Profenofos Removal by *Pseudomonas plecoglossicida* PF1 and *Acinetobacter baylyi* GFJ2 in Free and Immobilized Cell Forms and Their Motility

Miss Chutima Ploychankul

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Environmental Management
(Interdisciplinary Program)
Graduate School
Chulalongkorn University
Academic Year 2016

การกำจัดสารโพรฟีโนฟอสด้วย *Pseudomonas plecoglossicida* PF1 และ *Acinetobacter baylyi* GFJ2 ในรูปเซลล์อิสระและตรึง และสมบัติการเคลื่อนที่

นางสาวชุติมา พลอยจันทร์กุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาการจัดการสิ่งแวดล้อม (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2559

Thesis Title	Profenofos Removal by *Pseudomonas plecoglossicida* PF1 and *Acinetobacter baylyi* GFJ2 in Free and Immobilized Cell Forms and Their Motility
By	Miss Chutima Ploychankul
Field of Study	Environmental Management
Thesis Advisor	Associate Professor Sumana Ratpukdi, Ph.D.
Thesis Co-Advisor	Associate Professor Alisa Vangnai, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Graduate School
(Associate Professor Sunait Chutintaranond, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Ekawan Luepromchai, Ph.D.)
.....Thesis Advisor
(Associate Professor Sumana Ratpukdi, Ph.D.)
.....Thesis Co-Advisor
(Associate Professor Alisa Vangnai, Ph.D.)
.....Examiner
(Associate Professor Tawan Limpiyakorn, Ph.D.)
.....Examiner
(Professor Eakalak Khan, Ph.D.)
.....Examiner
(Associate Professor Onruthai Pinyakong, Ph.D.)
.....External Examiner
(Associate Professor Duangrat Inthorn, Ph.D.)

ชุติมา พลอยจันทร์กุล : การกำจัดสารโพรฟีโนฟอสด้วย *Pseudomonas plecoglossicida* PF1 และ *Acinetobacter baylyi* GFJ2 ในรูปเซลล์อิสระและตรึง และสมบัติการเคลื่อนที่ (Profenofos Removal by *Pseudomonas plecoglossicida* PF1 and *Acinetobacter baylyi* GFJ2 in Free and Immobilized Cell Forms and Their Motility) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. สุมนา ราษฎร์ภักดี, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. อลิสา วังใน, หน้า.

สารโพรฟีโนฟอสเป็นสารกำจัดศัตรูพืชที่นิยมใช้กันอย่างแพร่หลายและมีการรายงานการปนเปื้อนของสารชนิดนี้ในสิ่งแวดล้อม งานวิจัยนี้เป็นการศึกษาเกี่ยวกับการกำจัดสารโพรฟีโนฟอสโดยใช้เซลล์แบคทีเรียตรึง ซึ่งการศึกษานี้ยังครอบคลุมความสามารถในการเคลื่อนที่ของเซลล์ที่มีความสัมพันธ์กับศักยภาพการย่อยสลายทางชีวภาพด้วย แบคทีเรียที่ใช้ในการศึกษานี้ 2 สายพันธุ์ ได้แก่ *Pseudomonas plecoglossicida* PF1 (PF1) และ *Acinetobacter baylyi* GFJ2 (GFJ2) ซึ่งเป็นแบคทีเรียที่สามารถและไม่สามารถเคลื่อนที่ได้ตามลำดับ การศึกษาสามารถแบ่งออกได้เป็น 2 ขั้นตอน ขั้นตอนที่ 1 เป็นการอธิบายลักษณะการย่อยสลายสารโพรฟีโนฟอสทางชีวภาพด้วย PF1 และ GFJ2 และศึกษาผลของปัจจัยของสิ่งแวดล้อมที่มีผลต่อการย่อยสลาย อันได้แก่ ความเป็นกรดด่าง อุณหภูมิ และ ความเข้มข้นของสารโพรฟีโนฟอส สำหรับในขั้นตอนที่ 2 เป็นการประยุกต์ใช้เซลล์ตรึงในการทดลองแบบแบตช์ (อิทธิพลของขนาดของเซลล์ตรึง ชนิด และความเข้มข้นของเกลืออนินทรีย์) และคอลัมน์ (อิทธิพลของความเข้มข้นของสารโพรฟีโนฟอส) ผลการศึกษานี้เป็นการเพิ่มความรู้เกี่ยวกับความสัมพันธ์ระหว่างประสิทธิภาพการกำจัดสารและพฤติกรรมของจุลินทรีย์ที่ตรึงเปรียบเทียบกับเซลล์อิสระ การศึกษานี้ใช้แคลเซียมอัลจิเนตซึ่งเป็นวัสดุที่นิยมใช้ในการตรึงเซลล์แบบดักติด

จากผลการทดลองพบว่าทั้ง PF1 และ GFJ2 เป็นจุลินทรีย์มีความสามารถในการย่อยสลายสารโพรฟีโนฟอสได้สูงโดยมีร้อยละการกำจัดสารโพรฟีโนฟอส 60 ถึง 90 สภาวะที่เหมาะสมต่อการย่อยสลายสารโพรฟีโนฟอส ได้แก่ ความเป็นกรดด่าง 5.30-7.89 อุณหภูมิ 20-40 องศาเซลเซียส และความเข้มข้นของสารโพรฟีโนฟอส 10-200 มิลลิกรัมต่อลิตร จากการทดลองแบบแบตช์พบว่าขนาดของเม็ดเซลล์ตรึงมีผลต่อศักยภาพการกำจัดสารโพรฟีโนฟอส ขนาดของเม็ดที่เหมาะสม คือ เส้นผ่านศูนย์กลาง 4 มิลลิเมตร PF1 มีสมบัติการเคลื่อนที่โดย PF1 มีการชักนำโดยสารโพรฟีโนฟอสซึ่งส่งผลให้มีประสิทธิภาพในการกำจัดสารสูงด้วยเช่นกัน ส่วนผลการศึกษาในคอลัมน์พบว่าการดักติดเซลล์สามารถกักจุลินทรีย์ทั้งที่สามารถและไม่สามารถเคลื่อนที่ได้ นอกจากนี้ยังพบว่ามีการตรึงเซลล์ตามธรรมชาติบนทรายในชุดทดลองเซลล์อิสระเป็นเหตุให้ประสิทธิภาพในการกำจัดสารโพรฟีโนฟอสโดยชุดทดลองเซลล์อิสระและเซลล์ตรึงมีค่าใกล้เคียงกัน (ร้อยละ 60 ถึง 90) ในระยะยาวแบคทีเรียที่สามารถเคลื่อนที่ได้ (PF1) ทั้งในรูปเซลล์อิสระและเซลล์ตรึงทำงานได้ดีกว่า GFJ2

สาขาวิชา การจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5487765620 : MAJOR ENVIRONMENTAL MANAGEMENT
KEYWORDS: PROFENOFOS BIODEGRADATION, IMMOBILIZED CELL, RESPONSE SURFACE METHODOLOGY, SAND-FILTER COLUMN, CELL MOTILITY

CHUTIMA PLOYCHANKUL: Profenofos Removal by *Pseudomonas plecoglossicida* PF1 and *Acinetobacter baylyi* GFJ2 in Free and Immobilized Cell Forms and Their Motility. ADVISOR: ASSOC. PROF. SUMANA RATPUKDI, Ph.D., CO-ADVISOR: ASSOC. PROF. ALISA VANGNAI, Ph.D., pp.

Profenofos, a widely used pesticide, has been reported contamination in environment. This study investigated profenofos removal using the immobilized bacterial cells. Cell movement ability related to biodegradation performance was emphasized. Two profenofos-degrading strains, *Pseudomonas plecoglossicida* PF1 (PF1) and *Acinetobacter baylyi* GFJ2 (GFJ2) which were motile and non-motile bacteria, respectively were applied. The study divided into 2 parts. For the first part, characterization of profenofos biodegradation by PF1 and GFJ2 influencing by environmental conditions (effects of pH, temperature, and profenofos concentration) was performed. For the second part, the applications of the immobilized cells in batch (effects of the immobilized cell sizes and inorganic salts) and column (effect of profenofos concentrations) tests were examined to accomplish on the gap knowledge between the removal efficiency and behavior of the microorganisms after being immobilized in the matrices comparing to the free cell. The well-known cell entrapment matrix, calcium alginate (CA), was selected. The result showed that both PF1 and GFJ2 were the potential profenofos-degrading microorganisms with a high profenofos removal percentage (60-90%). Optimal conditions for profenofos biodegradation were at pHs of 5.30-7.87, temperatures of 20-40 °C, and profenofos concentrations of 10-200 mg/L. For the batch experiment, the bead size obviously affected the profenofos removal performance. The suitable bead size in this study was 4 mm-diameter. PF1 was positive in motility assays. PF1 got attraction by profenofos resulting in high removal efficiency. For the column experiment, the immobilization technique well retained both motile and non-motile cells. It was also found natural cell colonization on sand in the free cell columns led to similar profenofos removal performance by the free and immobilized cells (60-90%). For the long term, motile bacterium (PF1) either in free or immobilized cell forms performed better than GFJ2.

