd	0.008	0.013	nd	nd	nd	nd	nd	nd	nd	nd
72	nd	nd	nd	nd	nd	0.046	0.036	0.017	nd	nd	nd	nd	0.052	nd	nd	nd
78	nd	nd	nd	nd	nd	0.034	0.027	0.008	nd	nd	nd	nd	0.056	0.030	nd	nd
84	nd	nd	nd	nd	nd	nd	0.006	0.001	nd	nd	nd	nd	0.053	0.036	nd	nd

nd = not detectable

Table 3 Carbofuran metabolites, i.e. carbofuran phenol (CP) and 3-ketocarbofuran (3KC) (mg) observed in liquid phase during soil slurry phase reactor operation

Time	A		B		C		D		E		F		G		H	
(h)	CP	3KC	CP	3KC	CP	3KC	CP	3KC	CP	3KC	CP	3KC	CP	3KC	CP	3KC
0	nd	0.03	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
6	nd	nd	nd	0.092	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
12	0.413	0.110	0.264	0.113	0.050	0.136	0.054	0.052	nd	nd	nd	nd	nd	0.042	0.052	0.055
18	0.551	0.241	0.388	0.204	0.343	0.048	0.341	0.137	nd	nd	nd	nd	0.051	0.147	0.277	0.046
24	0.547	0.092	2.62	0.118	0.557	0.112	0.320	0.118	nd	nd	nd	nd	0.326	0.039	0. 441	0.007
30	nd	nd	0.55	0.044	0.350	0.207	0.187	0.148	nd	nd	nd	nd	0.240	0.148	0.335	0.013
36	nd	nd	0.43	0.181	0.210	0.138	0.160	0.089	nd	nd	nd	nd	0.255	0.142	0.211	nd
42	nd	nd	1.18	nd	0.102	nd	0.252	nd	nd	nd	nd	nd	0.188	nd	0.147	nd
48	nd	0.075	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.089	nd
54	0.284	0.050	nd	0.120	nd	0.036	0.170	0.056	nd	nd	nd	nd	0.322	0.089	nd	0.064
60	0.054	nd	1.22	0.013	0.090	0.073	0.106	0.06	nd	nd	nd	nd	nd	nd	nd	nd
66	nd	nd	1.89	nd	0.079	nd	0.155	0.087	nd	nd	nd	nd	0.197	0.091	0.022	0.035
72	nd	nd	0.70	0.020	nd	0.167	0.102	nd	nd	nd	nd	nd	0.213	0.065	nd	nd
78	nd	nd	nd	nd	0.057	0.083	0.084	0.103	nd	nd	nd	nd	0.153	0.039	nd	nd
84	nd	nd	nd	0.012	nd	0.095	0.111	0.046	nd	nd	nd	nd	0.077	0.079	nd	nd

nd = not detectable

3.2. Substrate partitioning between soil and liquid phases

Carbofuran partitioning in the soil and liquid phases during soil slurry phase reactor operation was investigated (Figs. 3 and 4). Substrate partitioning in the abiotic control reactor (reactor F) (Fig. 3) indicated the actual mechanism of carbofuran partitioning in the two phases of the slurry system was insignificant. After start-up of the reactor, carbofuran rapidly desorbed from the soil to the aqueous phase (Fig. 3). The partitioning of carbofuran approached steady state at 30 h of reactor operation with carbofuran concentrations in the soil and liquid phases of approximately 0.75 and 0.79 mg, respectively (Fig. 3). Reactor E, autoclaved soil with autoclaved corncob added, showed the same trend of carbofuran partitioning as reactor F (Fig. 3). The relatively high desorption of carbofuran from the soil into the liquid phase might be attributed to the hydroponic nature of carbofuran, i.e. its high water solubility of 351 mg/L at 25°C and low adsorption coefficient (Koc) of 22 (Hornsby and Wauchope, 1996). Desorption of carbofuran from the soil to the liquid phase could facilitate the mass transfer and increase contact between the microorganisms and carbofuran, which might result in an improvement in carbofuran degradation efficiency in the soil slurry phase reactor.

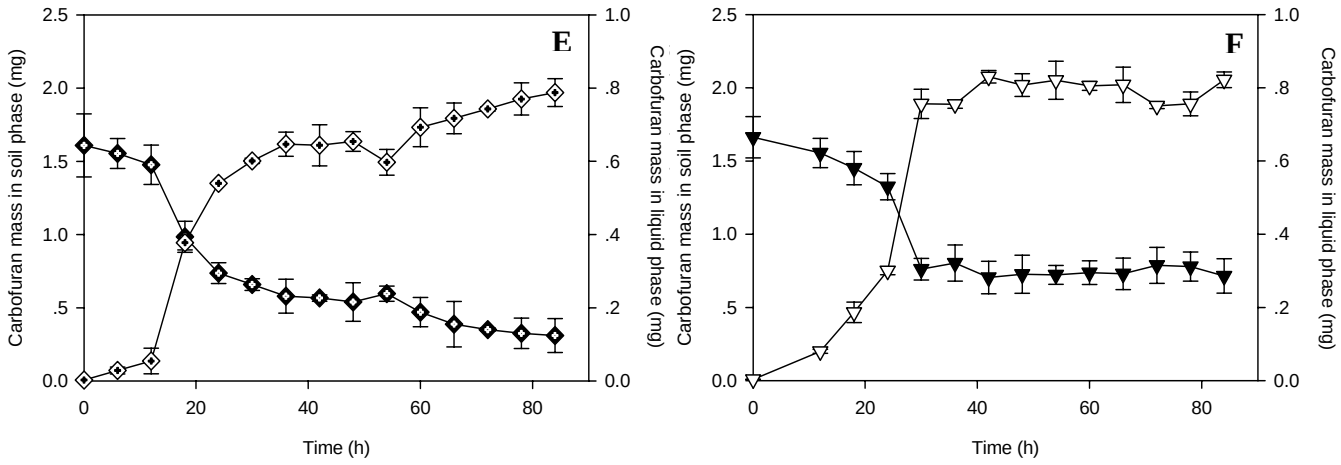

Fig. 3. Carbofuran partitioning between soil (close symbols) and liquid (open symbols) phases in abiotic control soil slurry phase reactors (reactor E: autoclaved soil slurry + autoclaved corncob; reactor F: autoclaved soil slurry)

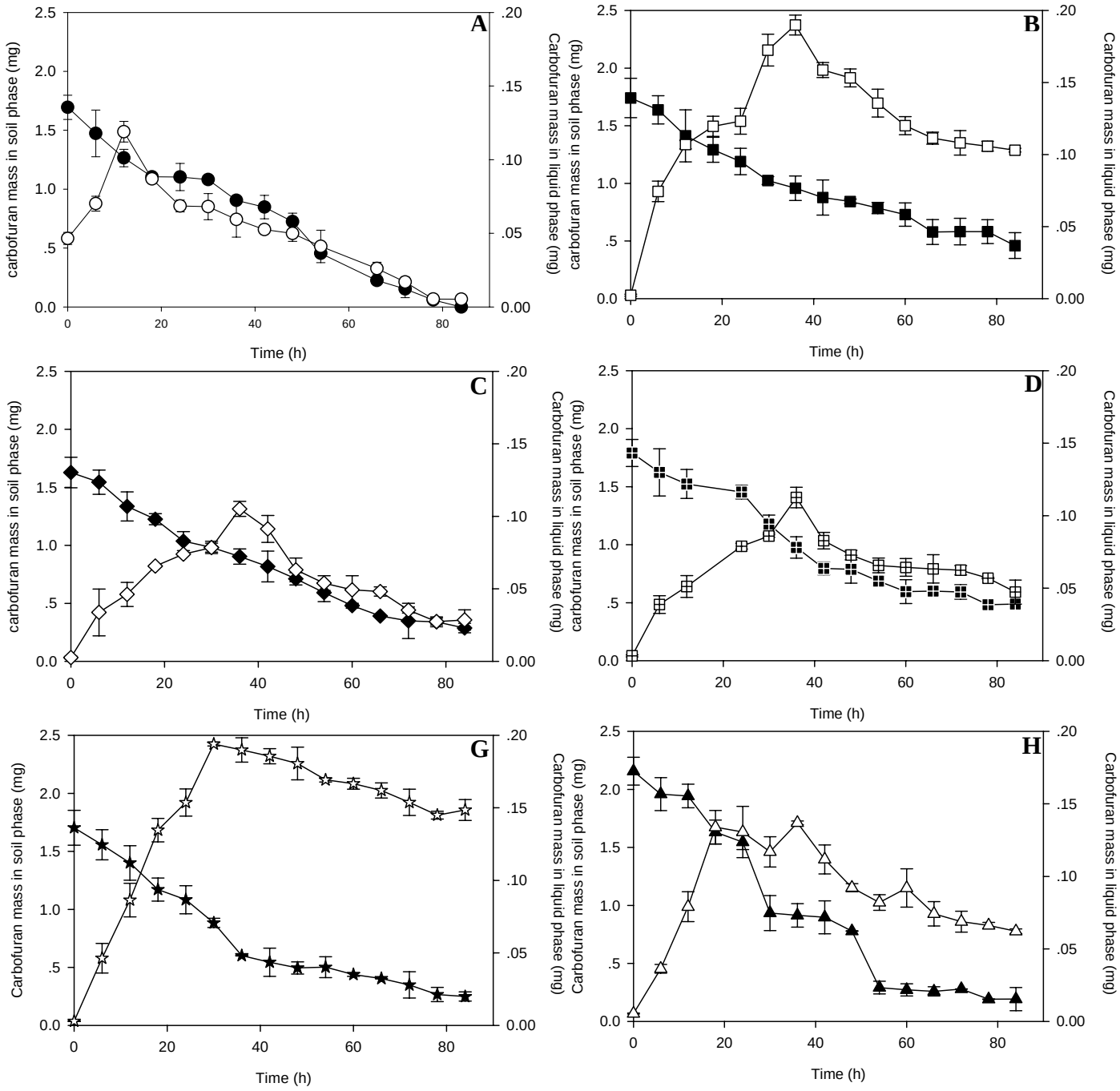

Fig. 4. Carbofuran partitioning between soil (close symbols) and liquid (open symbols) phases in soil slurry phase reactors with biological activity (reactor A: soil slurry + immobilized PCL3; reactor B: soil slurry; reactor C: autoclaved soil slurry + immobilized PCL3; reactor D: soil slurry + autoclaved corncob; reactor G: soil slurry + molasses; reactor H: soil slurry + immobilized PCL3 + molasses)

Carbofuran partitioning in the reactors with microbial activity (Fig. 4), i.e. bioaugmentation (reactors A, C and H), biostimulation (reactors G and H), and indigenous microorganisms (reactors B and D) was obviously different from that in the abiotic reactors (E and F). In reactors A and H, the carbofuran concentration in the liquid phase increased until 18 h of reactor operation, reaching maximum concentrations of 0.11 and 0.13 mg, respectively, and then rapid degradation of carbofuran was observed (Fig. 4). The continuous decrease in carbofuran concentration in the soil phase continued until the end of the experiment. This result indicated that desorbed carbofuran in the liquid phase was subjected to continuous biological degradation; therefore, desorption was enhanced due to the partitioning effect. The same partitioning patterns of carbofuran in the soil and liquid phases could be found in reactors B, D, C and G, with longer times of 30-36 h taken to approach the maximum carbofuran concentrations in the liquid phase of 0.11-0.19 mg (Fig. 4). As the carbofuran degradations in reactors A and H were more rapid and started earlier than in the other reactors, it could be concluded that PCL3 together with indigenous microorganisms in reactors A and H were more effective in enhancing carbofuran degradation than PCL3 (reactor C) or indigenous microorganisms (reactors B, D and G) alone in the reactor.

3.3. pH values and CFU variations

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

al., 2006). Reactors E and F showed relatively stable soil slurry pH values during operation (Fig. 5), which might due to the absence of microbial activity (Ventaka-Mohan et al., 2008).

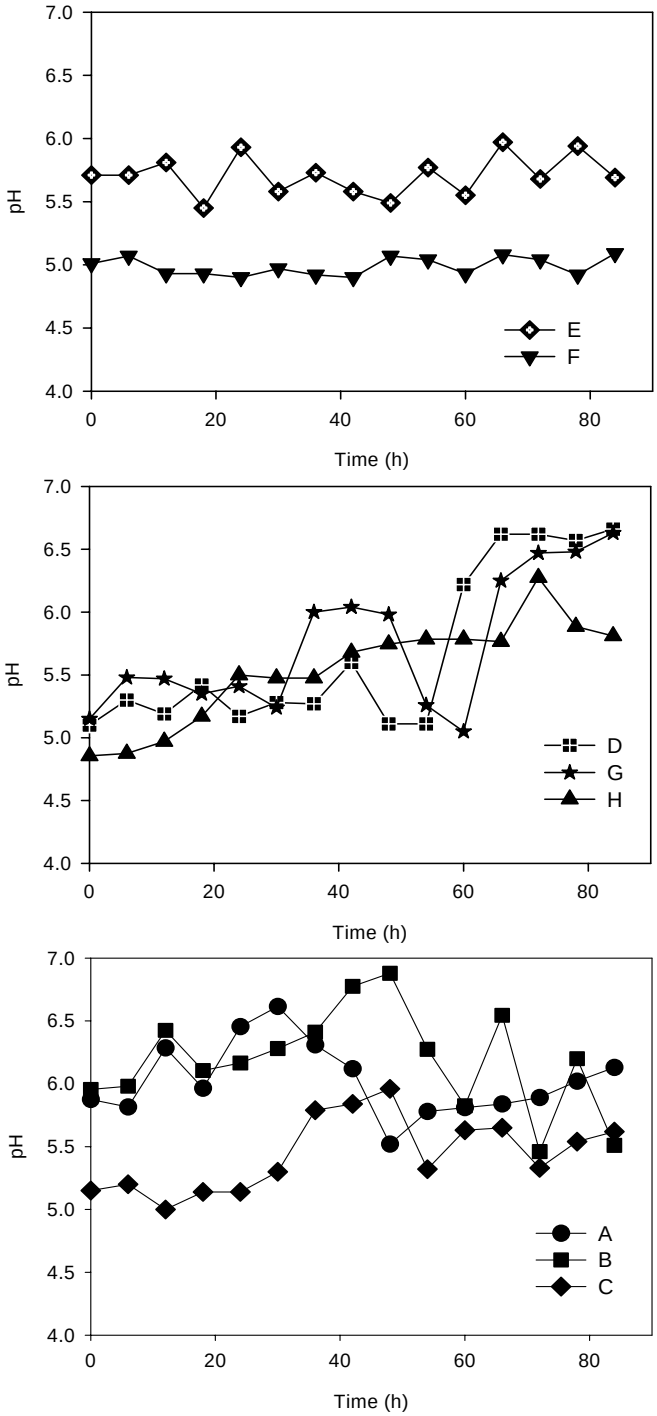

Fig. 5. The temporal variation of soil slurry phase pH during reactor operation

The number of carbofuran degraders in the soil slurry phase reactors with biological activity in terms of CFU (Fig. 6) was determined on a BSM agar plate coated with carbofuran as a sole carbon source. The results indicated the successful development of indigenous microbial numbers in the soil slurry phase reactor, which was shown by the marked increase in the numbers of carbofuran degraders (by one order of magnitude) in reactors operated with indigenous microorganisms alone (reactors B and D) (Fig. 6). However, the number of indigenous carbofuran degraders in the stimulated reactor (G) decreased by one order of magnitude, which might due to the toxic effects of by-products from molasses components and the metabolism of molasses by the indigenous microorganisms themselves. In the augmented reactor (reactor A), the number of carbofuran degraders in the soil slurry obviously varied over time. Though the CFU in reactor A decreased markedly by two orders of magnitude in some periods of operation (6-24 h and 42-66 h) (Fig. 6), the greatest carbofuran degradation efficiency was obtained in this reactor. This result implied that carbofuran degradation did not depend on the efficacy of free cell of carbofuran degraders suspended in the soil slurry, but might mainly depend on the immobilized PCL3 adsorbed on the support. In reactor H, to which was added both immobilized PCL3 and molasses, the number of carbofuran degraders increased by two orders of magnitude (Fig. 6). This result was different from the CFU variation in reactor G, which showed the negative effect of molasses on the growth of carbofuran degraders in the soil slurry. The explanation for this phenomenon is that PCL3 in immobilized cell form could be protected from the toxic effects of the by-products from molasses components or molasses metabolism (Bekatorou et al., 2004). In contrast, molasses might well be used by PCL3 as an energy source, resulting in continuous growth of the cell and leakage to the soil slurry due to the limited space in the support material. This was attributed to an increase in the number of carbofuran degraders in the soil slurry with a decrease in carbofuran degradation efficiency, as previously described.

Number of PCL3 in soil slurry of reactor C containing autoclaved soil slurry inoculated with immobilized PCL3 was greater than 10^5 CFU/mL throughout the experiment (Fig. 6) ensuring that the augmented degraders could survive during soil slurry phase reactor operation.