Field of Study: Environmental Management
Academic Year: 2016

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my deepest and sincerest appreciation to my advisor, Assoc. Prof. Dr. Sumana Ratpukdi for her dedication on my learning, kind suggestion, passionate, and encouragement throughout into this dissertation. She never gives up on me and tries to give some good advice about how to apply studies' life into real life. She always has a good decision in every problem occurred. I also would like to thank my co-advisor, Assoc. Prof. Dr. Alisa S. Vangnai for her precious time, kind guidance, and challenge support. She provided me an opportunity to work and study on molecular biology. In her laboratory, I've learned and obtained a lot on complexity molecular techniques. I am very grateful for her valuable suggestions and assistance. Furthermore, I would like to express my acknowledgement of Asst. Prof. Dr. Kitirote Wantala for his valuable advice and support along with this study.

I am very appreciated to Asst. Prof. Dr. Thunyalux Ratpukdi and Assoc. Prof. Dr. Pinthita Mungkarndee for all presentation comments on the clear idea of this experiment. In addition, I am very thankful to Assoc. Prof. Dr. Ekawan Luepromchai, chairman of dissertation committee including Assoc. Prof. Dr. Tawan Limpiyakorn, Prof. Dr. Eakalak Khan, Assoc. Prof. Dr. Onruthai Pinyakong, and Assoc. Prof. Dr. Duangrat Inthorn, members of dissertation committees. Without the important assistance of the Chairman and the committees, this dissertation would never be accomplished.

I am very thankful to all laboratory members at Room 604/1, Department of Biochemistry, Faculty of Science, Center of Excellence on Hazardous Substance Management (HSM), Chulalongkorn University, Department of Environmental Engineering, Faculty of Engineering, Khon Kaen University. I would like to thank The 90th Anniversary of Chulalongkorn University Scholarship and HSM program for financial support during the study.

Last but not least, I would like to express my deepest grateful to my mother, my father and Nareerat Tongtavol for their supporting. They always encourage me as the time pass by including bad or good time. Thanks for their hearts and loves. This work would not be succeeded without their moral support.

CONTENTS

Page
THAI ABSTRACT iv
ENGLISH ABSTRACT v
ACKNOWLEDGEMENTS vi
CONTENTS vii
LIST OF FIGURES 12
LIST OF TABLES 15
CHAPTER 1 Introduction 16
1.1 General introduction 16
1.2 Objectives 18
1.3 Scopes 18
1.4 Hypotheses 20
1.5 Experimental framework 20
CHAPTER 2 Literature Review 23
2.1 Profenofos 23
2.1.1 Properties of profenofos 23
2.1.2 Fate of profenofos in environment 23
2.1.3 Biodegradation of profenofos 25
2.2 Cell immobilization 27
2.3 Cell entrapment 29
2.3.1 Principle of cell entrapment 29
2.3.2 Types of cell entrapment materials 29
2.3.2.1 Calcium alginate 29
2.3.2.2 Carrageenan 29
2.3.2.3 Polyvinyl alcohol 30
2.3.2.4 Cellulose triacetate (CTA) 30
2.3.3 Applications of cell entrapment for environmental practices 30
2.3.3.1 Wastewater treatment 31
2.3.3.2 Bioremediation 31

Page

CHAPTER 3 Characterization of Profenofos Degradation by *Pseudomonas plecoglossicida* Strain PF1 Using Surface Response Methodology 34
3.1 Introduction 34
3.2 Materials and methods 35
3.2.1 Chemicals 35
3.2.2 Microorganism and cultivation 36
3.2.3 Profenofos biodegradation experiment 36
3.2.4 Analytical methods 38
3.3 Results and discussion 38
3.3.1 Profenofos biodegradation and identification of intermediate 38
3.3.2 Response surface methodology 42
3.3.2.1 Statistical characterization of profenofos biodegradation 42
3.3.2.2 Effects of pHs, temperatures, and profenofos concentrations on profenofos biodegradation 46
3.3.2.3 Interactions between pH, temperature, and profenofos concentration 47
3.4 Summary 50
CHAPTER 4 Profenofos Removal by *Acinetobacter baylyi* Strain GFJ2: Biodegradation Kinetics and Influence of Environmental Conditions 51
4.1 Introduction 51
4.2 Materials and methods 52
4.2.1 Chemicals 52
4.2.2 Microorganism and enrichment 52
4.2.3 Profenofos biodegradation kinetic test 53
4.2.4 Response Surface Methodology (RSM) 53
4.2.5 Analytical methods 55
4.3 Results and discussion 55
4.3.1 Profenofos biodegradation kinetics 55
4.3.2 Response surface methodology 59
4.3.2.1 Characterization of profenofos biodegradation 59

Page

4.3.2.2 Influence of pHs, temperatures, and initial profenofos concentrations on profenofos biodegradation.....62

4.3.2.3 Interactions of pHs, temperatures, and initial profenofos concentrations.....63

4.4 Summary.....65

CHAPTER 5 Profenofos Removal by Immobilized *Pseudomonas plecoglossicida* strain PF1 and *Acinetobacter baylyi* strain GFJ2 and Their Cell Retention Ability: Influence of Environmental Condition and Immobilized Cell Characteristics.....66

5.1 Introduction.....66

5.2 Materials and Methods.....68

5.2.1 Chemicals.....68

5.2.2 Bacterial cultivation and growth conditions.....68

5.2.3 Cell immobilization procedure.....69

5.2.4 Profenofos biodegradation and immobilized cell leaching assay.....69

5.2.5 Motility assay.....70

5.2.6 Analytical methods.....71

5.3 Results and discussion.....72

5.3.1 Profenofos biodegradation assay.....72

5.3.1.1 Influence of immobilized cell bead sizes on profenofos biodegradation.....72

5.3.1.2 Influence of profenofos concentrations on profenofos biodegradation.....75

5.3.1.3 Influence of inorganic salt types and concentrations on profenofos biodegradation.....85

5.3.2 Motility assay.....87

5.3.2.1 Immobilized cell leaching.....87

5.3.2.2 Swimming, swarming, twitching assays.....93

5.3.2.3 Capillary chemotaxis assay.....98

5.4 Summary.....101

CHAPTER 6 Profenofos Pesticide Removal by Immobilized Cells in Sand Column Experiment: Comparative Study of Motile and Non-motile Cells.....102

Page

6.1 Introduction....102
6.2 Materials and methods....103
6.2.1 Chemicals....103
6.2.2 Microorganisms and cultural condition....103
6.2.3 Cell immobilization procedure....104
6.2.4 Column experiment....105
6.2.4.1 Synthetic infiltrate....105
6.2.4.2 Sand and column preparation....105
6.2.4.3 Profenofos biodegradation and immobilized cell leaching in column experiment....107
6.2.5 Capillary chemotaxis assay....107
6.2.6 Analytical methods....108
6.2.6.1 Profenofos analysis....108
6.2.6.2 Cell viability....108
6.2.6.3 Immobilized cell morphology....109
6.3 Results and discussion....109
6.3.1 Profenofos biodegradation and immobilized cell leaching in column experiment....109
6.3.1.1 Profenofos biodegradation in column experiment....109
6.3.1.2 Immobilized cell leaching in column experiment....111
6.3.1.3 Immobilized cell morphology....112
6.3.2 Capillary chemotaxis assay of free and de-immobilized cells....113
6.3.3 Cell viability of free and de-immobilized cells....116
6.4 Summary....118
CHAPTER 7 Conclusions and Recommendations....119
7.1 Conclusions....119
7.2 Recommendations....120
....121
REFERENCES....121

Page

VITA 133

LIST OF FIGURES

Figure 1.1 Experimental frameworks.....21

Figure 2.1 The proposed pathway for PF degradation by *Pseudomonas aeruginosa* (Malghani et al., 2009).....26

Figure 3.1 Profenofos biodegradation (◆) and BCP production (**Δ**): (a) profenofos of 5 mg/L, (b) profenofos of 10 mg/L, and (c) profenofos of 20 mg/L.....41

Figure 3.2 Normal probability of standardized residual for profenofos degradation kinetic rates.....42

Figure 3.3 Graphical plots of profenofos degradation kinetic rates (h^{-1}) between experimental and predicted values.....45

Figure 3.4 Main effects for profenofos biodegradation kinetic rates.....45

Figure 3.5 Contour plot for profenofos degradation kinetic rates (h^{-1}) resulting from interaction of initial profenofos concentration (mg/L), pH, and temperature (°C): (a) interaction between pH and initial profenofos concentration, (b) interaction between temperature and initial profenofos concentration, and (c) interaction between pH and temperature.....49

Figure 4.1 Profenofos biodegradation at initial profenofos concentration of 20 mg/L (◆) and BCP production (**Δ**).....56

Figure 4.2 Profenofos biodegradation kinetics by GFJ2: (a) Michaelis-Menten plot and (b) Lineweaver-Burk plot. ■ represents experimental values while line plot (-) shows estimated values.....57

Figure 4.3 Normal percentage probability plot for profenofos biodegradation kinetic rates.....59

Figure 4.4 Parity plot of profenofos degradation kinetic rates (h^{-1}) between experimental and predicted values.....61

Figure 4.5 Main effects for profenofos biodegradation kinetic rates.....63

Figure 4.6 Contour plots for profenofos biodegradation kinetic rates (h^{-1}) resulting from interaction of initial profenofos concentration (mg/L), pH and temperature (°C): (a) pH and intial profenofos concentration, (b) temperature and initial profenofos concentration and (c) pH and temperature.....64

Figure 5.1 Schematic picture of double layer-immobilized cell.....70

Figure 5.2 Profenofos biodegradation of PF1 at different sizes of immobilized beads (Free cell (◆), 2-mm bead (▪), 4-mm bead (▲), and 6-mm bead (x)).....74

Figure 5.3 Profenofos biodegradation of GFJ2 at different sizes of immobilized beads (Free cell (◆), 2-mm bead (■), 4-mm bead (▲), and 6-mm bead (x)).....74

Figure 5.4 Profenofos biodegradation of PF1 at the different concentrations.....77

Figure 5.5 Profenofos biodegradation of GFJ2 at the different concentrations.....78

Figure 5.6 Profenofos degradation of immobilized PF1 and GFJ2 followed Michaelis-Menten equation 79

Figure 5.7 Profenofos degradation of immobilized PF1 and GFJ2 followed 80

Figure 5.8 Profenofos degradation of PF1 followed Michaelis-Menten equation: a) Free cell and b) Immobilized cell 81

Figure 5.9 Profenofos degradation of PF1 followed Lineweaver-Burk equation: a) Free cell and b) Immobilized cell 82

Figure 5.10 Profenofos degradation of GFJ2 followed Michaelis-Menten equation: a) Free cell and b) Immobilized cell 83

Figure 5.11 Profenofos degradation of GFJ2 followed Lineweaver-Burk equation: a) Free cell and b) Immobilized cell 84

Figure 5.12 Leaching cells from the immobilized PF1 beads in TSM medium.....89

Figure 5.13 Leaching cells from the immobilized GFJ2 beads in TSM medium.....89

Figure 5.14 SEM observation of the immobilized cell at the inner layer: a) PF1 and b) GFJ2.....90

Figure 5.15 SEM observation on the outer layer of the beads of PF1 after treated with profenofos at 20 mg/L for 48 h: a) magnification at x300 and b) magnification at x10,000 91

Figure 5.16 SEM observation on the outer layer of the beads of GFJ2 after treated with profenofos at 20 mg/L for 48 h: a) magnification at x300 and b) magnification at x10,000 92

Figure 5.17 Chemotactic response of PF1 and GFJ2 to profenofos at the different concentrations comparing with yeast extract as positive control at 90-s time period .99

Figure 5.18 Chemotactic response of PF1 and GFJ2 to profenofos at the different concentrations along with the time for the tests at: a) 20 mg/L and b) 100 mg/L.....100

Figure 6.1 Profenofos biodegradation in column experiment in free and immobilized cells at different concentration of profenofos.....106

Figure 6.2 Breakthrough curves of profenofos biodegradation in column experiments at profenofos of 20 mg/L (a, c, and e) and profenofos of 100 mg/L (b,

d, and f). Figures a and b were from the tests with only sand (◆) and calcium alginate beads (□). Figures c and d were from the tests with free (◆) and immobilized (□) PF1cells. Figures e and f were from the tests with free (◆) and immobilized (□) GFJ2 cells.....110

Figure 6.3 Leaching cells from free (light bar) and immobilized (dark bar) PF1 and GFJ2 in column: a) PF1 at 20 mg/L of profenofos, b) PF1 at 100 mg/L of profenofos, c) GFJ2 at 20 mg/L of profenofos, and d) GFJ2 at 100 mg/L.....112

Figure 6.4 SEM observations from the surface of free and immobilized cells at 0 PV (magnification at 5,000×): a) sand particles at 0 PV, b) sand particles of PF1 at 15 PV, c) sand particles of GFJ2 at 15 PV, d) immobilized PF1 at 0 PV, e) immobilized PF1 at 0 PV.....113

Figure 6.5 Chemotactic responses of PF1 and GFJ2 at the different substrates (yeast extract (light bar) and profenofos at 100 mg/L (dark bar)) : a) free cells and b) de-immobilized cells.....115

Figure 6.6 Percentage of damaged cells of free and de-immobilized PF1 (a) and GFJ2 (b) after testing for 15 PV 117

LIST OF TABLES

Table 2.1 Properties of profenofos 24

Table 2.2 Profenofos-degrading microorganisms 26

Table 2.3 Classification of the immobilized cell techniques (Tampion, 1987) 28

Table 2.4 Examples of immobilization technique is wastewater treatment application 32

Table 2.5 Examples of immobilization technique is bioremediation application 33

Table 3.1 Parameters and coded level used for the experimental design of profenofos biodegradation by PF1 ($\alpha = 1.5$). 37

Table 3.2 Profenofos biodegradation kinetic rates and removal efficiencies 40

Table 3.3 Regression coefficient for profenofos biodegradation by PF1 44

Table 3.4 Analysis of variance for profenofos degradation by PF1 44

Table 4.1 Parameters and factors applied in the experimental design of profenofos biodegradation by GFJ2 ($\alpha = 1.5$) 54

Table 4.2 Kinetic rates and removal efficiencies of profenofos biodegradation 58

Table 4.3 Regression coefficient for profenofos biodegradation by GFJ2 60

Table 4.4 Analysis of variance for profenofos degradation by GFJ2 61

Table 5.1 Profenofos biodegradation kinetic rates at the different sizes of the immobilized beads 73

Table 5.2 Profenofos biodegradation kinetic information 80

Table 5.3 Profenofos biodegradation kinetic rates with inorganic salt types and concentration from the free cells 86

Table 5.4 Profenofos biodegradation kinetic rates with inorganic salt types and concentration from the immobilized cells 87

Table 5.5 Motility assay of PF1 towards different profenofos concentrations 95

Table 5.6 Motility assay of GFJ2 towards different profenofos concentrations 96

Table 5.7 Motility assays of PF1 towards different profenofos concentrations 97

Table 5.8 Motility assays of GFJ2 towards different profenofos concentrations 97

CHAPTER 1
Introduction

1.1 General introduction

Profenofos is an organophosphate compound which is generally utilized in Southeast Asia (Irie, 2008 ; Toan, Sebesvari, Bläsing, Rosendahl, & Renaud, 2013)It has been widely used as an insecticide for controlling worms, plant bugs, flea hoppers, and whiteflies in cotton, mango, mangosteen, cabbage and other crucifer, tomato, watermelon, beans, and cotton fields (Irie, 2008 ; USEPA, 2006). The half-life of profenofos could be longer than 100 days leading to the presence of profenofos in soil, water, and air injuring the living organisms (NLM, 2002). Profenofos was also reported as a chemical most likely found in waterways at the Lumban and Pagsanjan regions, Philippines (Fabro & Varca, 2012).

Biodegradation of profenofos using microorganisms is one of the most cost effective methods to remediate the contaminated sites. The intermediate products that mostly found are 4-bromo-2-chlorophenol (BCP) and O-ethyl-S-propyl phosphorthioate (Jabeen, Iqbal, Anwar, & Parales, 2015; Siripattanakul-Ratpukdi, Vangnai, Sangthean, & Singkibut, 2014). There was profenofos-degrading microorganisms. For example, *Pseudomonas* sp. utilized parathion, diazinon, malathion, monocrotrophos, isofenphos, and profenofos (Horne, Harcourt, Sutherland, Russell, & Oakeshott, 2002; Karpouzas & Singh, 2006; Malghani, Chatterjee, Hu, & Zejiao, 2009; Mulbry, Kearney, Nelson, & Karns, 1987; B. K. Singh & Walker, 2006). According to a previous study, *Pseudomonas plecoglossicida* strain PF1 (PF1) was isolated for profenofos degradation (Siripattanakul-Ratpukdi et al., 2014). The strain utilized profenofos as the sole carbon source (90% removal efficiency in 2 days). Moreover, this strain well degraded other organophosphates, such as chlorpyrifos and dicrotophos (33-73% degradation). This advantage well fit to the practice in agricultural area. There were many groups of pesticides that were applied into the agricultural area such as organophosphates, organochlorines, carbamates, pyrethroids, and diazines (Fabro & Varca, 2012; Nair & Sujatha, 2012). Another potential degrading bacterium, *Acinetobacter baylyi* GFJ2 (GFJ2), was

introduced into this study due to its chlorinated compound degradation properties, such as 4-chloroaniline and 3,4-dichloroaniline(Hongsawat & Vangnai, 2011). From our preliminary study, GFJ2 obviously degraded profenofos which is a halogenated compound. Till now, the characterization of profenofos bioremediation by PF1 and GFJ2 has been limited.

There are several advantages of typical bioremediation, such as cost effective, environmental friendly, and ability to degrade substrates in a wide range. However, there are some limitation of bioremediation including cell leaching out of the contaminated area and sensitivity of microbial cells to environmental stresses. Cell immobilization technique was initiated for enhanced bioremediation. Microorganism was immobilized in a porous polymeric matrix; it was used for pollutant removal, such as nitrogen, carbon, herbicide, and other hazardous substances in wastewater and contaminated sites (Siripattanakul, Wirojanagud, McEvoy, Casey, & Khan, 2008). The entrapment matrices can increase biological activities and protect the cells from lethal effects by the intermediate toxic substances (Alonso, Rendueles, & Díaz, 2015; Siripattanakul et al., 2008). Research on profenofos biodegradation by the immobilized cells was limited. The work focused on profenofos degradation performance of immobilized cells (Talwar & Ninnekar, 2015).