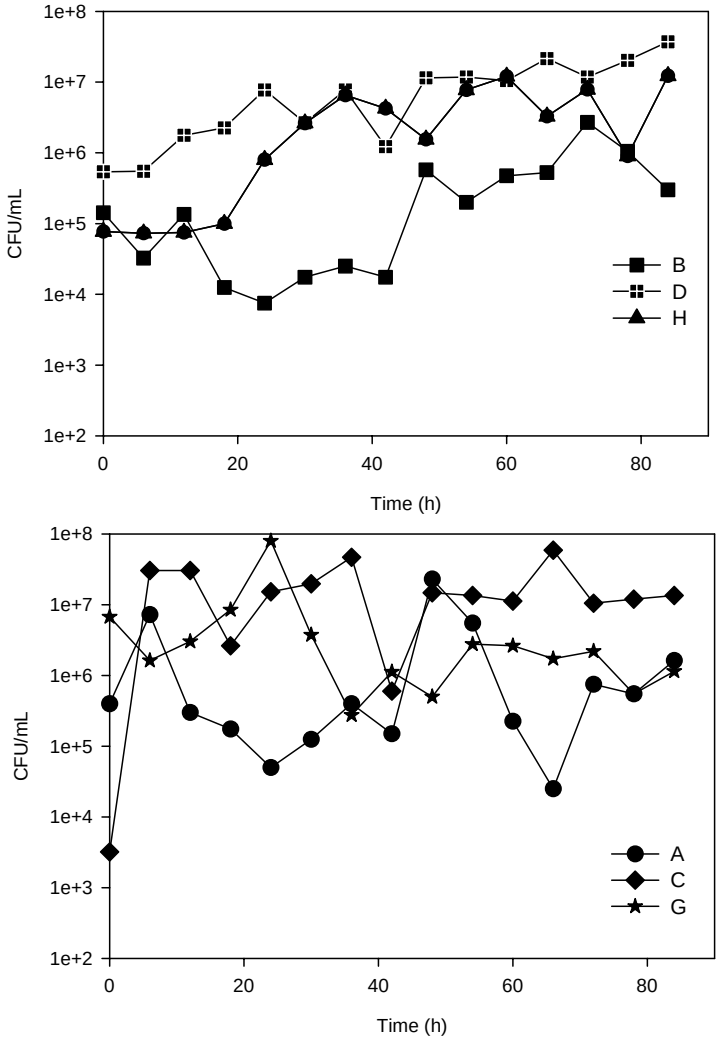

Fig. 6. The temporal CFU variation during soil slurry phase reactor operation

3.4. Kinetic degradation of carbofuran in soil slurry phase reactor

The kinetic aspect of carbofuran degradation in the soil slurry was examined in this study using zero-, first-, and second-order equations. The kinetic parameters of carbofuran degradation in the soil phase derived from analysis of the output of the three equations along with R^2 values are shown in Table 4. A reasonably good correlation was indicated by R^2 of more than 0.90. In the abiotic control reactor (F), the carbofuran degradation pattern did not follow any of the kinetic equations (R^2 = 0.64-0.66) (Table 4). This might be due to the fact that the desorbed carbofuran in the aqueous phase was not further degraded due to the absence of microorganisms in the reactor. Reactors A, C, E, and H showed a good correlation with the zero-order kinetic

Table 4 Kinetic parameters for carbofuran degradation in soil phase

Reactor	Order of equation	Degradation rate constant	Degradation half-life (h)[(1)]	Kinetic equation	R^2
A	Zero	0.020±0.0028 mg/h	39.75±5.57fg	$C_t = 1.59-0.020t$	0.98
	First	0.037±0.0021 /h	18.73 ±1.06cd	$C_t=2.43e^{-0.037t}$	0.85
	Second	0.083±0.0087 /(mg h)	7.11±0.75ab	$1/C_t=0.083t+0.59$	0.43
B	Zero	0.015±0.0012 mg/h	53.00±4.28i	$C_t = 1.59-0.015t$	0.95
	First	0.015 ± 0.0013 /h	46.20±4.00ghi	$C_t=1.71e^{-0.015t}$	0.99
	Second	0.017±0.0013 /(mg h)	27.06±2.07de	$1/C_t=0.017+0.46$	0.95
C	Zero	0.016±0.0013 mg/h	47.81±3.88ghi	$C_t = 1.53-0.016t$	0.97
	First	0.021±0.0015 /h	33.00±2.36ef	$C_t=1.79e^{-0.021t}$	0.96
	Second	0.034±0.0013 /(mg h)	5.88±0.22a	$1/C_t=0.034t+0.20$	0.91
D	Zero	0.016±0.0021 mg/h	52.50±6.89hi	$C_t = 1.68-0.016t$	0.93
	First	0.017±0.0014 /h	40.76±3.36fg	$C_t=1.84e^{-0.017t}$	0.97
	Second	0.019±0.0023 /(mg h)	21.58±2.61cd	$1/C_t=0.019t+0.41$	0.96
E	Zero	0.015±0.0013 mg/h	46.00±3.99ghi	$C_t = 1.38-0.015t$	0.96
	First	0.021±0.0022 /h	33.00±3.46ef	$C_t=1.50e^{-0.021t}$	0.93
	Second	0.031±0.0009 /(mg h)	15.16±0.44bc	$1/C_t=0.031t+0.47$	0.96
F	Zero	0.011±0.0014 mg/h	66.82±8.50j	$C_t = 1.47-0.011t$	0.66
	First	0.010±0.0015 /h	69.30±10.40j	$C_t=1.04e^{-0.010t}$	0.65
	Second	0.010±0.0017 /(mg h)	71.00±12.07j	$1/C_t=0.010t+0.71$	0.64
G	Zero	0.017±0.0017 mg/h	44.12±4.41gh	$C_t = 1.50-0.017t$	0.91
	First	0.023±0.0018 /h	30.13±2.36d	$C_t=1.72e^{-0.023t}$	0.98
	Second	0.039±0.0022 /(mg h)	5.13±0.29a	$1/C_t=0.039t+0.20$	0.93
H	Zero	0.026±0.0010 mg/h	39.42±1.52fg	$C_t = 2.05-0.025t$	0.93
	First	0.032±0.0020 /h	21.66±1.35cd	$C_t=2.67e^{-0.032t}$	0.93
	Second	0.061±0.0042 /(mg h)	7.38±0.51ab	$1/C_t=0.047t+0.45$	0.80

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

equation (R^2 = 0.93-0.98) (Table 4), which indicated that the degradation process in the soil phase of these reactors was independent of carbofuran concentration and the carbofuran degradation would not be inhibited by carbofuran concentration. Reactors B, D, and G showed a

good correlation with the first-order kinetic equation (R^2 = 0.97-0.99) (Table 4), revealing that the rate of degradation was directly proportional to the concentration of carbofuran in the soil phase of these reactors.

The half-lives of carbofuran were calculated and are depicted in Table 4. The half-life of carbofuran in the non-augmented reactor (B) was 46.2 h, which was longer than the half-lives of carbofuran in the reactors with bioremediation treatments (reactors A, C, D, G, and H) of 30.13-40.76 h (Table 4), indicating an improvement in carbofuran degradation by the bioaugmentation and/or biostimulation techniques. In this study the shortest half-life of carbofuran in the soil phase of 30.13 h was obtained in reactor G (Table 4), but the greatest efficiency of carbofuran removal was obtained in reactor A (96.97%), with a short half-life of carbofuran in the soil of 39.75 h (Table 4). To understand this phenomenon, we further investigated the kinetics of the degradation of carbofuran in the liquid phase after starting the degradation process in each reactor. The kinetic parameters along with R^2 for carbofuran degradation in the liquid phase of each reactor with the best correlation equations are presented in Table 5. The degradation of carbofuran in reactors E and F was insignificant; therefore the degradation kinetics were not examined in these reactors. The carbofuran half-life in the liquid phase of reactor G (148.53 h) was significantly longer than that in the liquid phase of reactor A (50 h) (Table 5). In addition, the carbofuran degradation process in reactor A started after 18 h of reactor operation, which was earlier than in reactor G (Fig. 4). Therefore, we could conclude that reactor A was the most

Table 5Kinetic parameters for carbofuran degradation in liquid phase

Reactor	Order of equation (best fitted)	Degradation rate constant	Degradation half-life (h)[(1)]	Kinetic equation	R^2
A	Zero	0.001±0.00009 mg/h	50.00±4.51b	$C_t = 0.10-0.001t$	0.98
B	First	0.011±0.0011 /h	63.00±6.32c	$C_t=0.24e^{-0.011t}$	0.93
C	First	0.027±0.0042 /h	25.67±3.97a	$C_t=0.253e^{-0.027t}$	0.94
D	First	0.011±0.0013 /h	63.00±7.44c	$C_t=0.12e^{-0.011t}$	0.90
G	Second	0.034±0.0003 /(mg h)	148.53±1.31c	$1/C_t=0.034t+5.05$	0.97
H	First	0.013±0.0014 /h	53.31±5.72bc	$C_t=0.17e^{-0.013t}$	0.98

[(1)] Comparison between treatment in column are significantly different (Duncan, p≤0.05) if marked with different small letters

effective treatment to remediate carbofuran, as indicated by the highest performance (96.97%) and shortest half-lives in the soil (Table 4) and the liquid phase (Table 5) of 39.75 h and 50 h, respectively. The carbofuran degradation pattern in the liquid phase of reactor B followed the second-order kinetics equation, indicating dependence on the concentrations of both the carbofuran and intermediates formed during the biodegradation process.

4. Conclusion

The soil slurry phase reactor was an effective technique for removing carbofuran from the soil. Desorption of carbofuran from the soil to the liquid phase offered favorable conditions for the biological degradation process of carbofuran. Bioaugmentation treatment could be applied together with the soil slurry phase bioreactor to enhance the carbofuran degradation efficiency in the soil. Variations of pH and CFU during the degradation process correlated with biological activity in the soil slurry phase reactors. Soil slurry augmented with immobilized PCL3 (reactor A) was the most effective treatment for bioremediating carbofuran giving the highest percentage of carbofuran removal (96.97%). Biostimulation (addition of molasses) treatment could improve the carbofuran degradation efficiency of indigenous microorganisms in soil, however, worsen the ability of PCL3 to degrade carbofuran.

Acknowledgements

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PART III

BIOAUGMENTATION OF CARBOFURAN RESIDUES IN SOIL BY *Burkholderia cepacia* PCL3: A SMALL-SCALE FIELD STUDIES

A manuscript prepared as short communication for submitting to New Biotechnology

ABSTRACT

The small-scale field studies were conducted in the plots with the dimension of 1 m x 1.25 m x 0.20 m in order to validate the *in situ* bioaugmentation by using free and immobilized *Burkholderia cepacia* PCL3 on corncob before implemented this technology on a large scale. In the soil with the presence of only indigenous microorganisms, the degradation of carbofuran was slow with the long $t_{1/2}$ of 127 d. Bioaugmented the soil with immobilized PCL3 could shorten the $t_{1/2}$ of carbofuran in soil to be 16 d. The significant longer $t_{1/2}$ of 28 d in soil was observed when the free cells of PCL3 were used in comparison to immobilized cells which suggested that bioaugmentation of carbofuran by using PCL3 in free cell form might not applicable. Growth and survival of carbofuran degraders in soil and in support materials were examined and the results indicated that the efficiency of carbofuran degradation directly correlated with the number of introduced carbofuran degrader surviving in the system.

Keywords: bioremediation, bioaugmentation, *Burkholderia cepacia* PCL3, carbofuran, field study

1. Introduction

Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate) is a broad-spectrum insecticide widely used in agriculture to control insects and nematodes on contact or after ingestion. Carbofuran is of environmental concern because it is soluble in water and highly mobile in soil, resulting in a high potential for groundwater contamination (Gapta, 1994). In 2003, Thailand imported carbofuran in solid and liquid forms up to 826.6 and 45.5 tons, respectively, for using in agriculture especially in rice fields (Department of Agriculture, 2003). Continuous use of carbofuran in the rice fields may subsequently exacerbate the risk of contamination of the soil and groundwater; thus, the removal of carbofuran is necessary.

One of the effective routes for pesticide removal is microbial degradation by a specific degrader and/or indigenous microorganisms. Our previous studies reported the discovery of microorganism capable of utilizing carbofuran as a sole C-source named *Burkholderia cepacia* PCL3 from phytoremidiated rhizosphere soil (Plangklang 2004). Since biodegradation of organic pollutants can be simply predicted by laboratory scale systems, the potential of PCL3 for bioaugmentation of carbofuran in soil had been firstly investigated in soil microcosm experiments with the carbofuran concentration in soil of 5 mg kg^{-1} (Plangklang and Reungsang, 2009). The results indicated that both free cells and immobilized cells of PCL3 on corncob and sugarcane bagasse could effectively degrade carbofuran in soil microcosms with the short half-lives ($t_{1/2}$) ranged between 13 and 19 d (Plangklang and Reungsang, 2009).

The present study attempted to validate an *in situ* bioaugmentation by using free and immobilized PCL3 in the small-scale field experiment before implemented this technology on a large scale. The depletion of carbofuran in the soil sample was investigated to determine the effectiveness of the bioaugmentation strategy. Quantification of microbial populations was carried out to examine the survival of the introduced microorganism in soil throughout the bioremediation treatment.

2. Materials and Methods

2.1 Culture medium and microorganism preparations

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

B. cepacia PCL3 (accession number of EF990634) (Plangklang, 2004) was used as the carbofuran degrader. It was grown in 100 mL Nutrient broth (NB) (Difco) containing 5 mg l^{-1} of carbofuran at 30 ^{o}C and 150 rpm for 36 h before harvested by centrifugation at 5,000 rpm for 10 min at 4 ^{o}C. The cell pellets were washed and re-suspended in BSM and was used as seed inoculum for free cell experiment and for cell immobilization. The immobilization of *B. cepacia* PCL3 on corncob were conducted followed the procedures described by Plangklang and Reungsang (2009). The internal cell density was approximately 10^{-8} CFU g^{-1} support.

2.2 Field Studies

A rice field soil, 0–15 cm depth, from Ban Nonmuang, Muang, Khon Kaen, Thailand where carbofuran had been applied during 1999 to 2009 was used for carrying out small-scale field studies. The levels of carbofuran contamination in the soil were approximately 1.63 mg kg^{-1} soil. The soil was classified as sandy loam soil which contained 0.89% organic carbon and 0.10% nitrogen with a pH of 6.7. Soil was sieved through a 2 mm mesh and kept at room temperature (28±2 ^{o}C) for 48 h prior the usage. In order to compare the carbofuran degradation efficiency with the previous microcosm work (Plangklang and Reungsang, 2009), the concentration of carbofuran in the soil was adjusted to be 5 mg kg^{-1} dry soil. Soil sample and analytical grade carbofuran (Sigma–Aldrich, USA) was mixed together with the help of a cement mixer before transported to the site of study. The plots measuring 1 m x 1.25 m and 20 cm deep were lined on all the sides by a plastic sheet in order to maintain the contents in the pots. Four experiments were setup as described in Table 1. Soil sample, 120 kg, and the contents in each treatment were thoroughly mixed with the help of a cement mixer before transferring to the plot. The moisture content of the soil was adjusted to be 15-18% and maintained

throughout the period of study by irrigating with watering the plots when required. The ambient temperature during the experiment fluctuated between 25 and 32 ^{o}C.

Table 1 Field trial experiments

Plot	Experimental setup
A	Soil mixed with 1.2 kg (dry basis) of PCL3 immobilized on corncob soaked in 2 L of BSM (equivalent to the final cell concentration of approximately 10^6 CFU g^{-1} dry soil).
B	Soil mixed with the free cells of PCL3 suspended in 2 L of BSM at the final cell concentration of approximately 10^6 CFU g^{-1} dry soil.
C	Soil mixed with 1.2 kg (dry basis) of corncob soaked in 2 L of BSM.
D	Soil mixed with 2 L of BSM.

At each sampling point, soil samples were collected from each plot at the depth of 10 cm using a hollow pipe of 1 in diameter from three different random positions, pooled, mixed thoroughly, and used for further analysis. Sampling was done in triplicate at 0, 5, 10, 15, 20, 25, 30, 40 and 60 d.

Carbofuran and its metabolites were extracted from the soil samples by an Accelerated Solvent Extractor ASE 100 (Dionex, USA) prior to analysis by HPLC following the conditions described by Plangklang and Reungsang (2008). The $t_{1/2}$ of carbofuran in the soil was calculated by fitting to a modified first-order kinetic model using SAS program (SAS Institute Inc., 1985). Data were analyzed by SPSS program Version 10.0 (SPSS Inc., Chicago, IL). The significance of treatments was set at p-value of less than or equal to 0.05 by the one-way ANOVA test.

Number of carbofuran degraders in the soil was determined by the drop-plate technique (Plangklang and Reungsang, 2010). The serial-diluted aliquots of the soil samples, 20 µl, were plated onto BSM agar coated with 5 mg l^{-1} of carbofuran and incubated at 30 ^{o}C until colonies appeared. A 10-g of wet immobilized cells were separated from the soil sample, washed and blended in 100 ml BSM for 3 min using blender. The suspension was then serial diluted and the enumeration of immobilized carbofuran degrader was then conducted by drop plate technique as described above.

3. Results and Discussions

The small-scale field studies were conducted in order to validate the *in situ* bioaugmentation by using free and immobilized PCL3 before implemented this technology on a large scale. Fig. 1 showed the dissipation of carbofuran in field soil bioaugmented with immobilized PCL3 on corncob and free cells along with their respective controls. The data were fitted to the modified 1st order kinetic model and the $t_{1/2}$ of carbofuran in soil in each plot was calculated. In the soil with only indigenous microorganisms (plot D), the degradation of carbofuran was slow with the long $t_{1/2}$ of 127 d. The addition of corncob into soil (plot C) ($t_{1/2}$ of 63 d) significantly increased the carbofuran degradation efficiency as compare to soil without organic amendment (plot D). Indigenous carbofuran degraders might use corncob as the energy source for their growth and/or the addition of corncob might increase the soil porosity and improve the oxygen transfer in soil which could stimulate carbofuran degradation activity (Mishra et al., 2001).