During the degradation process by immobilized cells, various factors, such as bacterial cell behaviors and environmental conditions influence efficiency of biodegradation. For bacterial cell behaviors, cell motility (by motile and non-motile microbial cells) and biodegradation performance were important factors leading to success of bioremediation by free cells (Lacal et al., 2011; Parales, Luu, Hughes, & Ditty, 2015). Cell motility is ability of cell to move through environment leading to opportunity of the cells reaching to substrate. The cell motility depended on cell manner and environment. It has been known that environmental conditions, for example, pH, temperature, and contaminant concentration were major effects on microbial degradation (Clarke et al., 2016; Lacal et al., 2011; Sampedro, Parales, Krell, & Hill, 2015; Witt, Dybas, Worden, & Criddle, 1999).

Thus far, there was no published work on the effect of environmental conditions to microorganisms and their profenofos biodegradability. The cell immobilization technique was applied for enhancing the profenofos biodegradation.

Within this context, the aim of this study is to investigate profenofos biodegradation by the immobilized cells compared to the free cells. Two effective cultures, PF1 and GFJ2, were chosen as model motile and non-motile microorganisms, respectively. The characterization of profenofos biodegradation by PF1 and GFJ2 influencing by environmental conditions (effects of profenofos concentration, pH, and temperature) was performed. Furthermore, the applications of the immobilized cells in batch (effects of the immobilized cell sizes and inorganic salts) and column (effect of profenofos concentrations) tests were examined to accomplish on the gap knowledge between the efficiency and behavior of the microorganisms after being entrapped into the matrices comparing to the free cell. The well-known cell entrapment matrix, calcium alginate (CA), was selected.

1.2 Objectives

The main objective of this work was to investigate profenofos biodegradation by the motile and non-motile bacteria in the free and immobilized cell forms. The specific objectives are as follows.

1.2.1 To characterize profenofos biodegradation by PF1 and GFJ2 influencing by pHs, temperatures, and initial profenofos concentrations using surface response methodology.

1.2.2 To investigate influence of immobilized cell preparation (bead sizes) and environmental condition (profenofos concentrations and inorganic salt types and concentrations) on profenofos removal in batch experiment.

1.2.3 To determine profenofos removal under different initial concentrations and cell movement in long term column experiment.

1.3 Scopes

This study has been held at the Environmental Biotechnology Laboratory, Department of Biochemistry, Faculty of Science, Chulalongkorn University and Department of Environmental Engineering, Faculty of Engineering, Khon Kaen University since 2013. The experiment was in a bench-scale test. The following details are the specific information on the scope of study.

1.3.1 A previously profenofos-degrading bacterium, PF1, isolated from profenofos-contaminated chili farm soil, Ubon Ratchathani, Thailand was utilized (Siripattanakul-Ratpukdi et al., 2014) whereas GFJ2 isolated from herbicide-contaminated soils was applied (Hongsawat & Vangnai, 2011).

1.3.2 Commercial profenofos in absolute ethanol (Syngenta Crop Protection Co., Bangkok, Thailand) was varied from 10-200 mg/L for experiment. Analytical profenofos in absolute ethanol (Dr. Ehrentorfer GmbH, LGC Standards, UK) were used for analysis and motility assays.

1.3.3 Gas Chromatograph with Electron Capture Detector (GC-ECD) from Shimadzu (GC-2014, Kyoto, Japan) was applied for profenofos analysis.

1.3.4 For the first part, influence of pHs (4-8), temperatures (15-45°C), and initial profenofos concentrations (5-20 mg/L) on profenofos biodegradation by PF1 and GFJ2 were investigated using surface response methodology. Profenofos biodegradation kinetics and interaction of the environmental conditions to profenofos degradation were focused.

1.3.5 Calcium alginate (sodium alginate of 3% (w/v) and calcium chloride of 3.5% (w/v)) was used as the immobilized matrix.

1.3.6 For the second part, the bead sizes of 2, 4, and 6 mm in diameter, profenofos concentrations of 20-200 mg/L, inorganic salt types (NaCl, $MgSO_4$, and $CaCO_3$), and inorganic salt concentrations of 100 and 1,000 mg/L were varied to investigate on influence of immobilized cell preparation and environmental condition. The profenofos biodegradation performance, cell motility, and immobilized cell micro-structural observation were focused.

1.3.7 Cell motility assays including plate (swimming, swarming, and twitching assay) and capillary chemotaxis tests were examined. Micro-structural observation of the immobilized cells using scanning electron microscope (SEM) was performed.

1.3.8 For long tern monitoring, profenofos concentrations of 20 and 100 mg/L were applied. Profenofos removal, cell growth and movement, cell viability based on Live/Dead BacLight test, and immobilization matrix durability were determined.

1.4 Hypotheses

1.4.1 At neutral pH, higher temperature (more than 35 °C), and increasing of initial profenofos concentrations introduced the increasing rate of biodegradation for both strains. A previously isolated PF1 was suitable strain which could work in wider ranges since it was more familiar to the contaminant.

1.4.2 Small bead size at high concentration of profenofos and high concentration of inorganic salt led to the low efficiency of profenofos biodegradation. The increasing of profenofos concentration induced the cell motility for PF1 (motile bacteria) but no influence on GFJ2 (non-motile bacteria).

1.4.3 The increasing of profenofos concentrations provided higher and slower substrate releasing to the microbial cells inside the immobilization matrices. This situation resulted in better profenofos biodegradation performance.

1.5 Experimental framework

Entire of this thesis could summarize in an experimental framework as shown in Figure 1.

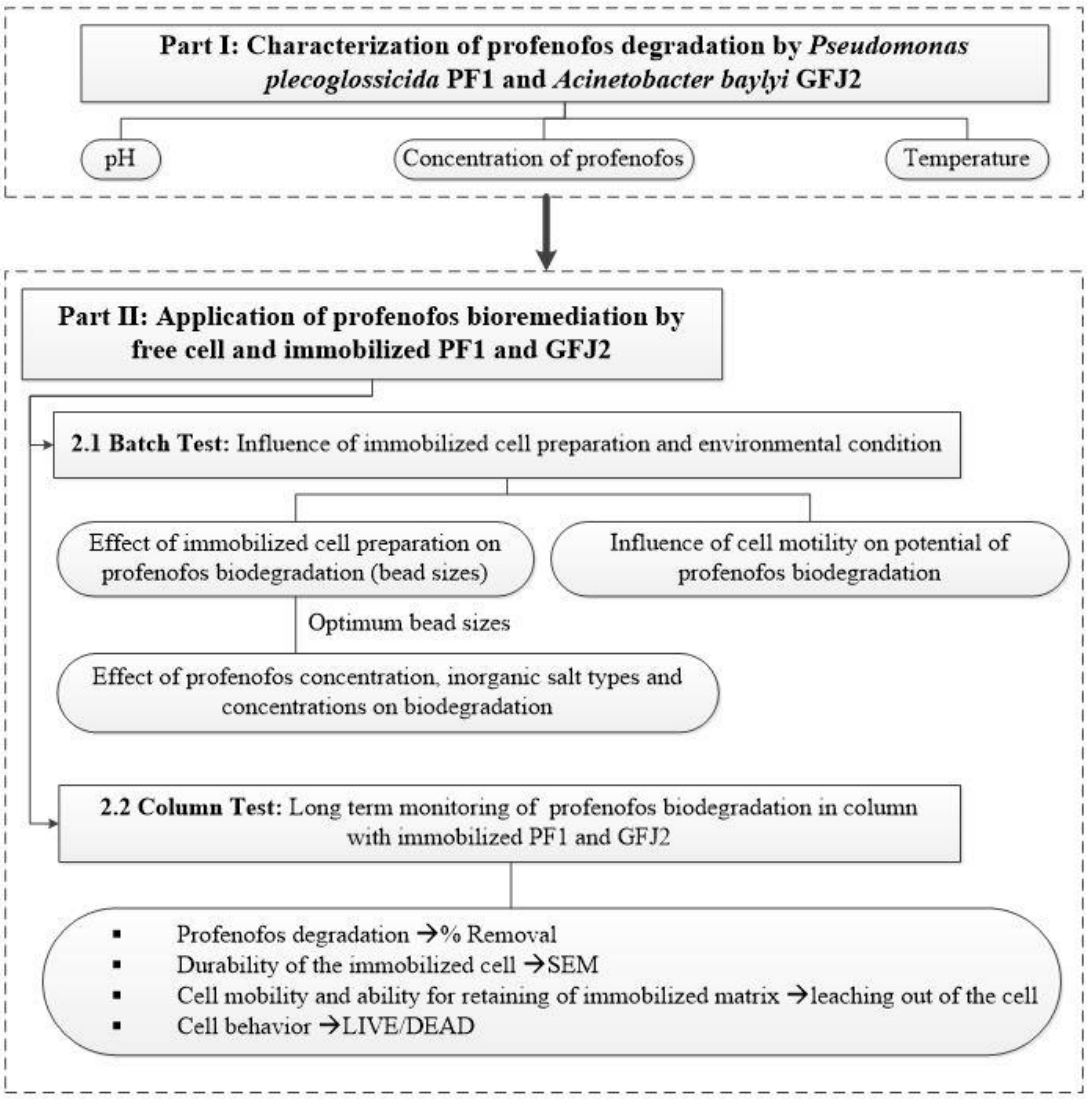


Figure 1.1 Experimental frameworks

Dissertation organization was classified based on framework (Figure 1.1). Details of each result chapter (chapters 3 to 6) were as follows.