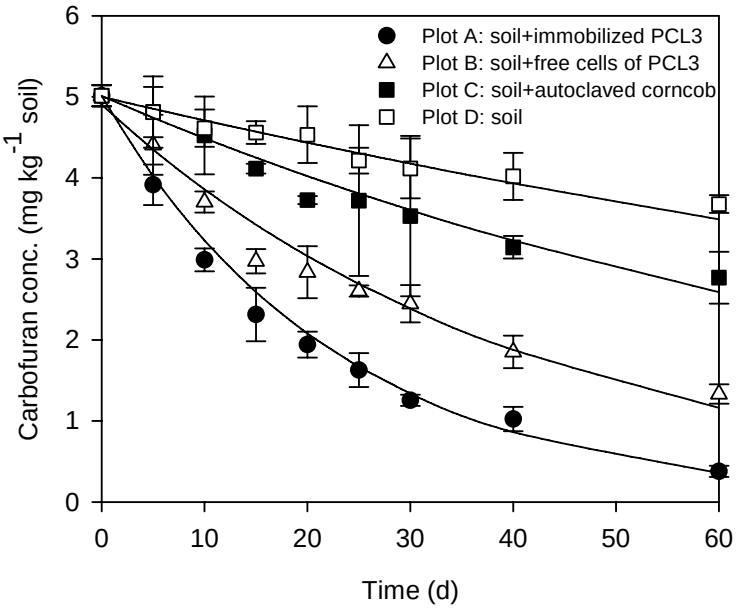

Fig.1 Carbofuran dissipation profiles in field soil; line indicated carbofuran degradation fitted to the modified 1st order kinetic model

Bioaugmented the soil with immobilized PCL3 (plot A) could shorten the $t_{1/2}$ of carbofuran in soil to be 16 d. The result implied that immobilized PCL3 on corncob has a potential to be used as the effective degrader for in situ bioaugmentation of carbofuran in large scale carbofuran contaminated sites. The significant longer $t_{1/2}$ of 28 d in soil was observed when the free cells of PCL3 were used (plot B) in comparison to immobilized cells (plot A) which suggested that bioaugmentation of carbofuran by using PCL3 in free cell form might not applicable.

The number of carbofuran degraders in soil and support material was determined during bioaugmentation treatment (Fig. 2). Results indicated that number of indigenous carbofuran degraders (plot D) was stable at approximately $4.7x10^5$ CFU g^{-1} soil during 60 d of field operation. The addition of corncob to the soil (plot B) resulted in 1 log unit increase in number of carbofuran degrader in the soil which might responsible for the greater carbofuran degradation efficiency as compared to soil without amendment.

In plot A, the number of carbofuran degrader in the support material was above 10^8 CFU g^{-1} support which equivalent to 10^6 CFU g^{-1} soil throughout the experiment and number of carbofuran degrader in soil increase by 1 log unit and remained stable after 20 d of bioaugmentation treatment (Fig. 2). These results indicated that the immobilization technique could improve the survival of PCL3 hence enhancing the carbofuran degradation efficiency in field soil. The support material could protect the inoculated degrader from predation and adverse environmental effects and could help in degradation by providing air pockets in the soil, thereby aerating the soil for prolonged survival and growth of the degrader (Labana et al., 2005; van Veen et al., 1997). In addition, the immobilized cells could act as the inoculum source during bioremediation operation in which the cells could grow and 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 and loss in degradation efficiency would not be observed in long term operation.

The number of carbofuran degraders in free cell experiment decreased from $5.3x10^6$ CFU g^{-1} soil to be approximately $4.4x10^5$ CFU g^{-1} soil after 10 d of field operation and stabilized thereafter (Fig. 2). The result indicated that PCL3 in free cell form could not survive in soil during field operation which could result in less effective carbofuran

degradation in the treatment with free cells (plot B) than in the treatment with immobilized cells (plot A) as described earlier.

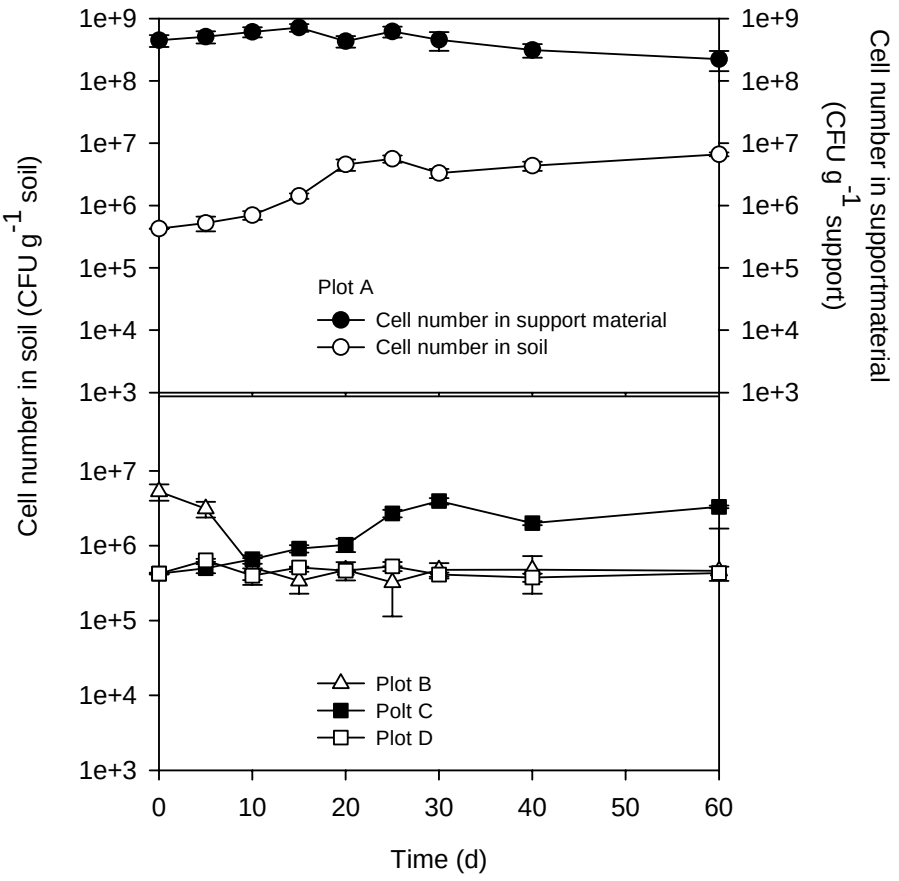

Fig.2 Variation in number of carbofuran degraders in soil and in support material during field operation.

Carbofuran phenol and 3-keto carbofuran were the metabolites found in soil during field operation (Table 2). The concentration of carbofuran phenol in soil increased overtime during 25 d of field operation and trended to decrease thereafter. Results revealed that carbofuran phenol could be metabolite by PCL3 and/or indigenous microorganisms which were coincided with the results from Plangklang and Reungsang (2009). The concentrations of 3-keto carbofuran fluctuated in between 0.01 and 0.25 mg kg^{-1} soil. Since PCL3 have not been reported to produce 3-keto carbofuran, we suggested

that 3-keto carbofuran is the metabolite from indigenous microorganism activity or abiotic degradation processes.

Table 2. Carbofuran metabolites detected in soil during field operation.

Time	carbofuran phenol (mg kg^{-1} soil)				3-keto carbofuran (mg kg^{-1} soil)			
(d)	Plot A	Plot B	Plot C	Plot D	Plot A	Plot B	Plot C	Plot D
0	nd	Nd	Nd	nd	nd	nd	nd	nd
5	0.38	0.21	0.05	0.07	0.01	0.01	nd	0.01
10	0.66	0.32	0.19	0.02	0.15	0.15	0.15	0.20
15	0.99	0.64	0.32	0.15	0.11	0.21	0.23	0.14
20	1.18	0.74	0.45	0.16	0.21	0.23	0.25	0.24
25	1.33	0.90	0.57	0.43	0.19	0.11	0.11	0.21
30	1.01	0.70	0.52	0.45	0.21	0.19	0.17	0.22
40	0.60	0.58	0.46	0.46	0.12	0.17	0.32	0.16
60	0.13	0.11	0.34	0.30	0.17	0.22	0.31	0.18

The comparison between the results from soil microcosms (Plangklang and Reungsang, 2009) and the present small-scale field experiments is shown in Table 2. In soil microcosms, the efficiency of carbofuran degradation by free cells and immobilized

Table 3 Comparison between the bioaugmentation results in soil microcosms and the present small-scale field experiments

Treatment	k_1 (d^{-1})	$t_{1/2}$ (d)	Number of carbofuran degraders in soil (CFU g^{-1})		Number of carbofuran degraders in support material (CFU g^{-1})		Reference
			Initial	Day 60	Initial	Day 60	
Microcosm scale							
Soil	0.009c±0.0003	74c ±2.0	$4.64x10^5$	$3.73x10^5$			Plangklang
Soil+free cells	0.052a±0.0163	13a±4.4	$4.75x10^6$	$2.45x10^5$			and Reungsang
Soil+ immobilized cell	0.044a±0.0068	16a±2.6	$4.64x10^5$	$7.51x10^7$	$6.7x10^7$	$7.46 x10^8$	(2008)
Field scale							
Soil	0.006c±0.0007	127d±16.3	$4.26x10^5$	$4.32x10^5$			This study
Soil+free cells	0.025b±0.0021	28b±2.5	$5.32x10^6$	$4.63x10^5$			
Soil+ immobilized cell	0.044a±0.0042	16a±1.5	$4.32x10^5$	$6.63x10^6$	$4.46x10^8$	$2.22x10^8$	

cells of PCL3 on corncob was not significantly different. In contrast, a longer $t_{1/2}$ of carbofuran in soil added with free cells than that with immobilized cells could be found in field experiment, though the numbers of carbofuran degraders in both experiments were in the same trend. The results indicated that there might be some factors appeared to limit carbofuran by free cell in the field system. The possible limit factors are less homogeneity and more fluctuating ambient temperature as compared to in soil microcosm experiment which were conducted under more controlled conditions. The survival of introduced effective carbofuran degrader, PCL3, is the most important key for successive removal of carbofuran from soil. However, the number of indigenous carbofuran degraders could survive above 10^{-5} CFU g^{-1} soil, hence, we could not indicate exactly the number of remained PCL3 in soil.

Conclusion

We were able to successfully demonstrate the bioremediation of carbofuran contaminated soil by bioaugmentation technique using immobilized cells of *B. cepacia* PCL3 as inoculum in small-scale field experiment. The short $t_{1/2}$ of carbofuran in soil of 16 d could be achieved and the introduced immobilized cells could survive over 60 d of field operation. Though the microcosm study demonstrated the effective degradation of carbofuran in soil by free cells of PCL3, some limit factors could cause the loss in their carbofuran degradation ability in field soil. This result suggested that bioaugmentation of carbofuran by using PCL3 in free cell form might not applicable in large area of contamination.

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PART IV

lux-MARKING AND APPLICATION OF CARBOFURAN DEGRADER *Burkholderia* cepacia PCL3

A manuscript prepared for submitting to New Biotechnology

ABSTRACT

The *luxAB*-mutant of carbofuran degrading bacterium *Burkholderia cepacia* PCL3*:luxAB*1 was constructed with the capability to emit the luminescence signal of $1.6x10^{-3}$ RLU cfu^{-1}. The mutant has the growth pattern and carbofuran degradation ability similar to PCL3 wild-type. The capability of tetracycline resistance was stable in PCL3:*luxAB*1 during repeated subculture over 120 generations. The luminescent emission by PCL3*:luxAB*1 is directly in correlation with the metabolic activity of the cells in which the exponential growth cells gave 100-fold higher value of light output in comparison to the cell in stationary phase. The optimal pH, temperature and n-decanal concentration on luminescence emission are 7.0, 35 ^{o}C and 0.01%, respectively. PCL3*:luxAB*1 could be used to assess the toxicity of carbofuran and carbofuran phenol in Basal salt medium (BSM) in which the difference sensitivity of the cells is depending on the biomass concentration. PCL3*:luxAB*1 was used to biodegrade carbofuran in BSM and soil. With the luciferase system, the degradative fraction of the augmented PCL3*:luxAB*1 and the difference between the active augmented PCL3*:luxAB*1 and indigenous microorganisms at the contaminated site could be indicated.

Keywords: bioremediation, bioaugmentation, *Burkholderia cepacia* PCL3, carbofuran, *luxAB*

1. Introduction

Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate) is a broad-spectrum insecticide widely used in agriculture to control insects and nematodes on contact or after ingestion. Carbofuran is of environmental concern because it is soluble in water and highly mobile in soil, resulting in a high potential for groundwater contamination (Gupta, 1994). In 2003, Thailand imported carbofuran in solid and liquid forms up to 826.6 and 45.5 tons, respectively, for using in agriculture especially in rice fields (Department of Agriculture (DOA), 2003). Continuous use of carbofuran in the rice fields may subsequently exacerbate the risk of contamination of the soil and groundwater; thus, the removal of carbofuran is necessary.

One promising routes for efficiently and cost-effectively removal of pesticide is microbial degradation by a specific degrader through the bioaugmentation technique. Previous studies reported the discovery of carbofuran degrading bacterium strain PCL3 affiliated with *Burkholderia cepacia* from carbofuran phytoremediated rhizosphere soil. The strain PCL3 utilizes carbofuran as a sole carbon source yielding carbofuran phenol as the metabolite. The studied results have shown that the strain PCL3 markedly enhanced the degradation of carbofuran in synthetic medium, soil as well as in the soil slurry phase suggesting that this strain may serve as the efficient *in-situ* and *ex-situ* bioremediation agents (Plangklang and Reungsang, 2008; 2009; 2010).

Effective bioaugmentation necessitates the survival and metabolic activity of the introduced degrader at the contaminated site. The genetic labeling systems have been extensive used to monitor the survival and metabolic activity of the microorganisms with the advantages of rapid detection and high sensitivity (Roda et al., 2004; Dorn et al., 2003; Ferguson et al. 1995). A frequently used genetic label is the bacterial luciferase which catalyzes the reaction of molecular oxygen, reduced flavin mononucleotide ($FMNH_2$) and long-chain aldehyde yielding the corresponding carboxylic acid, flavin mononucleotide (FMN), water and light presenting as the luminescence signal (490 nm) (Duncan et al., 1994). The luminescence signal has a dependency on the intracellular $FMNH_2$ concentration which is directly correlated with the metabolic activity of the living cell (Bachmann et al., 2007). Therefore, it can be used to indicate the viable degradative fraction of the augmented degrader and differentiate the augmented degrader

from the indigenous microorganisms (Mishra et al., 2004; Maoz et al., 2002). Two forms of luciferase have been used which are *luxAB* and *luxCDABE*. The *luxAB* is of our interest due to it does not require the energy of the labeled strain for producing the aldehyde substrate which can potentially inhibit microbes. By using *luxAB*, no aldehyde is present until the end of the bioremediation operation so any side effects of the compound on the system are thereby ruled out (Kristensen et al., 2006). In this study, we used an approach of transposon inserting the genetic marker *luxAB* into the genome of the strain PCL3 with the aim to obtain the transcongugant possessing the easily detectable phenotype suitable for monitoring its survival and growth during bioremediation treatment.

2. Materials and Methods

2.1 Plasmid, bacterial strains and cultivation

The plasmid and bacterial strains used in this study are listed in Table 1. *B. cepacia* PCL3 is the carbofuran degrader isolated from carbofuran phytoremediated rhizosphere

Table 1 Bacterial strains and plasmids used in this study.

Plasmid and bacterial strain	Genotype or characteristic	Source or reference
pUT *luxAB*	pUT derivative vector with the mini-Tn5-*luxAB* transposon consisting of a promoterless *luxAB* gene cassette from *Vibrio harveyi* upstream of tetracycline resistant gene	Biomedal, Spain
B. cepacia PCL3	16S rDNA is accessible in GenBank, accession no. EF990634	Plangklang and Reungsang (2009)
E. coli S17-1 λpir	*Tp*R *Sm*R *recA, thi, pro, hsd*R-M^{+}RP4: 2-Tc:Mu: Km Tn7 λpir	Biomedal, Spain
E. coli S17-1 λpir *luxAB*	*E. coli S17-1 λpir* carrying the pUT *luxAB* plasmid	This study
PCL3:*luxAB*1-5	Transconjugant of *B. cepacia* PCL3 carrying the mini-Tn5-*luxAB* transponson	This study

soil. *E. coli* S17-1 λpir was used to maintained pUT *luxAB* plasmid and proceed biparental matting with PCL3. *E. coli* strains were rutienly grown at 37 ^{o}C on Luria Bertani (LB) agar containing in g l^{-1}: tryptone, 10; yeast extract, 5; NaCl; 5, agar; 15, with the appropriate antibiotics (if required). For use as working stock culture, PCL3 was grown in Nutrient Broth (NB) containing with 5 mg l^{-1} of carbofuran at 30^{o}C in an orbital shaking incubator at 200 rpm. Bacterial strains were stored as glycerol stocks at -20^{o}C.

2.2 lux-Marking of B. cepacia PCL3

The *luxAB* cassette from *V. harveyi* was transformed into *B. cepacia* PCL3 by using bi-parental mating. A five-µl of overnight cultured *B. cepacia* PCL3 and *E. coli* S17-1 λpir *lux*AB was mixed together and dropped onto non-selective LB agar plate and incubated at 30^{o}C for 24 h. The colony formed were harvested and resuspended in 0.85% (w/v) NaCl and harvested by centrifugation at 10,000 rpm, 4 ^{o}C for 10 min. The cell pellets were resuspended in 100 µl of phosphate buffered saline buffer. The aliquoted cell suspension was spreaded onto LB agar containing 5 µg ml^{-1} of carbofuran and 5 µg ml^{-1} of tetracycline and incubated at 30 ^{o}C for 24 h. Bioluminescence transconjugants of PCL3 in which the mini-Tn5 *lux*AB cassette has inserted into downstream from the active promoter are tested for luminescence activity in the present of carbofuran. The absence of the pUT vector was confirmed by plasmid extraction using QIAprep Spin Miniprep Kit. The integration of the mini-Tn5 *lux*AB transposon was confirmed by *luxAB*-polymerase chain reaction (PCR) amplification using primers designed base upon the published sequences for *luxA* and *luxB* of *V. harveyi* (Waddell and Pope, 1999). The growth pattern and carbofuran degradation ability of *lux-AB* mutants were examined in Basal Salt Medium (BSM) (Mo et al., 1997) containing 5 mg l^{-1} of carbofuran in shake-flask experiment. The stability of the *luxAB-tet* cassette in the *lux-AB* mutants was monitored by repeated subculture in non selective NB containing 5 mg l^{-1} of carbofuran. Number of the mutants was counted on NA containing 5 µg ml^{-1} of tetracycline and non selective NA and the percentage of tetracycline resistant cells was calculated. The *luxAB*-mutant with remain the growth pattern and carbofuran degradation ability similar to wild-type strain and has the high stability of *luxAB-tet* cassette in the cells would be selected for use in subsequence experiments.

2.3 Relationship between luminescence emission and biomass concentration of luxAB-mutant

Relationship between the luminescence emission and biomass concentration was performed using the late exponential phase culture of *luxAB*-mutant grown in NB containing 5 mg l^{-1} of carbofuran in comparison to the late stationary phase culture. The cells were harvested at the biomass concentration late exponential phase and late stationary phase and centrifuged at 10,000 rpm for 5 min at 4 ^{o}C. The cell pellets were washed, resuspended and serial diluted in fresh NB medium containing 5 mg l^{-1} of carbofuran. The cell suspension with different biomass concentrations was immediately analyzed for bioluminescence activity with the addition of n-decanal substrate.

2.4 Effect of pH, temperature and n-decanal and carbofuran concentration on luminescence emission of luxAB-mutant

The effect of pH, temperature and n-decanal concentration on luminescence emission was performed using the late exponential phase culture of *luxAB*-mutant grown in NB containing 5 mg l^{-1} of carbofuran at the biomass concentration of approximately $5.4x10^{7}$ cfu ml^{-1}.

The effect of pH ranged between 3.0 and 9.0 was firstly investigated by suspended the cells in fresh carbofuran containing NB which was pH adjusted by using 2 M HCl or 1 M NaOH before adding the cells. After 1 h exposed to the different pH, the cell suspension was analyzed for bioluminescence activity with the addition of n-decanal substrate.

The optimal pH for luminescence emission of *luxAB*-mutant was further used to study the effect of temperature on luminescence emission of luxAB-mutant. The cell suspension in carbofuran containing NB with the optimum pH was incubated at the various temperatures ranged between 25 and 45 ^{o}C for 1 h before bioluminescence assay.

The effect of n-decanal concentration was then examined at the optimal pH and temperature. The cell suspension in carbofuran containing NB with the optimum pH was incubated at the optimul temperatures for 1 h before. After that, a 20-µl of n-decanal solution in 50% v/v ethanol in water was added to the cell suspension 5 min before bioluminescence assay.

2.5 Effect of carbofuran and carbofuran phenol concentration on the luminescence emission of luxAB-mutant

The effect of carbofuran and its metabolite i.e., carbofuran phenol concentrations on the luminescence emission of *luxAB-mutant* was determined in BSM supplemented with carbofuran or carbofuran phenol as the sole C-source. The mutant was grown overnight in NB containing 5 mg l^{-1} of carbofuran at 30 ^{o}C and 200 rpm. The cells were harvested by centrifugation at 10,000 rpm for 5 min at 4 ^{o}C, washed and resuspended in BSM before being used as seed inoculum. The solutions of carbofuran (0-300 mg l^{-1}) or carbofuran phenol (0-100 mg l^{-1}) were inoculated with different cell concentration ranged of 10^5 to 10^7 cfu ml^{-1} and incubated at the optimum temperature for light emission. The luminescence signal was initially analyzed before and after 6 h exposed to carbofuran at different concentrations.

2.6 Carbofuran biodegradation and survival of luxAB-mutant in synthetic medium and soil

Degradation of carbofuran in BSM by *luxAB*-mutant was conducted in a 500-ml shake flask in batch experiments. C-limited BSM, 200 ml, containing 20 mg l^{-1} of carbofuran as a sole carbon source, was added into the flasks before inoculation with approximately 10^6 cfu ml^{-1} of mutant seed inoculum. Flasks were then incubated at room temperature and shaken at 150 rpm. The cultured medium were sampled at the interval time for bioluminescence assay and to determine the biomass concentration in the culture media by drop plate on BSM agar coated with 5 mg l^{-1} of carbofuran. The carbofuran and its metabolite were extracted from the medium and quantitative analyzed by HPLC. Control was BSM with inoculation. The experiments were performed in triplicates.

The soil microcosm experiments were conducted in in 425 cm3 glass jars capped with plastic lids. Carbofuran solution was spiked into 20 g dry weight of soil in each glass jar, at the final concentration of 20 mg kg^{-1} dry soil and well mixed by hand stirring. The seed inoculum of the *luxAB*-mutant was added into the soil or autoclaved soil at the initial cell concentration of 10^6 cfu g^{-1} dry soil. The initial moisture content of the soil samples was adjusted to 15 to 18% before incubation at an average room temperature of 29±2 ^{o}C avoiding sunlight. Soil samples were sacrificed at the interval time and further extracted and analyzed for carbofuran and its metabolite concentration by HPLC. At each sampling

time, total numbers of carbofuran degraders in the soil were counted by drop plate technique on carbofuran-coated BSM agar. Three sets of control were included, i.e., soil with no inoculation, autoclaved soil with inoculation and autoclaved soil with no inoculation. The experiments were performed in triplicates.

2.7 Analytical methods

2.7.1 Analysis of carbofuran and its metabolite concentrations

Carbofuran and its metabolites were extracted of from culture media using a liquid-liquid partitioning method and from soil using Accelerated Solvent Extractor (ASE100) and analyzed by HPLC followed the protocol described by Planklang and Reungsang (2009). The half-lives of carbofuran in the soil were calculated by fitting to a modified first-order kinetic model using SAS program (SAS Institute Inc. 1985; Plangklang and Reungsang 2008).

2.7.2 Bioluminescence assay

Bioluminescence assay was conducted in a Fluostar Optima Micro plate reader operated in well mode. The 20-µl n-decanal substrate solution in 50% v/v ethanol in water was added to 200 µl of sample solution kept in black 96-well microplate. The concentration of stock n-decanal solution was varied to obtain the expected final concentration. Bioluminescence signal was record for 10 s starting 5 min after n-decanal addition and expressed as relative light units (RULs). All experiments were performed on triplicate samples.

2.7.3 Dose response analysis

Dose response curve were use to interpret the inhibitory concentration (IC) of carbofuran and carbofuran phenol on *luxAB*-mutant. The results are reported as IC_{50} value, the concentration that caused a 50% reduction in bioluminescence activity (Weitz et al. 2001) which was calculated by non-linear regression using Sigma Plot 9.0.

2.7.4 Statistical analysis

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

3. Materials and Methods

3.1 lux-Marked B. cepacia PCL3

Five *luxAB*-mutants named PCL3:*luxAB*1, 2, 3, 4 and 5 was obtained as the transconjugants of *B. cepacia* PCL3 carrying the mini-Tn5-*luxAB-tet* transponson. The PCR amplification of the genomic DNA isolated from PCL3:*luxAB* using the primers base on the nucleotide sequences of *luxAB* gene from *V. harveyi* confirmed that the chromosomal insertion was established (data not shown). PCL3:*luxAB*1 and 5 gave the stronger luminescence signal than the mutants 2, 3 and 4. All transconjugants displayed the same growth pattern in NB as PCL3 wild type, however, PCL3:*luxAB*2, 4 and 5 did not perform the degradation of carbofuran while PCL3:*luxAB*1 and 3 maintained the ability to degrade carbofuran (5 mg l^{-1}) in BSM (data not shown). The stability of the *luxAB-tet* cassette in the transconjugants was monitored by repeated subculture in non-selective NB (Fig. 1). The results demonstrated that the capability of tetracycline resistance was stable in PCL3:*luxAB*1 and 2 during repeated subculture over 120 generations. There was significant loss (30%) in tetracycline resistance ability of PCL3:*luxAB*3, 4 and 5 after 30 generations subculture in non selective medium. Base upon the obtained results, PCL3:*luxAB*1 was selected to be used in all subsequence experiments.

The relationship between the luminescence emission and biomass concentration of PCL3:*luxAB*1 at late exponential and late stationary growth phase were compared (Fig. 2). The result revealed that the luminescence emission was directly proportional to biomass concentration. The exponential growth cells gave the value of light output of $1.6x10^{-3}$ RLU cfu^{-1} and the luminescence signal decreased to the background value (17 RUL ml^{-1}) when the cell concentration was approximately $3.7x10^{3}$ cfu ml^{-1}. The value of light output decreased to $8x10^{-5}$ RLU cfu^{-1} with the higher detection limit of approximately $6.42x10^{4}$ cfu ml^{-1} when the cell entered the late stationary phase which is most likely due to the decrease in metabolic activity of the cells (Wiles et al., 2005). The results suggested that the *luxAB* label can be used as the reporter gene to represent the active viable cells as the degradative fraction of PCL3:*luxAB*1 in the bioaugmentation system.

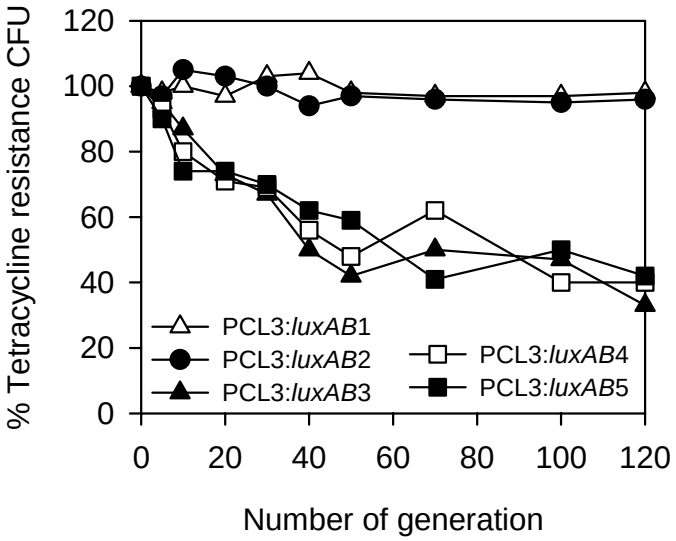

Fig. 1 Stability of *luxAB-tet* gene constructed in triplicate culture of PCL3:*luxAB*1-6, showing the percentage of tetracycline resistant cells during repeated subculture in non selective medium.

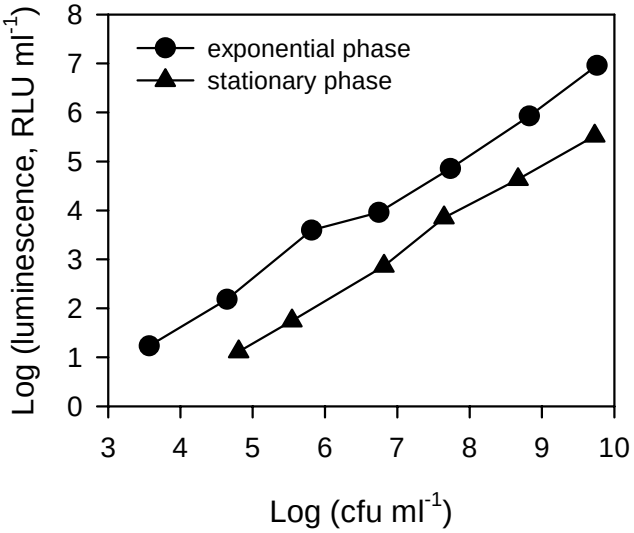

Fig. 2 Relationship between luminescence and biomass concentration during late exponential and late stationary phase of PCL3:*luxAB*1.

4.2 Effect of pH, temperature and n-decanal concentration on luminescence emission of PCL3:luxAB

A clearly different of luminescence signal was observed after incubated PCL3:*luxAB*1 for 1 h in the NB with different pH values (Fig. 3a). The signal remained above $4.5x10^5$ RLU ml^{-1} at the pH ranged between 5.5 and 8.5. The luminescence signal decreased 10 fold after 1 h incubation at the pH of 4.0 and dropped down to the background level after incubation at the pH of 3.0 for 1 h. The decrease in luminescence signal (2.6 fold) after incubation PCL3:*luxAB*1 at the pH of 9.0 could also be observed. Luciferase released by the function of *luxAB* gene had been reported as the acidic sensitive enzyme. The decrease in pH of the liquid medium from 6.8 to 2.75 resulted in 10 fold decrease in luminescence signal of *luxAB*-marked *Mycobacterium smegmatis* (Wiles et al., 2005). The wild range of pH from neutral to alkaline condition were suitable for the activity of luciferase from many strain such as *Photobacterium* sp. and *Vibrio harveyi* (Wongratana et al., 2005). In addition, the strong acidic or alkaline condition might toxic to the cells hence reducing the luminescence activity.

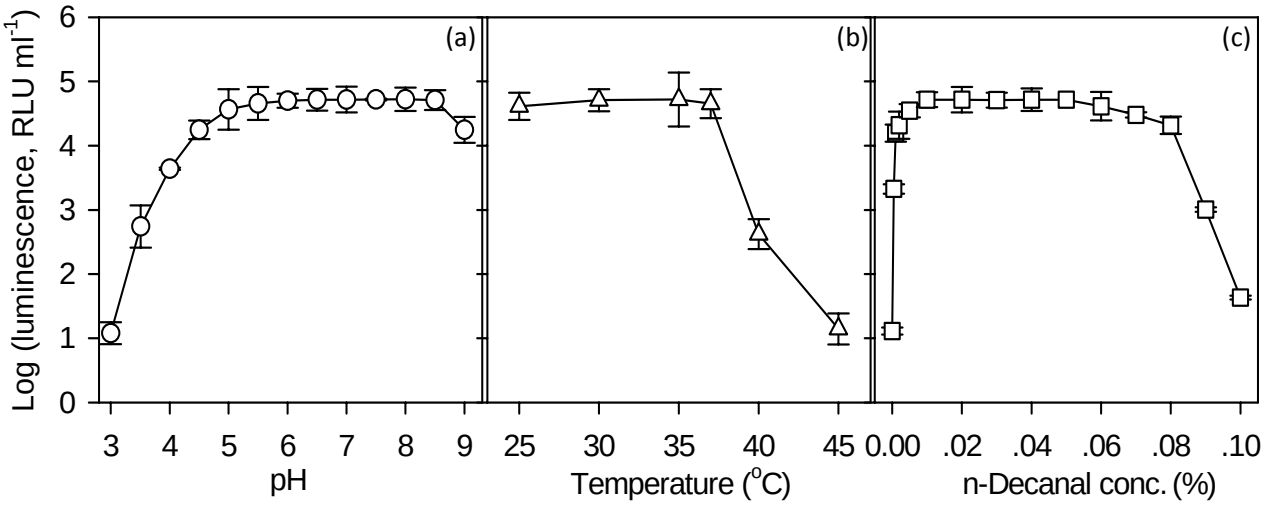

Fig. 3 Effect of pH, temperature and n-decanal concentration on bioluminescence emission of PCL3:*luxAB*1.

The effect of temperature ranged between 25 and 45 ^{o}C on luminescence emission of PCL3:*luxAB*1 was investigated at pH 7.0 after incubation for 1 h (Fig. 3b). The results indicated that the luminescence signal of greater than 4.1x10^{5} RLU ml^{-1} could be detected at the temperature range of 25 to 37 ^{o}C. The 100 fold reduction in luminescence signal was observed after incubation at 40 ^{o}C and the luminescence activity of PCL3:*luxAB*1 was completely inhibited after incubation at the 45 ^{o}C for 1 h. The limited expressed temperature range of *lux* genes are varied depending on the type of microorganism. The *lux* genes of *V. fisheri* and *V. harveyi* were reported to express at the temperatures of lower than 30 ^{o}C and 37 ^{o}C, respectively, while *P. luminesencs lux* gene can function at temperatures as high as 45 ^{o}C (Weitz et al., 2001; Winson et al., 1998).

PCL3:*luxAB*1 was further examined for the effect of n-decanal concentration on its luminescence activity (Fig. 3c). At the concentration of n-decanal betweem 0.01 and 0.05%, the luminescence signal was approximately 5.2x10^{4} RLU ml^{-1}. n-Decanal equal to and above 0.06% negatively affected (20% signal reduction) the luminescence emission of PCL3:*luxAB*1. The luminescence signal decrease when the concentration of n-decanal was decreased to equal and lower than 0.05%. The optimum n-decanal concentration (0.01%) for luminescence emission of PCL3:*luxAB*1 as well as the optimum pH of 7.0 and temperature of 35 ^{o}C were selected for luminescence analysis in the further experiments.