Chapter 3: Characterization of profenofos degradation by *Pseudomonas plecoglossicida* strain PF1 using surface response methodology. This result was from experimental framework part 1. The results in this chapter included the characterization of profenofos biodegradation by PF1 influencing by pHs, temperatures, and initial profenofos concentrations using surface response methodology. Profenofos biodegradation kinetics and interaction of the environmental conditions to profenofos degradation were focused.

Chapter 4: Profenofos removal by *Acinetobacter baylyi* strain GFJ2: biodegradation kinetics and influence of environmental conditions. The results in this chapter included the influence of environmental parameters as pHs, temperature, and initial profenofos concentration on profenofos biodegradation using surface response methodology by GFJ2.

Chapter 5: Profenofos pesticide degradation and movement of immobilized PF1 and GFJ2. This result was from experimental framework part 2.1. The results in this chapter included the investigation on the influence of immobilized cell preparation (bead size) and environmental condition (profenofos concentrations and inorganic salt types and concentrations) on profenofos removal in batch experiment. Profenofos biodegradation performance and cell motility were focused.

Chapter 6: Profenofos pesticides removal by immobilized cells in sand column experiment: comparative study of motile and non-motile cells. This result was from experimental framework part 2.2. The results in this chapter included the monitoring of profenofos removal in long term column experiment. The influence of profenofos concentrations on biodegradation performance and cell leaching and motility were focused.

CHAPTER 2
Literature Review

Organophosphorus compounds (OPs) are widely used in many products as pesticides, plastics, lubricants, fire retardants, and fuel additives. The most notoriously of OPs is applied as pesticides since 1937 (Karpouzas & Singh, 2006). The compounds mainly contain esters or thiols from phosphoric, phosphonic, phosphinic or phosphooramidic acid (B. K. Singh & Walker, 2006).

2.1 Profenofos

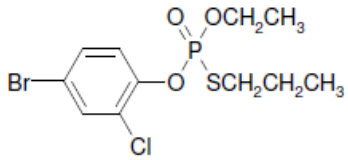
2.1.1 Properties of profenofos

Profenofos is the insecticides that utilized for manage worms, plant bugs, flea hoppers, and whiteflies (USEPA, 2006). It has solubility in water only 20 mg/L, but is instantly soluble in organic solvents (ethanol, acetone, toluene, and n-hexane) at 25 °c. According to US Enironmental Protection Agency, profenofos is classified as a restricted use pesticide and has an acute effect on oral and dermal administration. In addition, profenofos is highly toxic to aquatic ecosystems (Malghani et al., 2009; USEPA, 2006). The properties of profenofos were presented in Table 2.1.

2.1.2 Fate of profenofos in environment

Pesticide could be presented in soil, water, and air in the vadose zone. Profenofos absorbed into suspended solids and sediment according to the K_{oc} values (869-3,162). The volatilization of profenfos in soil surface is not an essential fate (Henry's Law constant of 2.2 x 10^{-8} atm-m^2/mole. At alkaline soil, the half-life of profenfos is 2 days. The hydrolysis of profenofos is slower in neutral and acid soils (NLM, 2002) (108 days at pH 5, 62 days at pH 7, and 0.33 days at pH 9). Similarly Malghani et al. (2009) reported that profenofos is unstable under alkaline solution (50% of hydrolysis degradation of 5.7 hours at pH 9, compared with 14.6 day at pH 7).

Table 2.1 Properties of profenofos

Properties	**Expression**
Structure formula	
Chemical name	O-(4-bromo-2-chlorophenyl) O-ethyl S-propyl phosphorothioate
Chemical family	Organophosphate
Color and form	Amber oily liquid
Formula	$C_{11}H_{15}O_3PSBrCl$
Molecular mass	373.6 g/mol
Density	1.46 g/cm^3 at 20 °C
Octanol-water partition coefficient	log K_{ow} = 4.44 at 25 °C
Solubility in water at 22 °c	28 mg/L at pH 6.9
Vapor pressure	9x10^{-7} mmHg at 25 °C

Profenofos can be persisted on soil, water, and living organisms. The half-life of profenofos can be varied from 2 to 7 days (He, Fan, & Liu, 2010; Ondo Zue Abaga et al., 2011; Romeh, Mekky, Ramadan, & Hendawi, 2009). For example, the residues of profenofos on tomato fruits can be persisted with the half-life at 5 days. For the soil phase, the half-life of profenofos existed for 7-15 days. The concentration of profenofos in water on the seasoning was higher in May than in December resulting from the dilution of the rain after raining season in December (Nasrabadi, Bidhendi, Karbassi, Grathwohl, & Mehrdadi, 2011). The pesticides were washed out along the watershed of river (Southern Caspian Sea basin, Haraz River). There was a lower precipitation rate in May resulting in the high concentration of profenofos was discovered. The texture of the soil and the elevation of upstream and downstream also are an influence on the fate of profenofos. For instance, gravel and sandy soils which have low rate of inter-connected structure of the pores were less absorbed pesticide than clayey and loamy soils. The deeply slope elevation in upstream cause a high rate run-off of pesticides into the lower level of downstream (Blanchoud, Moreau-Guigon,

Farrugia, Chevreuil, & Mouchel, 2007; Comoretto et al., 2008; Nasrabadi et al., 2011). The toxicity on non-targeted species and the contaminated in ground water are the main problems of profenofos occurring. It can be detected in the cotton soil after 60 days (H. Liu, Guo, Liao, & Wang, 2012).

2.1.3 Biodegradation of profenofos

Biodegradation is a process to remove toxicity or transformation of the substance by using microorganisms. This process is cost effective and environmental friendly. Profenofos can be degraded by microorganisms in both soil and liquid medium.

Pseudomonas putida and *Burkholderia gladioli* were isolated from soil at Hubei, China degraded profenofos (200 µg/g) at pH 5.5-7.2 with temperature range from 28 °c to 36 °c (Malghani et al., 2009). In the study, biodegradation of profenofos was investigated in both liquid media and soil. Profenofos had been applied into the liquid media as a sole carbon source. *P. putida* degraded 92.37% of profenofos while *B. gladioli* utilized 87.5% of profenofos in 96 h. In the soils samples, 70% of profenofos started to degrade in 5 days and reach 99% with 25 days in both cultures. *P. aeruginosa* also degraded 86.81% of profenofos at 200 µg/g within 48 h (Malghani et al., 2009). 4-bromo-2-chlorophenol (BCP) was discovered as the intermediated substance during the degradation of profenofos (Figure 2). Similarly, Salunkhe et al. (2013) reported that profenofos at concentration 5µg/mL can be degraded by *Bacillus subtilis* strains isolated from grapevines, grape rhizosphere, grape berries, and vineyard soil. The half-life of proefenofos was 2-4 days. BCP was also found. According to Li et al. (2009), *Arthrobacter* sp. Strain scl-2 degraded profenofos with other OPs by using isofenphos-methyl hydrolase. Methyl parathion hydrolase from *Sphingomonas* sp. Dsp-2 degraded profenofos, chlorpyrifos, parathion, methyl-parathion, and fenitrothion (R. Li et al., 2009; X. Li, He, & Li, 2007).

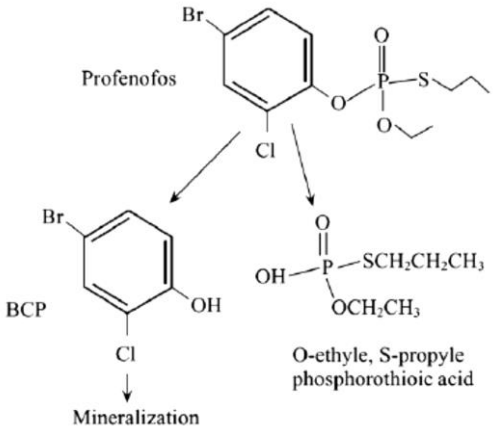


Figure 2.1 The proposed pathway for PF degradation by *Pseudomonas aeruginosa* (Malghani et al., 2009)

Table 2.2 Profenofos-degrading microorganisms

Microorganisms	**Source**	**Optimal condition**	**Concentration**	**Incubation time (h)**	**Reference**
Pseudomonas putida	Soil from Hubei, China	28-36 °C, pH 5.5-7.2	200 µg/g	102	Malghani et al. (2009)
Pseudomonas aeruginosa	Profenofos-polluted soil	30 °C, pH 5.5-7.2	100 mg/L	100	Malghani et al. (2009)
Burkholderia gladioli	Soil from Hubei, China	28-36 °C, pH 5.5-7.2	200 µg/g	102	Malghani et al. (2009)
Bacillus subtilis DR-39	Grapevines	28 °C, pH 7.66	5 µg/mL	4.03 (DT_{50} days)	Salunkhe et al. (2013)
Bacillus subtilis CS-126	Grapevines	28 °C, pH 7.66	5 µg/mL	3.57 (DT_{50} days)	Salunkhe et al. (2013)
Bacillus subtilis TL-171	Grapevines	28 °C, pH 7.66	5 µg/mL	2.87 (DT_{50} days)	Salunkhe et al. (2013)
Bacillus subtilis TS-204	Grapevines	28 °C, pH 7.66	5 µg/mL	2.53 (DT_{50} days)	Salunkhe et al. (2013)