4.3 Effect of carbofuran and carbofuran phenol concentrations on survival of PCL3:luxAB1

The effect of carbofuran and its metabolite i.e., carbofuran phenol concentrations was determined in BSM supplemented with carbofuran or carbofuran phenol as the sole C-source after incubation for 6 h. The luminescence signal was initially analyzed before exposed to carbofuran at different concentrations and noted as the maximum value. The luminescence emitted after incubation for 6 h was measured and expressed as the percentage of maximum luminescence value. The induction of luminescence by carbofuran was not observed in *PCL3:luxAB*1. When the cell concentration of 5.5x10^{7} cfu ml^{-1} was used in the test (Fig. 4), *PCL3:luxAB*1 was significantly less sensitive to carbofuran (IC_{50} of 166.73 mg l^{-1}) than carbofuran phenol (IC_{50} of 54.33 mg l^{-1}) (Table 2). The remained luminescence signal at above 95% of maximum value indicated that the

sample inhibition did not occur at the carbofuran and carbofuran phenol concentrations up to 75 and 20 mg l^{-1}, respectively. The complete inhibitory of carbofuran and carbofuran phenol on *PCL3:luxAB*1 was found at the concentration above 250 and 83 mg l^{-1}, respectively.

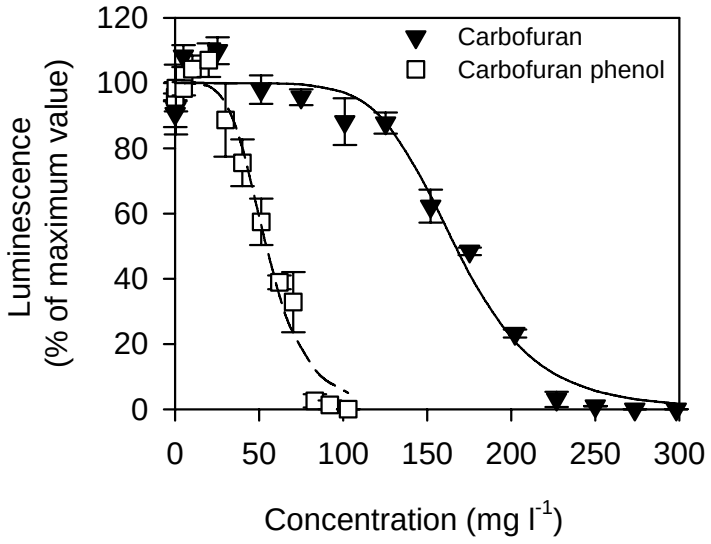

Fig. 4 Dose-response curves showing the effect of carbofuran and carbofuran phenol concentration on bioluminescence emission of PCL3:*luxAB*1 ($5.5x10^7$ cfu ml^{-1}) exposed for 6 h to carbofuran and carbofuran phenol in BSM.

Table 2 IC_{50} for PCL3:*luxAB*1 at different cell concentration exposed for 6 h to carbofuran and carbofuran phenol in BSM.

Cell concentration (cfu ml^{-1})	IC_{50} (mg l^{-1})	
	Carbofuran	Carbofuran phenol
$5.5x10^7$	166.73±4.73a	54.33±5.49a
$3.5x10^6$	136.20±5.11b	46.28±2.11b
$4.4x10^5$	84.15±7.11c	32.11±4.67c

The difference sensitivity of the cells is depending on the biomass concentration in which the IC_{50} value decreased with the decrease in biomass concentration (Table 2). The

higher cell density might cause a reduction in the ability of cell accession to the pollutant hence reducing the sensitivity of the cells (Rasmussen et al. 1997).

The degradation of carbofuran in BSM at the various initial concentrations by PCL3 (initial concentration of $6.43x10^{6}$ cfu ml^{-1}) was previously investigated (Plangklang 2004). The substrate inhibition model was used to predict carbofuran degradation behavior of PCL3. Carbofuran degradation ability of PCL3 was significant decreased at the carbofuran concentration above 100 mg l^{-1} and at the concentration of equal to and above 249 mg l^{-1} completely inhibited carbofuran degradation ability of PCL3 (Plangklang, 2004). Since the values carbofuran concentrations inhibiting the degradation and luminescence signal were not markedly different, the results implied that the carbofuran degradation and tolerance to carbofuran are the related characteristic of PCL3. The different phenomena was reported by Weitz et al. (2001) in which the toluene degradation and tolerance to toluene are unrelated characteristic of the toluene degrading bacterium *Pseudomonas putida* F1.

The use of *lux* gene for rapid and sensitive toxicity bioassay of heavy metals (Cu and Zn) and toxic hydrocarbon compounds (toluene and 3,5-dichlorophenol) had been proven by previous researchers (Nybroe et al., 2008; Weitz et al., 2001). The present study also underlines the range of using *lux* gene as the reporter system for toxicity bioassay responding to pesticide and the degradative metabolite.

4.4 Biodegradation of carbofuran in synthetic medium and soil by PCL3:luxAB1

The degradation of carbofuran by PCL3*:luxAB*1 in BSM and soil was investigated at the initial carbofuran concentration of 20 mg l^{-1} and 20 mg kg^{-1} soil, respectively. Results indicated that the mutant degraded carbofuran with the half-lives in BSM and soil of 3.61 and 12.15 d, respectively (Table 3). Carbofuran phenol was the metabolite detected during carbofuran degradation in BSM and soil bioaugmented with PCL3*:luxAB*1, and the accumulation of carbofuran phenol was not observed at the end of incubation. When compare the obtained results from the present study with previous results of carbofuran biodegradation by PCL3 wild-type (Plangklang and Reungsang, 2009), it could be concluded that there was not different between carbofuran degradations pattern of PCL3*:luxAB*1 and wild-type.

Table 3 Degradation rate constants (k_1) and half-lives ($t_{1/2}$) of carbofuran in the in BSM and soil

Treatment	k_1 (d^{-1})	$t_{1/2}$ (d)	r^2
BSM+PCL3:*luxAB*1	0.192±0.0130	3.61±0.25	0.97
Control: BSM	0.016±0.0030	43.31±8.47	0.90
Soil+PCL3:*luxAB*1	0.057±0.0065	12.15±1.41	0.98
Autoclaved soil+ PCL3:*luxAB*1	0.051±0.0077	13.79±2.10	0.97
Control: Soil	0.011±0.0027	65.77±16.61	0.95
Control: Autoclaved soil	0.006±0.0007	111.92±13.69	0.89

4.5 Survival of PCL3:luxAB1 in synthetic medium and soil

The number of viable carbofuran degrader(s) compared to luminescence emission during carbofuran degradation in BSM and soil was depicted in Fig. 5 and 6, respectively. During carbofuran degradation in BSM (Fig. 5), number of PCL3*:luxAB*1 increased 100 fold after 6 d of incubation. The biomass concentration remained above 10^8 cfu ml^{-1} until the end (20 d) of incubation, while the luminescence signal decreased rapidly after 8 d. The results implied that the PCL3*:luxAB*1 could survive throughout the

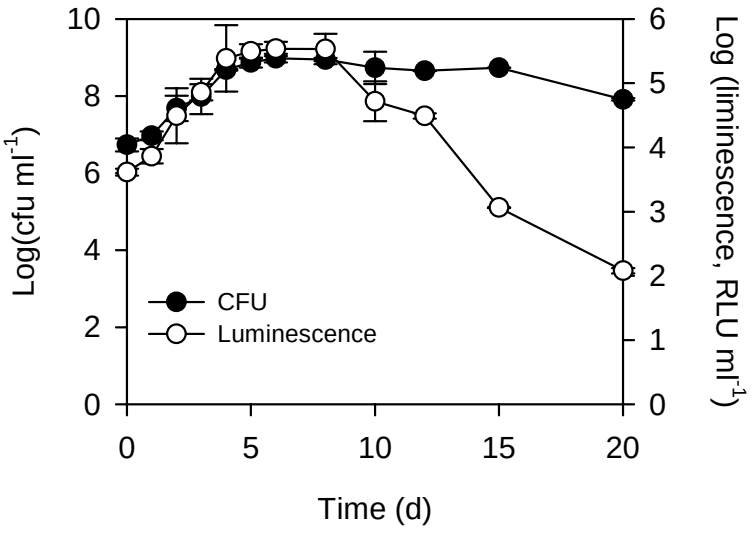

Fig. 5 Variations of biomass concentration and luminescence emission during carbofuran degradation in BSM.

experiment but its metabolic activity decreased after 8 days of incubation which might be due to the limit in carbon source i.e., carbofuran in the system as a result of degradation process.

Slightly increase in number of carbofuran degraders in control soil microcosm (soil with no inoculation) after 50 day of incubation implied that the adaptation of indigenous microorganisms for biodegradation activity after exposed to carbofuran was occurred. Throughout the experiment, the background luminescence in soil was constant at approximately 32 RLU g^{-1} soil (Fig. 6).

The number of carbofuran degraders and luminescence signal in bioaugmented soil and autoclaved soil slightly increased (10 fold) after 5 d of incubation. The biomass concentration in bioaugmented soil could be maintained above 10^6 cfu g^{-1} soil throughout the experiment while in the bioaugmented autoclaved soil tended to decrease after 40 d of incubation (Fig. 6). The luminescence signal decreased rapidly after 20 d of microcosm operation to be lower than the initial value (at d 0) after 60 d of incubation and drop down to the background level at the end (90 d) of microcosms operation. Since the luminescent

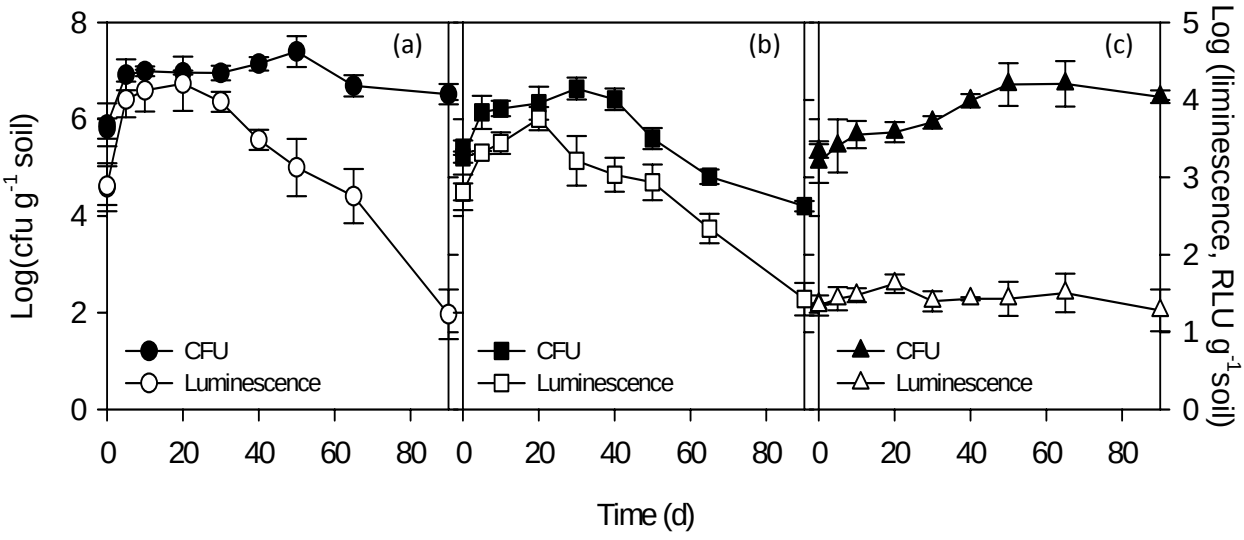

Fig. 6 Variations of biomass concentration and luminescence emission during carbofuran degradation in PCL3:*luxAB*1 augmented soil (a), PCL3:*luxAB*1 augmented autoclaved soil (b) and soil with inoculation (c).

emission by PCL3*:luxAB*1 is directly in correlation with the metabolic activity of the cells, it could be concluded that PCL3:*luxAB*1 could survive and active within the period of 60 d in soil microcosm, after that the microbial activity of the cells was reduced. The indigenous microorganisms or non active cells of PCL3:*luxAB*1 might be included to the viable cells counted which could be the results of non-reduction of the cell concentration in the soil bioaugmented treatment over the period of microcosm operation.

4. Conclusion

The *luxAB*-mutant of carbofuran degrading bacterium *Burkholderia cepacia* PCL3*:luxAB*1 was constructed with the capability to emit the luminescence signal of $1.6x10^{-3}$ RLU cfu^{-1}. The mutant has the growth pattern and carbofuran degradation ability similar to PCL3 wild-type. The optimal pH, temperature and n-decanal concentration on luminescence emission are 7.0, 35 ^{o}C and 0.01%, respectively. PCL3*:luxAB*1 could be used to assess the toxicity of carbofuran and carbofuran phenol in Basal salt medium (BSM) in which the difference sensitivity of the cells is depending on the biomass concentration. The results clearly demonstrated that the luminescent emission by PCL3*:luxAB*1 is directly in correlation with the metabolic activity of the cells. This characteristic gives a capability to indicate the degradative fraction of the augmented PCL3*:luxAB*1 as well as to differentiate the active augmented PCL3*:luxAB*1 from the indigenous microorganisms at the contaminated site. The ease, rapid and sensitive detection of the luminescence emitted from PCL3*:luxAB*1 would facilitate the monitoring of its microbial activity representing the carbofuran degradation efficiency in the contaminated site and the fast response to obtain the effective bioremediation of carbofuran can be performed.

Acknowledgements

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ผลงานที่ได้จากงานวิจัย (Output)

1. **นักศึกษาระดับปริญญาเอกด้านการกำจัดสารอันตรายโดยวิธีทางชีวภาพ 1 คน**
   นางสาวเพ็ญศรี ปลั่งกลาง สำเร็จการศึกษาจาก หลักสูตรสหสาขาวิชาการจัดการสิ่งแวดล้อมและของเสียอันตราย จุฬาลงกรณ์มหาวิทยาลัย

2. **ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการระดับนานานาชาติ**

   2.1 ผลงานที่ได้รับการตีพิมพ์ 1 เรื่อง

Plangklang, P. and Reungsang, A. Bioaugmentation of carbofuran by *Burkholderia cepacia* PCL3 in a bioslurry phase sequencing batch reactor. Process Biochemistry. 2010; 45:230-238. Impact factor = 2.336 (ที่มา: Journal Citation Reports, 2009)

   2.2 ผลงานที่ส่งตีพิมพ์แล้ว อยู่ในสถานะ under review 1 เรื่อง

Biodegradation of Carbofuran in Sequencing Batch Reactor Augmented with the Immobilized *Burkholderia cepacia* PCL3 on Corncob (Manuscript number PRBI-D-10-00364 submitted to Process Biochemistry – Under Review)

   2.3 ผลงานที่อยู่ในระหว่างดำเนินการส่งตีพิมพ์ 2 เรื่อง

Bioaugmentation of carbofuran residues in soil by *Burkholderia cepacia* PCL3: A small-scale field studies (short communication) (Manuscript prepared for submitting to New Biotechnology)

lux-Marking and application of carbofuran degrader *Burkholderia* cepacia PCL3 (Manuscript prepared for submitting to New Biotechnology)

3. **ผลงานวิจัยอื่น ๆ**
   นำเสนอผลงานวิจัยในงานประชุมวิชาการระดับนานาชาติ ณ ต่างประเทศ 1 เรื่อง

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

4. จำนวนและรายละเอียดการได้รับเชิญไปเป็นวิทยากร

รศ.ดร. อลิศรา เรืองแสง: Invited Speaker งานประชุม "The 2nd BMB Conference: Biochemistry and Molecular Biology for Regional Sustainable Development" หัวข้อ "Bioremediation of Carbofuran Contaminated Soil by *Burkholderia* sp. PCL3" ในวันที่ 7 พฤษภาคม 2552 ณ โรงแรมโฆษะ จังหวัดขอนแก่น

น.ส. เพ็ญศรี ปลั่งกลาง: Invited Speaker งานประชุม "Interdisciplinary Symposium Biological Response to Chemical Contaminant: From Molecular to community level" หัวข้อ "Biodegradation of carbofuran in sequencing batch reactor augmented with immoblized *Burkholderia cepacia* PCL3" ในวันที่ 2-4 กันยายน 2552, ณ The University of Aveiro เมือง Aveiro ประเทศ Portugal

5. การเชื่อมโยงทางวิชาการกับนักวิชาการอื่น ๆ ทั้งในและต่างประเทศ

ได้มีการเชื่อมโยงงานวิจัยกับอาจารย์จากจุฬาลงกรณ์มหาวิทยาลัยที่มีความเชี่ยวชาญทางด้านเทคนิคการบำบัดสารพิษทางชีวภาพ และเทคนิคทางอณูชีววิทยา โดย ได้แลกเปลี่ยนแนวคิดและประสบการณ์การวิจัย เพื่อให้เนื้อหางานวิจัยสมบูรณ์ยิ่งขึ้น นอกจากนี้งานวิจัยนี้ในขั้นตอนการส่งถ่าย (transform) ยีน *lux*CDABE เข้าสู่ *Burkholderia* sp. PCL3 ได้รับการอนุเคราะห์สถานที่ทำการวิจัย อุปกรณ์เครื่องมือ และสารเคมีบางส่วนจาก Center for Marine Environmental Studies, Ehime University, Bunkyo-3, Matsuyama, 790-8577 Japan ภายใต้ความร่วมมือกับ Prof. Dr. SATORU SUZUKI โดยนักวิจัยภายใต้โครงการ (น.ส. เพ็ญศรี ปลั่งกลาง) ได้เดินทางไปทำการวิจัยในระหว่างวันที่ 15 มกราคม 2551 ถึงวันที่ 15 เมษายน 2551 โดยได้รับทุนสนับสนุนจากสำนักงานคณะกรรมการการอุดมศึกษา ทุนแลกเปลี่ยนอาจารย์/นักศึกษาโครงการเครือข่ายเชิงกลยุทธ์เพื่อการผลิตและพัฒนาอาจารย์ในสถาบันอุดมศึกษา นอกจากนี้ยังได้รับความอนุเคราะห์ใช้เครื่องมือต่างๆ เช่น เครื่องมือวเคราะห์ค่า luminescence จากศูนย์เทคโนโลยีหลังการเก็บเกี่ยว (Postharvest Technology Centre) มหาวิทยาลัยเกษตรศาสตร์ วิทยาเขตกำแพงแสน

6. การเชื่อมโยงทางวิชาการกับนักวิจัยภายในสถาบันเดียวกัน

ได้มีการเชื่อมโยงทางวิชาการกับนักวิจัยภายในมหาวิทยาลัยขอนแก่น โดยในแต่ละหน่วยงานที่ทีมผู้วิจัยสังกัดอยู่ได้มีการอนุเคราะห์เครื่องมือที่ใช้ในการทดลอง และเครื่องมือวิเคราะห์ต่างๆ ที่ทางหน่วยงานหลักไม่มี อีกทั้งยังได้มีการแลกเปลี่ยนแนวคิดและประสบการณ์การวิจัย เพื่อหาแนวทางสานต่องานวิจัย และเพื่อให้ใช้ประโยชน์จากงานวิจัยได้สูงสุด

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Bioaugmentation of carbofuran by *Burkholderia cepacia* PCL3 in a bioslurry phase sequencing batch reactor

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ABSTRACT

The effectiveness of bioremediation technology in the removal of carbofuran from contaminated soil using a bioslurry phase sequencing batch reactor (SBR) was investigated. A 2-L laboratory glass bottle was used as a bioreactor with a working volume of 1.5 L at room temperature (27 ± 2 °C). One total cycle period of the SBR was comprised of 1 h of fill phase, 82 h of react phase, and 1 h of decant phase. The carbofuran concentration in the soil was 20 mg/kg soil. A carbofuran degrader isolated from carbofuran phytoremediated soil, namely *Burkholderia cepacia* PCL3 (PCL3) immobilized on corncob, was used as the inoculum. The results revealed that bioaugmentation treatment (addition of PCL3) gave the highest percentage of carbofuran removal (96.97%), followed by bioaugmentation together with biostimulation (addition of molasses) treatment (88.23%), suggesting that bioremediation was an effective technology for removing carbofuran in contaminated soil. Abiotic experiments, i.e. autoclaved soil slurry with corncob and no PCL3 treatment and autoclaved soil slurry with no PCL3 treatment, could adsorb 31.86% and 7.70% of carbofuran, respectively, which implied that soil and corncob could act as sorbents for the removal of carbofuran.