Microorganisms	Source	Optimal condition	Concentration	Incubation time (h)	Reference
Arthrobacter sp. Scl-2	Isocarbophos-polluted soil	30 °C	100 mg/L	18	Li et al. (2009)
Sphingomonas sp. Dsp-2	Chlorpyrifos-polluted soil	30 °C, pH 7.0	100 mg/L	24	Li et al. (2007)

2.2 Cell immobilization

Immobilized cell is widely utilized in the field of environmental sciences to develop treatment processes including the flocculation of microorganisms in activated sludge, adsorption of microorganisms on supporters, and entrapment of microorganisms which mostly used in bioreactor, and production of the useful compounds such as amino acids, organic acids, antibiotics, steroids, and enzymes (Cassidy, Lee, & Trevors, 1996). According to Tampions (1987) introduced the advantages of immobilized cell which are long-term stability of biocatalyst, low leakage of cells, high resistance to abrasion, resistance to microbial degradation, low diffusional limitation, high surface area, cheap support materials, and non-toxic materials (Table 3). The limitations of the immobilized cells are differentiated of cell morphology between inner and outer part on the immobilization matrices, cell activities, bacterial growth, and substrate diffusion. The application of immobilized cell is used in agriculture, bio-control, pesticide application, and pollutant biodegradation in contaminated soil or groundwater (Hu, Korus, Levinson, & Crawford, 1994; Lozinsky & Plieva, 1998).

Table 2.3 Classification of the immobilized cell techniques (Tampion, 1987)

Technique	Types of material	Advantages	Disadvantages
Adsorption	Neutral supports	Cheap	• Cell leakage • Sensitive to pH changes
		Mild	
		Reusable	
		Simple	
	Charged supports	Mild	
		Reusable	
		Simple	
Flocculation	-	Simple	• Cell leakage • Diffusional limitations
	-	Mild	
Entrapment	Natural polymers	Mild	Diffusional limitations
		Simple	
	Synthetic polymers	Mild	• Toxicity • Expensive • Diffusional limitations
		Simple	
		Reusable	
Covalent coupling	-	Permanent	• Toxicity • Expensive
Containment	-	Mild	• Diffusional limitations • Expensive
	-	Simple	
	-	Reusable	

2.3 Cell entrapment

2.3.1 Principle of cell entrapment

Cell entrapment is technique that cells immobilized inside polymeric materials such as polysaccharide and synthetic polymers to avoid direct contact between cell and environment. Therefore, this technique is applied in many fields such as food industries, medical purposes, pharmaceutical uses, and environmental studies. The cell entrapment was performed with two consecutive procedures. The first step is mixing of entrapment material and cells. Later, gelation of the mixture is taken place. Normal shapes of the entrapped cells are sphere or cubic by gelation using dropping or platting techniques, respectively.

2.3.2 Types of cell entrapment materials

Two types of entrapment materials are natural and synthetic polymers. Natural matrices are polysaccharides made from algae or seaweed, such as calcium alginate, carrageenan, agarose, and gelatin. On the other hand, the synthetic agents are polymers, such as polyvinyl alcohol, cellulose triacetate, and polyacrylamide.

2.3.2.1 Calcium alginate

Calcium alginate is a cross-linking of alginate with divalent cation, such as Ca^{2+}. Alginate is a non-toxic natural polysaccharide from brown algae, such as *Macrocystis pyrifera*, *Laminaria digitata*, *Laminaria hyperborea*, and *Eklonia cava* and some bacteria, principally *Azotobacter vinelandii* (Fett et al., 1986). Alginate is provided as a sodium salt of alginate. While the sodium alginate viscous solution contacts with a Ca^{+} solution, a gel is suddenly formed (Siripattanakul & Khan, 2010).

2.3.2.2 Carrageenan

Carrageenan is produced from red algae, mainly *Chondrus crispus*, *Eucheuna cottonii*, *Gigartina stellata* and *G. radula*. The gelation of carrageenan depends on temperature and gelation chemicals containing K^{+},or Al^{+3} ions.

2.3.2.3 Polyvinyl alcohol

Polyvinyl alcohol is a non-toxic synthetic polymer. The characteristics of raw PVA are white and free-flowing granule. There are several gelation techniques for producing PVA gels for immobilized cell; for instance, boric acid-PVA (BPVA), freezing and thawing of PVA (FPVA), and phosphorylated-PVA (PPVA) methods. The BPVA technique is a one-step droplet gelation method by a cross-linking of boron-PVA (Furukawa, Ike, Ryu, & Fujita, 1993). The FPVA technique is a physical cross-linking during temperature-induced condition (Lozinsky & Plieva, 1998). Recent developed technique, PPVA method, is less time consuming and cell damage than BPVA. The PPVA technique is a two-step droplet gelation method that is spherical bead formation and hardening. The spherical bead formation is the first step by cross-linking of the PVA-boron similar to BPVA. After that, the spherical beads are transferred to a sodium phosphate solution for bead hardening process. A sodium phosphate increases the surface gel strength through PVA phosphorylation.

2.3.2.4 Cellulose triacetate (CTA)

The other natural polymer used for matrix of entrapped cell is cellulose containing a chain of glucose molecules. The modification of cellulose with esterification and etherification are applied for the entrapped cell. The entrapped cells of CTA are prepared by the plated gelation (Siripattanakul & Khan, 2010). The 10% (w/v) powder of CTA is dissolved in methylene chloride. Concentrated suspension cells are mixed with CTA solution. After that, the mixture is plated and soaked into toluene solution for hardening. The hardened CTA is cut to small cube and washed with water.

2.3.3 Applications of cell entrapment for environmental practices

As a result of the immobilized cells properties, it is broadly used in many industries for increasing metabolic activity and metabolite production, protecting cells from toxic substances, and increasing plasmid stability.

2.3.3.1 Wastewater treatment

The immobilization technique was mostly introduced into wastewater treatment system on the industrial waste, municipal sewage, and agricultural wastewater treatment systems to reduce or detoxify toxic substance before releasing to receiving water. Most applications on the immobilized cell for nitrification and sulfate reduction in wastewater were achieved (Pramanik & Khan, 2008). For example, Yan and Hu (2009) investigated on partial nitrification of immobilized nitrifying bacteria at wide range of pH and temperature. The result showed that the immobilized cell supported the partial nitrification process in pH range from 6.0 to 8.5 with temperature at 15 to 35 °C comparing to free cell (Yan, 2009). The immobilized also removed COD for 60-70%. Table 4 presents examples of immobilization technique is wastewater treatment application.

2.3.3.2 Bioremediation

Bioremediation is the biodegradation of contaminants which required suitable conditions for growth and bacterial activities. These conditions were varied depending on nature of contaminated sites and degrading cultures. Bioremediation occurred in many environmental media, such as soil and groundwater. It is difficult to maintain the activity of microorganisms. Therefore, the immobilization technique was initiated to lessen these problems, such as reducing of direct contact to toxic compound. Pino et al. (2016) investigated bioaugmentation of immobilized consortium on polychlorinated biphenyl (PCB)-contaminated soil (Pino, Muñera, & Peñuela, 2016). The result showed that the removal of PCB by the alginate-immobilized cells was greater than that by free cells. In addition, Pradeep and Subbaiah (2015) examined the repeating use of the immobilized *P. putida* for chlorpyrifos degradation. The result showed that the immobilized cell could degrade 70% of chlorpyrifos and was able to reuse for 50 times with less cell leakage (112 x 10^3 CFU/mL) (Pradeep & Subbaiah, 2015). Table 5 presents examples of immobilization technique is bioremediation application.