1. Introduction

Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate) is a broad-spectrum insecticide widely used in agriculture to control insects and nematodes on contact or after ingestion. Carbofuran is of environmental concern because it is soluble in water and highly mobile in soil, resulting in a high potential for groundwater contamination [1]. In 2003, Thailand imported carbofuran in solid and liquid forms up to 826.6 and 45.5 tons, respectively, for using in agriculture especially in rice fields [2]. Continuous use of carbofuran in the rice fields may subsequently exacerbate the risk of contamination of the soil and groundwater; thus, the removal of carbofuran is necessary.

One of the effective routes for pesticide removal is microbial degradation by a specific degrader and/or indigenous microorganisms. Previous studies reported the discovery of microorganisms capable of degrading carbofuran and other pesticides from contaminated natural matrices [3–5]. These degraders could use the pesticide as their energy source, i.e. C- or N- or C- and N-sources. The addition of microbial cultures capable of degrading pesticide, the so-called bioaugmentation technique, is reported to be an effective bioremediation approach for improving pesticide degradation in contaminated soils and water that lack indigenous microbial activity [6]. In addition to bioaugmentation, biostimulation is another bioremediation treatment to remove pesticides contamination in the environment. This treatment stimulates the activity of the indigenous microorganisms by adding the organic and/or inorganic additives such as N or P, etc. The amendments added would be used by the indigenous microorganisms for cell growth resulting in an increase in cell number as well as their activities to degrade the pesticides. In addition, the amendments could be necessary as the enzyme-inducers and/or the co-metabolic substrates in the pesticide degradation pathways [7].

Bioaugmentation and biostimulation can both be applied *in situ* by directly adding degraders and/or additives to the contaminated areas and *ex situ* through biodegradation in a bioreactor. Though *in situ* treatment has the advantages of simplicity and cost-effectiveness, it requires a long time period to complete the degradation and can be restrained by some limiting conditions such as low permeability and heterogeneity of the contaminated matrices [8]. Therefore, bioreactor technology which can be specially designed in a variety of configurations to maximize

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microbial activity has drawn our attention as a way to bioremediate carbofuran.

Soil slurry phase reactors comprised of 10–40% soil in liquid (water) are a relatively new application for soil and sediment bioremediation. It is an alternative technology for decontamination of soil and sediment which minimizes the limiting effect of mass transfer on biodegradation [6,9,10]. Contaminant in a soil-slurry treatment system can partition itself to the soil and liquid phases and can be utilized by both indigenous microorganisms in the soil and inoculated specific degraders. Therefore, the degradation process can take place in the soil, water, and/or soil–water phases, resulting in an enhancement of contaminant degradation [11]. Published data have indicated successful bioremediation of hazardous substances, especially pesticides such as pendimethalin [12] and hexachlorocyclohexane isomers [13–15], using a soil slurry phase reactor. In addition, it has been reported that the efficiency of remediation of contaminated soil could be effectively improved by using a bioslurry reactor augmented with specific microorganisms capable of degrading the pesticide of interest. However, to the best of our knowledge, there has been no report on carbofuran remediation in a bioaugmented soil slurry phase reactor.

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

2. Materials and methods

2.1. Immobilization of B. cepacia PCL3

2.1.1. Microorganism preparation

B. cepacia PCL3 (accession number of EF990634) was used as the carbofuran degrader [16]. It was grown in 100 mL nutrient broth (NB) containing 5 mg/L of carbofuran at 30 °C and 150 rpm for 36 h and was used as seed inoculum for immobilization.

2.1.2. Support material preparation

Corncob was used as a support material to immobilize *B. cepacia* PCL3. This material has high matrix porosity and a pore size that could enhance the cell adsorption capability during immobilization. Corncob was cut into 0.7 cm × 0.7 cm × 0.7 cm pieces using a knife and then 300 g of cut corncob was boiled in 3 L of 1% NaOH for 3 h to remove lignin and fibers inside the materials which might react with the cells [17]. The alkaline-boiled corncob was washed 3 times with 3 L of distilled water, soaked in distilled water overnight, and then sterilized by autoclaving at 121 °C for 15 min and kept at 4 °C prior to usage.

2.1.3. Cell immobilization

Adsorption was used as the immobilization method in this study. This method is typically performed when porous media are used as support materials with the advantage of ease of operation. The immobilization technique was conducted by adding 75 g of sterile corncob to 250 mL of sterile NB containing 5 mg/L of carbofuran before inoculating with PCL3 (10^6 CFU/mL). The flask was then incubated at 150 rpm, at room temperature, for 48 h. After incubation, the support material was transferred to fresh NB containing 5 mg/L of carbofuran and incubated, as previously described, before harvesting by filtration through a Buchner filter funnel and washing with 0.85% NaCl by an aseptic technique. This process was repeated 2 times. Immobilized cells were kept at 4 °C until use in further experiments. The internal cell density on the corncob after immobilization was approximately 10^7 CFU/g dry material. The procedures for cell immobilization followed the method of Plangklang and Reungsang [18].

2.2. Soil

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

2.3. Soil slurry preparation

Air-dried soil was spiked with carbofuran at a concentration of 20 mg/kg dry soil, well mixed by hand stirring, and kept at 4 °C for 24 h to induce the homogenous sorption of carbofuran over the soil particles. For soil slurry preparation, the impregnated soil was added to distilled water at a ratio of 1 g soil:20 mL distilled water before feeding to the reactor. The method of soil slurry preparation was adapted from Venkata-Mohan et al. [11].

2.4. Soil slurry phase reactor configuration

A 2-L laboratory glass bottle was used as a reactor in this study with a working volume of 1.5 L and a suitable inlet and outlet arrangement as shown in Fig. 1. To prevent sorption of carbofuran on tubing system, the glass tubes were used inside the reactor. Due to the biodegradation of carbofuran was mostly occurred by aerobic microorganisms, the reactor was operated with aerobic condition in this study. Oxygen was supplied from an air compressor through an air diffuser at the flow rate of 600 mL/min at the bottom of the bottle. The contents of the reactor were continuously mixed by a magnetic stirrer in which the magnetic bar with 0.4 cm in diameter and 4 cm in length was placed on the center of the bottom of the reactor. This mixing system was set to facilitate the uniform diffusion of air from the bottom of the reactor towards upflow direction and keep the soil slurry phase in a suspension form.

2.5. Soil slurry phase reactor operation

In this experiment, eight soil slurry phase reactors (A–H) (Table 1) were operated with an initial carbofuran concentration of 20 mg/kg dry soil. The soil slurry was prepared as described above and fed to the reactor at a flow rate of 25 mL/min using a peristaltic pump. For the bioaugmented reactors (A, C, and H), 100 g wet corncob immobilized with *B. cepacia* PCL3 (10^7 CFU/g dry corncob) were added to the reactor before feeding in the soil slurry. For the biostimulated reactors (G and H), molasses (1500 mg COD/L) was mixed with the soil slurry before feeding to the reactor. Each reactor was aerobically operated in sequencing batch mode with a total cycle period of 84 h (HRT). The cycle period consisted of 1 h of fill phase, 82 h of react phase, and 1 h of decant phase. The cycle period was fixed according to the results from our previous study in which the half-life of carbofuran in liquid medium (5 mg/L) degraded by *B. cepacia* PCL3 was 3 d [18]. Aerobic conditions in the reactor were maintained by pumping air through an oxygen diffuser at an air flow rate of 600 mL/min. During the react phase, the reactor was continuously stirred using a magnetic stirrer to homogenize the contents and maintain air diffusion in the reactor. The soil slurry was sampled every 6 h via the sampling port using a peristaltic pump. The concentrations of carbofuran and its metabolites, i.e. carbofuran phenol and 3-keto carbofuran, in both the soil and liquid phases were determined by extraction using a liquid–liquid partitioning method followed by HPLC. The pH values of the soil slurry were measured using a digital pH meter (Sartorius, Germany). The number of carbofuran degraders in the soil slurry was enumerated by a drop-plate technique.

2.6. Analysis method

2.6.1. Extraction of carbofuran in soil slurry

In order to extract carbofuran and its metabolites from the soil and liquid phases, the soil slurry was centrifuged at 6000 rpm and 25 °C for 15 min to separate the liquid from the soil. The liquid phase was filtered through cellulose acetate paper number 1 (Whatman, England) prior to the liquid–liquid partitioning extraction procedure [15]. Briefly, 2 mL of methanol were added to 2 mL of liquid sample and then the mixture was sonicated twice for 10 min on a 50/60 voltage cycle. After sonication, carbofuran and its metabolites were extracted in a separation funnel with dichloromethane. This extraction was performed 3 times with 4, 2, and 2 mL of dichloromethane. The organic fractions from the extractions were collected, pooled, and evaporated in the fume hood, then re-dissolved in 4 mL of 60% methanol and passed through a 0.45 μm nylon membrane syringe filter before analysis by HPLC.

The soil phase was air dried at room temperature (30 ± 2 °C) and weighed in an HDPE tube, and 60% methanol was added at a ratio of 1:200 (w/v) soil:methanol. The tube was horizontally shaken at 200 rpm for 30 min and further centrifuged at 6000 rpm and 10 °C for 10 min to separate the supernatant. This step was repeated twice and the supernatants were pooled and filtered through cellulose acetate paper number 1 prior to the liquid–liquid partitioning extraction procedure described above. The final volume of the extract was adjusted to 0.4 mL and then filtered through a nylon membrane filter disc before analysis by HPLC. Percentage recoveries of this procedure were 94.4, 93.2 and 94.0% for carbofuran, carbofuran phenol and 3-ketocarbofuran, respectively.

2.6.2. HPLC analysis of carbofuran and its metabolites

The extracts were analyzed using a Shimadzu 10-A HPLC equipped with a 4.6 mm × 150 mm Lunar 0.5 μm C-18 column (Phenomenex, USA), a UV detector operating at 220 nm, and a 20 μL injector loop. The HPLC operating parameters were: mobile phase, methanol–water (60:40); flow rate, 1 mL/min; ambient temperature. External standard linear calibration curves for carbofuran, carbofuran phenol, and 3-keto carbofuran were used to quantify their concentrations in the aqueous phase. The observed concentrations were characterized by peak areas.

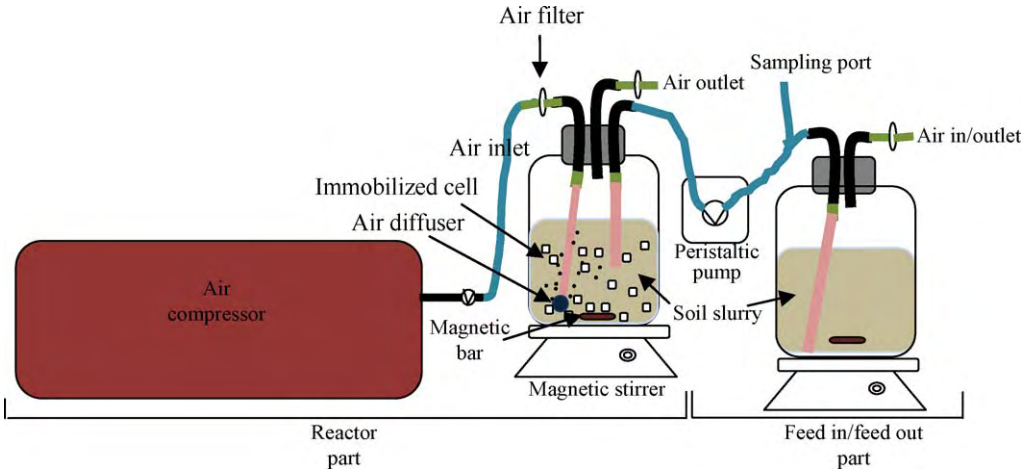

Fig. 1. Schematic diagram of soil slurry phase reactor (not subject to scale).

2.6.3. Kinetic analysis of carbofuran degradation in soil phase

The experimental data obtained from soil slurry phase reactor operation were studied by fitting to zero-, first-, and second-order kinetic equations as described in Eqs. (1)–(3), respectively, in order to understand the kinetic aspect of carbofuran degradation:

$$C = C_0 - k_0 t \quad (1)$$

$$C_t = C_0 e^{-k_1 t} \quad (2)$$

$$\frac{1}{C_t} = \frac{1}{C_0} + k_2 t \quad (3)$$

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

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

$$t_{1/2}, 0^0 = \frac{C}{2k_0} \quad (4)$$

$$t_{1/2}, 1^0 = \frac{0.693}{k_1} \quad (5)$$

$$t_{1/2}, 2^0 = \frac{1}{k_2 C} \quad (6)$$

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

2.6.4. Enumeration of carbofuran degraders in the soil slurry by the drop-plate technique

The number of carbofuran degraders in the soil slurry was determined by the drop-plate technique. The serial-diluted aliquots of the soil slurry samples, 20 μL, were plated onto basal salt medium (BSM) agar coated with 5 mg/L of carbofuran and incubated at 30 °C until colonies appeared. BSM agar [19], pH 7, contains (in g/L): 5.57, $NaHPO_4$; 2.44, KH_2PO_4; 2.00, NH_4Cl; 0.20, $MgCl_2{\cdot}6H_2O$; 0.0004, $MnCl_2{\cdot}4H_2O$; 0.001, $FeCl_3{\cdot}6H_2O$; 0.001, $CaCl_2$, and 1.5% agar was added to the medium before autoclaving at 121 °C for 15 min. Carbofuran solution in sterile distilled water, as the sole C-source, was coated on the BSM agar at a concentration of 5 mg/L using a glass spreader prior to use.

3. Results and discussions

3.1. Soil slurry phase reactor performance

3.1.1. Non-augmented and abiotic reactors

The degradation profiles and degradation efficiency of carbofuran in the soil slurry for the non-augmented reactors (B and D) and abiotic control reactors (E and F) as a function of operation time are depicted in Fig. 2(a). Reactor B, which had only indigenous microorganisms in the soil, exhibited marked carbofuran degradation (Fig. 2(a)) with a relatively high efficiency of 67.69%. This indicated that there might be some carbofuran degraders presented as native microflora in the soil. The rice field where the soil samples were collected had a history of carbofuran application with a background carbofuran concentration of 0.06 mg/kg dry soil; therefore, microorganisms in the soils would have been able to adapt to use carbofuran as an energy source [20].

The addition of autoclaved corncob to the soil slurry (reactor D) increased the carbofuran degradation efficiency to 70.12%. In addition, the abiotic experiments (i.e. autoclaved soil slurry with corncob and no additional PCL3 treatment) demonstrated an adsorbance of 31.86% of carbofuran, which implied that corncob could act as a sorbent for the removal of carbofuran. However, it should be noted that the sorption of carbofuran onto the support material could lead to a higher degradation rates when the

Table 1
Soil slurry phase reactor treatment.