Table 2.4 Examples of immobilization technique is wastewater treatment application

Microorganisms	**Contaminants**	**Immobilization techniques**	**Degradation**	**References**
Decolorization strains	Azo dyes	50 mL of gel PVA	Complete mineralization and reusable of the beads for more than 30 cycles	(Fang, Wenrong, & Yuezhong, 2004)
Nitrifying bacteria Denitrifying bacteria	Ammonia in anaerobic sludge	2% (w/v) alginate	Partial nitrification (to nitrile) was occurred	(Hill & Khan, 2008)
Activated sludge	Ammonium-rich organic wastewater	5% (w/v) alginate, 10% (w/v) PVA with 2% (w/v) alginate, 2% (w/v) carboxymethylcellulose, 3% chitosan	Alginate was the suitable immobilized material	(Yan, 2009)
Rhodobacter shaeroide S and NR-3	Cooking oil	2% (w/v) alginate with 2% (w/v) agar	96% removal of oil from wastewater	(Takeno, Yamaoka, & Sasaki, 2005)

Table 2.5 Examples of immobilization technique is bioremediation application

Microorganisms	Contaminants	Immobilization techniques	Degradation	References
Pseudomonas sp. UG14Lr	Phenanthrene	5% (w/v) of alginate	Twice higher degradation by the entrapped cells was found.	(Weir, Dupuis, Providenti, Lee, & Trevors, 1995)
Alcaligenes faecalis	Phenol	4% (w/v) of alginate	20% increasing of degradation by the entrapped cells was found.	(Bastos, Cassidy, Trevors, Lee, & Rossi, 2001)
Pseudomonas sp. *Stenotrophomonas* sp.	Polychlorinated biphenyl	2% (w/v) of alginate	30% increasing of degradation by the entrapped cells was found.	(Pino et al., 2016)
Streptomyces sp.	Lindane	3% agar, 5% (w/v) PVA with 0.8% (w/v) alginate, 9-cm-long silicone tube, Cloth sachets	The cells with silicone tube was able to remove 80% of lindane.	(J. M. Saez, Benimeli, & Amoroso, 2012)
Micrococcus sp. CPN 1	Cypermethrin	Polyurethane foam, 4% (w/v) alginate, polyacrylamide, agar	Polyurethane foam was the suitable matrices for immobilization in term of operational stability.	(Tallur, Mulla, Megadi, Talwar, & Ninnekar, 2015)
Pseudomonas fluorescens	Endosulfan	4% (w/v) alginate	The entrapped cells was able to degrade 350 µg/L of endosulfan within 6 days	(Jesitha, Nimisha, Manjusha, & Harikumar, 2015)
Pseudomonas putida	Chlorpyrifos	3% (w/v) alginate	The entrapped cells was able to degrade 65% of chlorpyrifos with 50 cycle duration time of the beads	(Pradeep & Subbaiah, 2015)
Pseudoxanthomonas suwonensis strain HNM	Profenofos	4% (w/v) alginate, 6% (w/v) PVA with alginate, bentonite clay with alginate	Cells in bentonite with alginate matrix had the highest rate of degradation. The cells well reused for more than 30 cycles.	(Talwar & Ninnekar, 2015)

CHAPTER 3
Characterization of Profenofos Degradation by *Pseudomonas plecoglossicida* Strain PF1 Using Surface Response Methodology

3.1 Introduction

Profenofos is one of common organophosphorus pesticides applied for cotton, mango, cabbage, and chili production. Extensive profenofos utilization caused contamination in the environment. For example, Harnpicharnchai et al. reported the surface water contaminated profenofos concentration of 0.32-0.95 mg/L in Khon Kaen, Thailand which exceeded the concentration of 0.3 µg/L for drinking water standard (Hamilton et al., 2003; Harnpicharnchai, Chaiear, & Charerntanyarak, 2013). Profenofos is toxic to birds, mammals, and aquatic invertebrates leading to damage nervous system. It was listed as a restricted used pesticide by US Environmental Protection Agency (USEPA, 2006). The profenofos contamination in water could be a problematic issue for environment and organisms.

Microbial degradation was an effective technique for pesticide removal in environment. The technique is environmental friendly, cost effective, and high removal efficiency. Generally, the products (intermediate or end products) from microbial process are less toxic. However, for bioremediation practice, the degrading microorganisms require a suitable condition for effective activities, such as pH, temperature, and substrate concentration (Deng et al., 2015; John & Shaike, 2015; B. K. Singh, 2009). For profenofos biodegradation, *Pseudomonas plecoglossicida* strain PF1 (PF1) was previously isolated from profenofos-contaminated chili farm soil (Siripattanakul-Ratpukdi et al., 2014). The strain successfully degraded profenofos in water at a wide range of concentrations (up to 100 mg/L). PF1 also degraded other organophosphorus pesticides including chlorpyrifos and dicrotophos. Thus far, information of profenofos degradation by PF1 was limited. There was no published study on the characterization of profenofos biodegradation by PF1 under influence of environmental parameters.

Conventional method for characterization of biodegradation consumed long process time and high cost. Statistical method using response surface methodology (RSM) was introduced for characterization and optimization for the biodegradation. The RSM method reduced amount of experiments and errors resulting in less time and cost consuming. The RSM method also used to analyze and investigate the interaction effect among the tested parameters. In addition, the statistical experimental design as Central Composite Design (CCD) was successfully used to find the key parameters influencing the biodegradation (Chin-Pampillo, Ruiz-Hidalgo, Masís-Mora, Carazo-Rojas, & Rodríguez-Rodríguez, 2015; Jabeen et al., 2015).

Aim of the present work was to characterize profenofos degradation by PF1. The removal of profenofos contaminated in water was emphasized. The RSM with CCD method was applied to investigate the effects of key environmental parameters including pH, temperature, and initial profenofos concentration on profenofos biodegradation performance. The interaction of each parameter was determined. The ranges of tested parameters (pHs of 5-8, temperatures of 15-45°C, and the profenofos concentrations of 5-20 mg/L) were chosen based on environmental values. The profenofos biodegradation kinetic rate and primary degradation intermediate (4-bromo-2-chlorophenol, BCP) monitoring also carried out. The biodegradation performance information for treating profenofos-contaminated water will be helpful for both academics and bioremediation practices in the future.

3.2 Materials and methods

3.2.1 Chemicals

Commercial grade profenofos (50%, w/v, Syngenta Crop Protection Co., Thailand) used for entire of experiment was obtained from a local pesticide distributer. Analytical grade profenofos (Supelco, Sigma Chemical, Singapore) and BCP (Dr. Ehrenstorfer GmbH, LGC Standards, UK) were obtained for chemical analysis. Other chemicals for entire experiment were purchased from Himedia (India), Ajax (Australia), and RCI Labscan (Australia).

3.2.2 Microorganism and cultivation

Pseudomonas plecoglossicida strain PF1 (GenBank accession number KJ620776), a previously isolated bacterium, was studied (Siripattanakul-Ratpukdi et al., 2014). The bacterial strain was firstly activated in minimal salt medium (MSM) supplementing with profenofos of 20 mg L^{-1} every 4 d for 3 times before used. Formulation of MSM included $Na_2HPO_4 \cdot 2H_2O$ 6.82 g, KH_2PO_4 3 g, NaCl 0.5 g, NH_4Cl 2 g, and $MgSO_4 \cdot 7H_2O$ 0.51 g in 1000 m/L of phosphate buffer at pH 6.80 ($NaH_2PO_4 \cdot 2H_2O$ 0.083% (w/v) and $Na_2HPO_4 \cdot 2H_2O$ 0.17% (w/v)) (Siripattanakul-Ratpukdi et al., 2014).

For PF1 enrichment, the active culture (10% by volume) was inoculated into fresh MSM medium with 0.1% (w/v) yeast extract. The enrichment conditions were shaking at 150 rpm, 24 h, and 30°C. The enriched suspension (OD_{600} = 1) was harvested by centrifugation (5,000 rpm, 20 min, and 15°C). The pellet was twice washed by a NaCl solution of 0.85% (w/v). The, the washed pellet was re-suspended in MSM medium (no yeast extract) to obtain PF1 of approximately 10^{12} CFU/mL.

3.2.3 Profenofos biodegradation experiment

For profenofos biodegradation experiment, three replicate tests with the 30-mL MSM medium supplemented with different initial profenofos concentrations and pHs were carried out in 125-mL serum bottles. The experiment was operated under shaking conditions of 160 rpm and different incubation temperatures. The MSM medium without the cell suspension was conducted as a control. The profenofos biodegradation kinetic rate was calculated using Equation (3.1):

$$C_t = C_0 \times e^{-kt} \qquad \text{Equation (3.1)}$$

where C_0 and C_t are the initial and final concentration of profenofos (mg/L), respectively. The k value is the kinetic rate of profenofos biodegradation (h^{-1}) whereas t is time (h).

Based on literatures, the environmental conditions including pHs (6.50-8.50), temperatures (20.00-40.00°C), and profenofos concentrations (0.32-1.00 mg/L) were found in surface water of agricultural area (Chandrasekar et al., 2014; Jaipieam et al.,

2009; Lee, 2006; Mansouriieh, Sohrabi, & Khosravi, 2016; Singaraja et al., 2014; Yasukawa et al., 2009). The interaction of each parameter to a response was simplified as variables in central composite design (CCD) employed using Minitab 16 statistical software (Minitab, Inc., Pennsylvania, USA) (Table 3.1). In this experiment, the environmental parameter ranges were inserted into Minitab16 software. The program predicted the five level of variation according to CCD model (α=1.5). Based on experimental practice, pHs of at 4.64, 6.25, and 7.87 were adjusted to 4.60, 6.30, and 7.90, respectively. The profenofos biodegradation kinetic rate was selected as the response in this study.

Table 3.1 Parameters and coded level used for the experimental design of profenofos biodegradation by PF1 (α = 1.5).