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

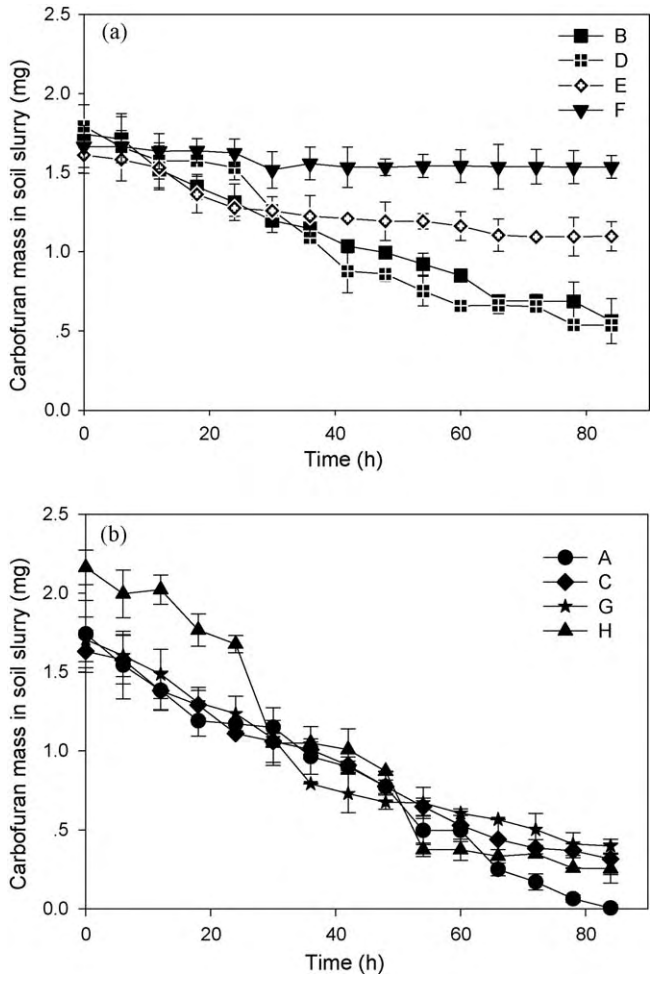

Fig. 2. Degradation profiles of carbofuran in soil slurry phase reactors. (a) Without bioremediation treatment (reactor B: soil slurry; reactor D: soil slurry + autoclaved corncob; reactor E: autoclaved soil slurry + autoclaved corncob; reactor F: autoclaved soil slurry); (b) with bioremediation treatments (reactor A: soil slurry + immobilized PCL3; reactor C: autoclaved soil slurry + immobilized PCL3; reactor G: soil slurry + molasses; reactor H: soil slurry + immobilized PCL3 + molasses).

immobilized PCL3 was used. Abiotic control (reactor F, autoclaved soil slurry) showed an insignificant carbofuran degradation profile (Fig. 2(a)). However, a relatively low carbofuran degradation efficiency of 7.70% was found (Fig. 2(a)). This might have resulted from oxidation and volatilization processes due to the continuous aeration during reactor operation. Although the oxidation and volatilization are not as important as microbial degradation to carbofuran dissipation, they were contributing to dissipation processes which could be found in abiotic control as reported in previous study [21].

3.1.2. Effect of bioremediation treatments

The effects of bioaugmentation, biostimulation, and bioaugmentation together with biostimulation treatments on carbofuran degradation in the soil slurry phase reactor were investigated in this experiment. The carbofuran degradation profiles in soil slurry phase reactors with bioremediation treatments as a function of operation time are depicted in Fig. 2(b). A significant improvement in carbofuran degradation in the soil slurry could be found when these bioremediation techniques were compared to non-augmented experiments. The highest carbofuran degradation of 96.97% could be achieved with the bioaugmentation treatment (addition of immobilized PCL3, reactor A), followed by degradation of 88.23% by bioaugmentation together with biostimulation treatments (addition of PCL3 and molasses, reactor H) and degradation of 76.70% by biostimulation treatment (addition of molasses; reactor G).

Molasses used as the organic amendment in the biostimulation treatment (reactor G) could improve the carbofuran degradation efficiency of the indigenous microorganisms in which the carbofuran degradation was increased from 67.69% to 76.70% as compared to reactor B (Fig. 2). However, the reduction in carbofuran degradation efficiency (8.74%) after the addition of molasses to the augmented reactor could be observed (reactor H compared to reactor A). This might due to the fact that PCL3 preferred to use sugars in molasses than carbofuran since sugars have less complex structures and are easier to be metabolized [18]. In addition, there might be some adverse effects from substances contained in molasses, such as metal ions [22] or by-products of molasses metabolism, on PCL3 augmented to the reactor.

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

Our previous research [20] investigated carbofuran degradation in soil and rhizosphere soil microcosms in which soils were collected from the same rice field as presented in this study. The experiments were conducted using soil microcosms with an initial carbofuran concentration of 5 mg/kg dry soil. The bioaugmenta-

Table 2
Carbofuran metabolites, i.e. carbofuran phenol (CP) and 3-ketocarbofuran (3KC) (mg) observed in soil phase during soil slurry phase reactor operation.

Time (h)	Reactor A		Reactor B		Reactor C		Reactor D		Reactor E		Reactor F		Reactor G		Reactor H	
	CP	3KC	CP	3KC	CP	3KC	CP	3KC	CP	3KC	CP	3KC	CP	3KC	CP	3KC
nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
6	nd	nd	nd	0.046	0.051	0.047	0.052	nd	nd	nd	nd	nd	0.058	0.043	nd	nd
12	0.188	0.135	0.077	0.025	0.064	0.066	0.054	0.125	nd	nd	nd	nd	0.078	0.075	0.083	nd
18	0.285	0.057	0.068	0.036	0.095	0.135	0.095	0.060	nd	nd	nd	nd	0.064	0.116	0.058	0.047
24	0.400	nd	0.075	0.030	0.062	0.033	0.051	0.031	nd	nd	nd	nd	0.114	0.085	0.052	0.015
30	nd	nd	0.053	nd	0.054	0.037	nd	0.070	nd	nd	nd	nd	0.134	0.151	nd	0.06
36	0.131	nd	0.060	nd	0.059	0.023	0.059	0.100	nd	nd	nd	nd	0.124	0.147	0.053	nd
42	nd	0.050	0.056	0.034	0.052	0.034	0.059	nd	nd	nd	nd	nd	0.108	0.092	0.050	0.010
48	nd	nd	nd	nd	nd	nd	nd	0.006	nd	nd	nd	nd	nd	0.039	nd	nd
54	nd	nd	nd	nd	nd	nd	nd	0.007	nd	nd	nd	nd	0.055	0.047	nd	nd
60	nd	nd	nd	nd	nd	0.041	nd	nd	nd	nd	nd	nd	0.052	0.056	nd	nd
66	nd	nd	nd	nd	nd	nd	0.008	0.013	nd	nd	nd	nd	nd	nd	nd	nd
72	nd	nd	nd	nd	nd	0.046	0.036	0.017	nd	nd	nd	nd	0.052	nd	nd	nd
78	nd	nd	nd	nd	nd	0.034	0.027	0.008	nd	nd	nd	nd	0.056	0.030	nd	nd
84	nd	nd	nd	nd	nd	nd	0.006	0.001	nd	nd	nd	nd	0.053	0.036	nd	nd

nd = not detectable.

Table 3
Carbofuran metabolites, i.e. carbofuran phenol (CP) and 3-ketocarbofuran (3KC) (mg) observed in liquid phase during soil slurry phase reactor operation.

Time (h)	A		B		C		D		E		F		G		H	
	CP	3KC	CP	3KC	CP	3KC	CP	3KC	CP	3KC	CP	3KC	CP	3KC	CP	3KC
0	nd	0.03	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
6	nd	nd	nd	0.092	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
12	0.413	0.110	0.264	0.113	0.050	0.136	0.054	0.052	nd	nd	nd	nd	nd	0.042	0.052	0.055
18	0.551	0.241	0.388	0.204	0.343	0.048	0.341	0.137	nd	nd	nd	nd	0.051	0.147	0.277	0.046
24	0.547	0.092	2.62	0.118	0.557	0.112	0.320	0.118	nd	nd	nd	nd	0.326	0.039	0. 441	0.007
30	nd	nd	0.55	0.044	0.350	0.207	0.187	0.148	nd	nd	nd	nd	0.240	0.148	0.335	0.013
36	nd	nd	0.43	0.181	0.210	0.138	0.160	0.089	nd	nd	nd	nd	0.255	0.142	0.211	nd
42	nd	nd	1.18	nd	0.102	nd	0.252	nd	nd	nd	nd	nd	0.188	nd	0.147	nd
48	nd	0.075	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.089	nd
54	0.284	0.050	nd	0.120	nd	0.036	0.170	0.056	nd	nd	nd	nd	0.322	0.089	nd	0.064
60	0.054	nd	1.22	0.013	0.090	0.073	0.106	0.06	nd	nd	nd	nd	nd	nd	nd	nd
66	nd	nd	1.89	nd	0.079	nd	0.155	0.087	nd	nd	nd	nd	0.197	0.091	0.022	0.035
72	nd	nd	0.70	0.020	nd	0.167	0.102	nd	nd	nd	nd	nd	0.213	0.065	nd	nd
78	nd	nd	nd	nd	0.057	0.083	0.084	0.103	nd	nd	nd	nd	0.153	0.039	nd	nd
84	nd	nd	nd	0.012	nd	0.095	0.111	0.046	nd	nd	nd	nd	0.077	0.079	nd	nd

nd = not detectable.

tion technique, free cell of PCL3 inoculation, was applied to both kinds of soil in order to improve the carbofuran degradation efficiency. When compared to carbofuran degradation in soil microcosms, the soil slurry phase bioreactor showed a better performance in carbofuran degradation. The shortest time to achieve 90% carbofuran degradation in the bioaugmented soil microcosms was 50 d [20], which was 14.3 times longer than using the soil slurry phase treatment (3.5 d) (Fig. 2(b)), even though the initial carbofuran concentration in the soil microcosms was 4 times lower than in the soil slurry phase reactor. These findings indicate that the bioslurry phase reactor could be applied for the purpose of significantly enhancing carbofuran degradation efficiency in the soil. The aeration together with mechanical mixing might be responsible for this trend by improving the mass transfer rate and contact among microorganisms, carbofuran, and nutrients, and hence increasing the rates of carbofuran biodegradation. This finding was similar to the results from previous studies [9,11,13,14].

Carbofuran phenol and 3-keto carbofuran were observed to be the metabolites in both soil and liquid phases in the reactors with biological activity (reactors A–D, G and H) (Tables 2 and 3). In the soil phase, carbofuran phenol and 3-keto carbofuran could be observed in reactors A–D and H from 6 to 42 h while, in reactors D and G, these metabolites could be found until the end of reactor operation. Higher concentrations of carbofuran metabolites were found in the liquid phase than in the soil phase, and the accumulation of carbofuran phenol and 3-keto carbofuran in the liquid phase could be found in all bioreactors until the end of reactor operation except for reactors A and H. A decrease in carbofuran metabolites during reactor operation implied that the microorganisms in the soil slurry phase reactors might be able to metabolize carbofuran metabolites as their energy sources, as was reported in a study by Yan et al. [3].

3.2. Substrate partitioning between soil and liquid phases

Carbofuran partitioning in the soil and liquid phases during soil slurry phase reactor operation was investigated (Figs. 3 and 4). Substrate partitioning in the abiotic control reactor (reactor F) (Fig. 3) indicated the actual mechanism of carbofuran partitioning in the two phases of the slurry system was insignificant. After start-up of the reactor, carbofuran rapidly desorbed from the soil to the aqueous phase (Fig. 3). The partitioning of carbofuran approached steady state at 30 h of reactor operation with carbofuran concentrations in the soil and liquid phases of approximately 0.75 and 0.79 mg, respectively (Fig. 3). Reactor E, autoclaved soil with autoclaved corncob added, showed the same trend of carbofuran partitioning as reactor F (Fig. 3). The relatively high desorption of carbofuran from the soil into the liquid phase might be attributed to the hydroponic nature of carbofuran, i.e. its high water solubility of 351 mg/L at 25 °C and low adsorption coefficient (Koc) of 22 [23]. Desorption of carbofuran from the soil to the liquid phase could facilitate the mass transfer and increase contact between the microorganisms and carbofuran, which might

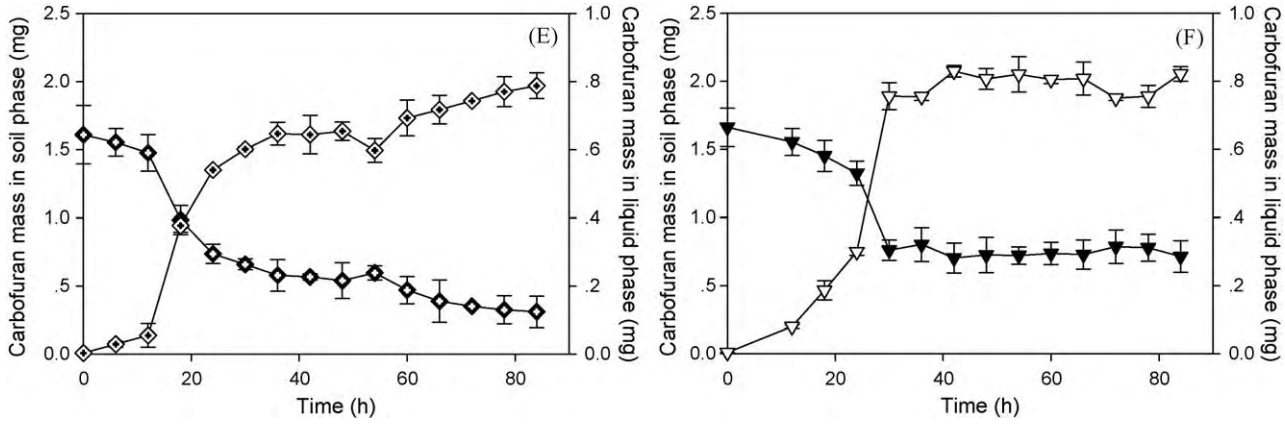

Fig. 3. Carbofuran partitioning between soil (close symbols) and liquid (open symbols) phases in abiotic control soil slurry phase reactors (reactor E: autoclaved soil slurry + autoclaved corncob; reactor F: autoclaved soil slurry).

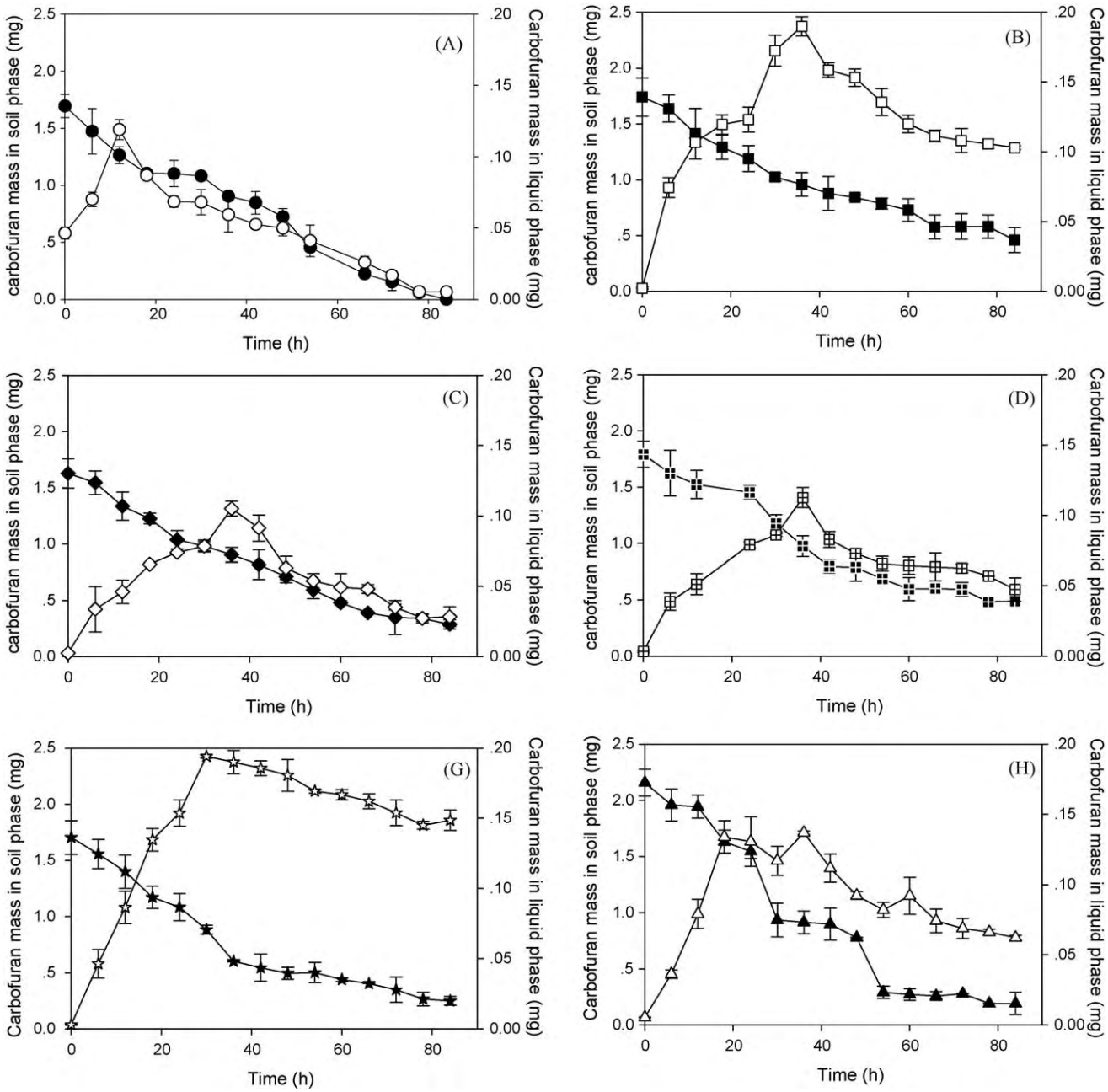

Fig. 4. Carbofuran partitioning between soil (close symbols) and liquid (open symbols) phases in soil slurry phase reactors with biological activity (reactor A: soil slurry + immobilized PCL3; reactor B: soil slurry; reactor C: autoclaved soil slurry + immobilized PCL3; reactor D: soil slurry + autoclaved corncob; reactor G: soil slurry + molasses; reactor H: soil slurry + immobilized PCL3 + molasses).

result in an improvement in carbofuran degradation efficiency in the soil slurry phase reactor.

Carbofuran partitioning in the reactors with microbial activity (Fig. 4), i.e. bioaugmentation (reactors A, C and H), biostimulation (reactors G and H), and indigenous microorganisms (reactors B and D) was obviously different from that in the abiotic reactors (E and F). In reactors A and H, the carbofuran concentration in the liquid phase increased until 18 h of reactor operation, reaching maximum concentrations of 0.11 and 0.13 mg, respectively, and then rapid degradation of carbofuran was observed (Fig. 4). The continuous decrease in carbofuran concentration in the soil phase continued until the end of the experiment. This result indicated that desorbed carbofuran in the liquid phase was subjected to continuous biological degradation; therefore, desorption was enhanced due to the partitioning effect. The same partitioning patterns of carbofuran in the soil and liquid phases could be found in reactors B–D and G, with longer times of 30–36 h taken to approach the maximum carbofuran concentrations in the liquid phase of 0.11–0.19 mg (Fig. 4). As the carbofuran degradations in reactors A and H were more rapid and started earlier than in the other reactors, it could be concluded that PCL3 together with indigenous microorganisms in reactors A and H were more effective in enhancing carbofuran degradation than PCL3 (reactor C) or indigenous microorganisms (reactors B, D and G) alone in the reactor.

3.3. pH values and CFU variations

Soil slurry pH and CFU (colony forming unit) were investigated during soil slurry phase reactor operation as important parameters

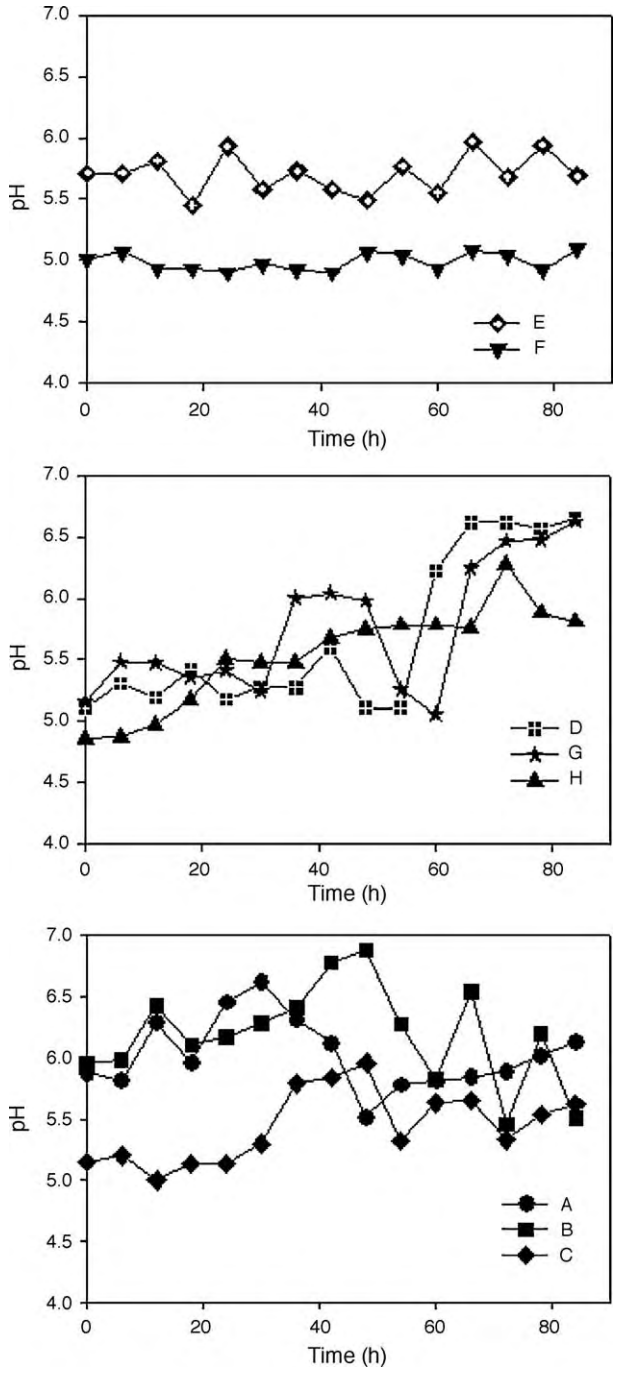

Fig. 5. The temporal variation of soil slurry phase pH during reactor operation.

indicating microbial activity. The results indicated that pH variation in the reactors with biological activity (reactors A–D, G, and H) was more obvious compared to the abiotic control reactors (E and F) (Fig. 5). In reactors A–C a slight increase in the pH of the soil slurry was observed during the early period of reactor operation and a further decrease in the soil slurry pH was found after 28 h (reactor A) and 42 h (reactors B and C) of reactor operation. A tendency of the soil slurry pH to increase was observed in reactors D, G, and H throughout reactor operation. The variation in soil slurry pH could result from the products obtained from biodegradation activity. An increase in the soil slurry pH might be due to the alkaline nature of by-products formed during the metabolism of carbofuran and organic matter by the microorganisms inhabiting the soil slurry [8]. Subsequently, a

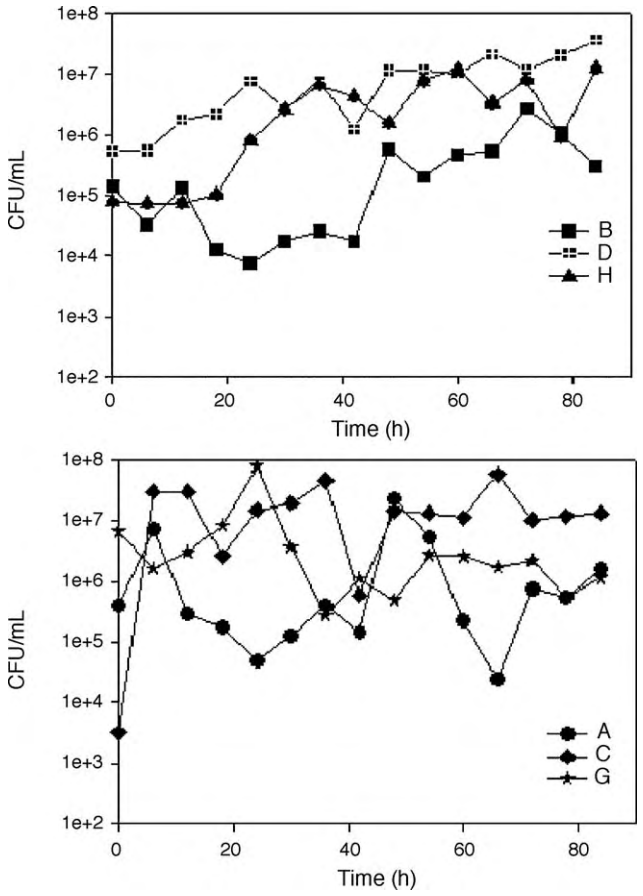

Fig. 6. The temporal CFU variation during soil slurry phase reactor operation.

decrease in the soil slurry pH in reactors A, B, and C might due to the formation of CO_2 from mineralization of the metabolic intermediates formed [11]. Reactors E and F showed relatively stable soil slurry pH values during operation (Fig. 5), which might due to the absence of microbial activity [24].

The number of carbofuran degraders in the soil slurry phase reactors with biological activity in terms of CFU (Fig. 6) was determined on a BSM agar plate coated with carbofuran as a sole carbon source. The results indicated the successful development of indigenous microbial numbers in the soil slurry phase reactor, which was shown by the marked increase in the numbers of carbofuran degraders (by one order of magnitude) in reactors operated with indigenous microorganisms alone (reactors B and D) (Fig. 6). However, the number of indigenous carbofuran degraders in the stimulated reactor (G) decreased by one order of magnitude, which might due to the toxic effects of by-products from molasses components and the metabolism of molasses by the indigenous microorganisms themselves. In the augmented reactor (reactor A), the number of carbofuran degraders in the soil slurry obviously varied over time. Though the CFU in reactor A decreased markedly by two orders of magnitude in some periods of operation (6–24 and 42–66 h) (Fig. 6), the greatest carbofuran degradation efficiency was obtained in this reactor. This result implied that carbofuran degradation did not depend on the efficacy of free cell of carbofuran degraders suspended in the soil slurry, but might mainly depend on the immobilized PCL3 adsorbed on the support. In reactor H, to which was added both immobilized PCL3 and molasses, the number of carbofuran degraders increased by two orders of magnitude (Fig. 6). This result was different from the CFU variation in reactor G, which showed the negative effect of molasses on the growth of carbofuran degraders in the soil slurry. The explanation for this phenomenon is that PCL3 in immobilized

Table 4
Kinetic parameters for carbofuran degradation in soil phase.

Reactor	Order of equation	Degradation rate constant	Degradation half-life (h)[a]	Kinetic equation	R^2
A	Zero	0.020 ± 0.0028 mg/h	39.75 ± 5.57fg	$C_t = 1.59 - 0.020t$	0.98
	First	0.037 ± 0.0021 /h	18.73 ±1.06cd	$C_t = 2.43e^{-0.037t}$	0.85
	Second	0.083 ± 0.0087 /(mg h)	7.11 ± 0.75ab	$1/C_t = 0.083t + 0.59$	0.43
B	Zero	0.015 ± 0.0012 mg/h	53.00 ± 4.28i	$C_t = 1.59 - 0.015t$	0.95
	First	0.015 ± 0.0013 /h	46.20 ± 4.00ghi	$C_t = 1.71e^{-0.015t}$	0.99
	Second	0.017 ± 0.0013 /(mg h)	27.06 ± 2.07de	$1/C_t = 0.017 + 0.46$	0.95
C	Zero	0.016 ± 0.0013 mg/h	47.81 ± 3.88ghi	$C_t = 1.53 - 0.016t$	0.97
	First	0.021 ± 0.0015 /h	33.00 ± 2.36ef	$C_t = 1.79e^{-0.021t}$	0.96
	Second	0.034 ± 0.0013 /(mg h)	5.88 ± 0.22a	$1/C_t = 0.034t + 0.20$	0.91
D	Zero	0.016 ± 0.0021 mg/h	52.50 ± 6.89hi	$C_t = 1.68 - 0.016t$	0.93
	First	0.017 ± 0.0014 /h	40.76 ± 3.36fg	$C_t = 1.84e^{-0.017t}$	0.97
	Second	0.019 ± 0.0023 /(mg h)	21.58 ± 2.61cd	$1/C_t = 0.019t + 0.41$	0.96
E	Zero	0.015 ± 0.0013 mg/h	46.00 ± 3.99ghi	$C_t = 1.38 - 0.015t$	0.96
	First	0.021 ± 0.0022 /h	33.00 ± 3.46ef	$C_t = 1.50e^{-0.021t}$	0.93
	Second	0.031 ± 0.0009 /(mg h)	15.16 ± 0.44bc	$1/C_t = 0.031t + 0.47$	0.96
F	Zero	0.011 ± 0.0014 mg/h	66.82 ± 8.50j	$C_t = 1.47 - 0.011t$	0.66
	First	0.010 ± 0.0015 /h	69.30 ± 10.40j	$C_t = 1.04e^{-0.010t}$	0.65
	Second	0.010 ± 0.0017 /(mg h)	71.00 ± 12.07j	$1/C_t = 0.010t + 0.71$	0.64
G	Zero	0.017 ± 0.0017 mg/h	44.12 ± 4.41gh	$C_t = 1.50 - 0.017t$	0.91
	First	0.023 ± 0.0018 /h	30.13 ± 2.36d	$C_t = 1.72e^{-0.023t}$	0.98
	Second	0.039 ± 0.0022 /(mg h)	5.13 ± 0.29a	$1/C_t = 0.039t + 0.20$	0.93
H	Zero	0.026 ± 0.0010 mg/h	39.42 ± 1.52fg	$C_t = 2.05 - 0.025t$	0.93
	First	0.032 ± 0.0020 /h	21.66 ± 1.35cd	$C_t = 2.67e^{-0.032t}$	0.93
	Second	0.061 ± 0.0042 /(mg h)	7.38 ± 0.51ab	$1/C_t = 0.047t + 0.45$	0.80

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

cell form could be protected from the toxic effects of the by-products from molasses components or molasses metabolism [25]. In contrast, molasses might well be used by PCL3 as an energy source, resulting in continuous growth of the cell and leakage to the soil slurry due to the limited space in the support material. This was attributed to an increase in the number of carbofuran degraders in the soil slurry with a decrease in carbofuran degradation efficiency, as previously described.

Number of PCL3 in soil slurry of reactor C containing autoclaved soil slurry inoculated with immobilized PCL3 was greater than 10^5 CFU/mL throughout the experiment (Fig. 6) ensuring that the augmented degraders could survive during soil slurry phase reactor operation.

3.4. Kinetic degradation of carbofuran in soil slurry phase reactor

The kinetic aspect of carbofuran degradation in the soil slurry was examined in this study using zero-, first-, and second-order equations. The kinetic parameters of carbofuran degradation in the soil phase derived from analysis of the output of the three equations along with R^2 values are shown in Table 4. A reasonably good correlation was indicated by R^2 of more than 0.90. In the abiotic control reactor (F), the carbofuran degradation pattern did not follow any of the kinetic equations (R^2 = 0.64–0.66) (Table 4). This might be due to the fact that the desorbed carbofuran in the aqueous phase was not further degraded due to the absence of microorganisms in the reactor. Reactors A, C, E, and H showed a good correlation with the zero-order kinetic equation (R^2 = 0.93–0.98) (Table 4), which indicated that the degradation process in the soil phase of these reactors was independent of carbofuran concentration and the carbofuran degradation would not be inhibited by carbofuran concentration. Reactors B, D, and G showed a good correlation with the first-order kinetic equation (R^2 = 0.97–0.99) (Table 4), revealing that the rate of degradation was directly proportional to the concentration of carbofuran in the soil phase of these reactors.

The half-lives of carbofuran were calculated and are depicted in Table 4. The half-life of carbofuran in the non-augmented reactor (B) was 46.2 h, which was longer than the half-lives of carbofuran in the reactors with bioremediation treatments (reactors A, C, D, G, and H) of 30.13–40.76 h (Table 4), indicating an improvement in carbofuran degradation by the bioaugmentation and/or biostimulation techniques. In this study the shortest half-life of carbofuran in the soil phase of 30.13 h was obtained in reactor G (Table 4), but the greatest efficiency of carbofuran removal was obtained in reactor A (96.97%), with a short half-life of carbofuran in the soil of 39.75 h (Table 4). To understand this phenomenon, we further investigated the kinetics of the degradation of carbofuran in the liquid phase after starting the degradation process in each reactor. The kinetic parameters along with R^2 for carbofuran degradation in the liquid phase of each reactor with the best correlation equations are presented in Table 5. The degradation of carbofuran in reactors

Table 5
Kinetic parameters for carbofuran degradation in liquid phase.

Reactor	Order of equation (best fitted)	Degradation rate constant	Degradation half-life (h)[a]	Kinetic equation	R^2
A	Zero	0.001 ± 0.00009 mg/h	50.00 ± 4.51b	$C_t = 0.10 - 0.001t$	0.98
B	First	0.011 ± 0.0011 /h	63.00 ± 6.32c	$C_t = 0.24e^{-0.011t}$	0.93
C	First	0.027 ± 0.0042 /h	25.67 ± 3.97a	$C_t = 0.253e^{-0.027t}$	0.94
D	First	0.011 ± 0.0013 /h	63.00 ± 7.44c	$C_t = 0.12e^{-0.011t}$	0.90
G	Second	0.034 ± 0.0003 /(mg h)	148.53 ± 1.31c	$1/C_t = 0.034t + 5.05$	0.97
H	First	0.013 ± 0.0014 /h	53.31 ± 5.72bc	$C_t = 0.17e^{-0.013t}$	0.98

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

E and F was insignificant; therefore the degradation kinetics were not examined in these reactors. The carbofuran half-life in the liquid phase of reactor G (148.53 h) was significantly longer than that in the liquid phase of reactor A (50 h) (Table 5). In addition, the carbofuran degradation process in reactor A started after 18 h of reactor operation, which was earlier than in reactor G (Fig. 4). Therefore, we could conclude that reactor A was the most effective treatment to remediate carbofuran, as indicated by the highest performance (96.97%) and shortest half-lives in the soil (Table 4) and the liquid phase (Table 5) of 39.75 and 50 h, respectively. The carbofuran degradation pattern in the liquid phase of reactor B followed the second-order kinetics equation, indicating dependence on the concentrations of both the carbofuran and intermediates formed during the biodegradation process.

4. Conclusion

The soil slurry phase reactor was an effective technique for removing carbofuran from the soil. Desorption of carbofuran from the soil to the liquid phase offered favorable conditions for the biological degradation process of carbofuran. Bioaugmentation treatment could be applied together with the soil slurry phase bioreactor to enhance the carbofuran degradation efficiency in the soil. Variations of pH and CFU during the degradation process correlated with biological activity in the soil slurry phase reactors. Soil slurry augmented with immobilized PCL3 (reactor A) was the most effective treatment for bioremediating carbofuran giving the highest percentage of carbofuran removal (96.97%). Biostimulation (addition of molasses) treatment could improve the carbofuran degradation efficiency of indigenous microorganisms in soil, however, worsen the ability of PCL3 to degrade carbofuran.

Acknowledgements

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