Factors	**Parameters**	**Units**	**Level**				
			-α	**-1**	**0**	**+1**	**+α**
A	pH	-	4.64	5.30	6.25	7.20	7.87
B	Temperature	°C	14.70	21.00	30.00	39.00	45.30
C	Profenofos concentration	mg/L	4.85	8.00	12.50	17.00	20.15

The kinetic rate of profenofos biodegradation was applied in the mathematical model designated by the full quadratic equation (Equation (3.2)). Analysis of variance (ANOVA) was applied for calculating Fisher test (*F*), its associated probability (*p*) and the coefficient of determination (R^2) which indicated the goodness of fit to the regression model. The contour plots were constructed by fitting the quadratic equation from regression analysis, holding one parameter at the optimum value, and changing the other two parameters. The plots were used to analyze the interaction between the significant parameters.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{i \neq j}^{3} \beta_{ij} x_{ij} \qquad \text{Equation (3.2)}$$

Y is the response of interest (profenofos biodegradation kinetic rate). x_i and x_j are the parameters as showing pH, temperature, and profenofos concentration. β is the regression coefficient values of the model.

3.2.4 Analytical methods

Profenofos remaining and BCP increasing concentrations were analyzed using a gas chromatography (GC) with electron capture detector (Agilent 4890, Agilent, USA) with liquid/liquid extraction technique. *N*-hexane with 0.01% (by volume) acetic acid was added into 500 µL of the samples from the batch reactors with sample and extraction solvent ratio 1:1. The mixture was vigorously mixed for 10 min and centrifuged at 10,000 rpm for 5 min. The organic phase was filtered by 0.22 µm filter nylon.

One µL of the filtered sample was injected into the GC with a HP-5 column (30-m length, 0.25-mm i.d., and 0.25-µm film thickness). The GC condition was splitless mode, injection temperature of 240°C, and helium gas flow of 1.5 mL min^{-1}. The GC temperature program was 10 min period. The program started at 180 °C and hold for 2.00 min, increased to 250 °C with the rate of 40 °C min^{-1} and hold for 6.25 min. Profenofos and BCP peaks came out at 8.78 min and 3.08 min, respectively.

3.3 Results and discussion

3.3.1 Profenofos biodegradation and identification of intermediate

The profenofos biodegradation by PF1 at the different initial concentrations (5, 10, and 20 mg/L) was shown in Figure 3.1. The biodegradation trends were similar for all tests. Profenofos concentration quickly decreased in the first 12 h and gradually reduced later on. The profenofos removal percentages were 50-90% with the utilization rates of 0.17-0.78 mg/L/h as presented in Table 3.2. The profenofos degradation well fit the first order kinetic model ($R^2 > 0.88$). This indicated that the initial profenofos concentrations influenced the degradation performance. The primary profenofos intermediate, BCP (less than 1 mg/L), was detected (Figure 3.1). The detected BCP concentration was much lower than the removed profenofos concentration.

The profenofos biodegradation result showed that PF1 was an efficient profenofos-degrading microorganism. It was able to use profenofos as the sole carbon source for its catabolic activity. Previously, it was reported that typical primary intermediate product of profenofos biodegradation is BCP (Jabeen et al., 2015; Siripattanakul-Ratpukdi et al., 2014). Siripattanakul-Ratpukdi et al. (2014) found

potential of profenofos and BCP removal by a microbial consortium containing PF1 (Siripattanakul-Ratpukdi et al., 2014). The result from this study confirmed that PF1 simultaneously degraded profenofos and BCP. At low profenofos concentration (5 mg/L), BCP decreased along with the time as shown in Figure 3.1(a). During the experiment with higher profenofos concentrations (10-20 mg/L), it was found slight BCP accumulation of less than 1 mg/L (Figure 3.1(b) and 3.1(c)). As mentioned earlier, the contaminated concentration normally is less than 1 mg/L (Harnpicharnchai et al., 2013). Consequently, it could state that PF1 is a potential bacterial strain for remediating profenofos-contaminated situations. Moreover, PF1 well removed BCP which was reported as a toxic intermediate product of profenofos biodegradation (Jabeen et al., 2015).

Table 3.2 Profenofos biodegradation kinetic rates and removal efficiencies

Run	pH	Temperature (°C)	Profenofos concentration (mg/L)	*k* (h^{-1} x 10^{-2})	Profenofos removal efficiency (%)	Profenofos utilization rate (mg /L/h)
1	7.20	39.00	17.00	9.10	84.25	0.69
2	6.25	30.00	12.50	9.30	81.89	0.69
3	6.25	14.70	12.50	1.00	71.81	0.38
4	7.20	21.00	8.00	6.70	70.76	0.28
5	5.30	21.00	8.00	4.30	57.53	0.18
6	5.30	39.00	8.00	4.00	54.83	0.17
7	4.64	30.00	12.50	6.30	63.26	0.38
8	7.87	30.00	12.50	8.60	78.23	0.48
9	6.25	30.00	12.50	9.50	84.36	0.76
10	7.20	39.00	8.00	7.00	64.57	0.32
11	6.25	30.00	12.50	9.30	84.77	0.72
12	6.25	30.00	12.50	9.80	83.81	0.74
13	6.25	30.00	20.15	9.40	85.20	0.78
14	7.20	21.00	17.00	7.80	77.27	0.69
15	6.25	30.00	12.50	8.90	83.14	0.75
16	6.25	30.00	12.50	8.20	81.01	0.66
17	6.25	30.00	4.85	4.80	55.91	0.17
18	6.25	45.30	12.50	5.20	63.64	0.33
19	5.30	39.00	17.00	8.90	83.07	0.91
20	5.30	21.00	17.00	7.60	76.22	0.65

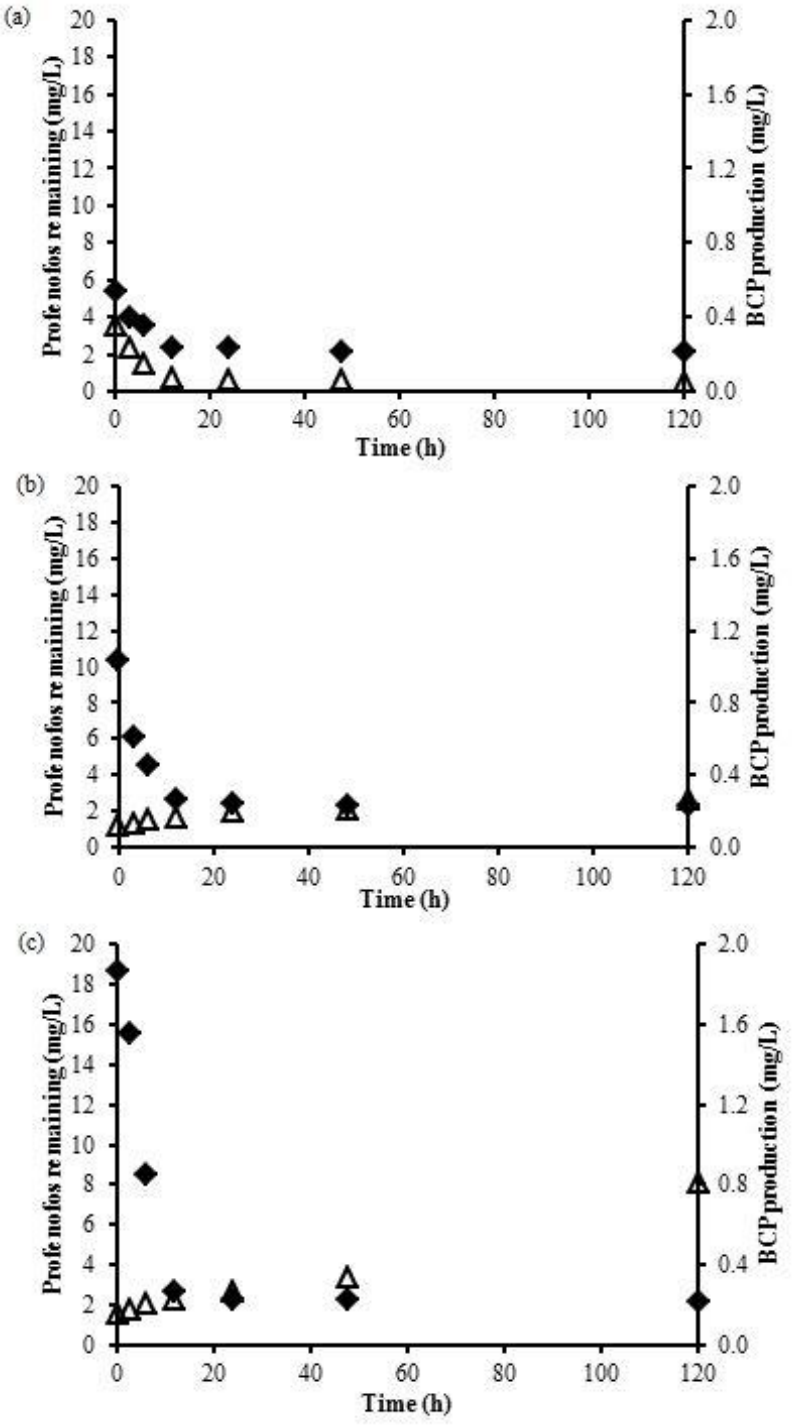


Figure 3.1 Profenofos biodegradation (◆) and BCP production (Δ): (a) profenofos of 5 mg/L, (b) profenofos of 10 mg/L, and (c) profenofos of 20 mg/L

3.3.2 Response surface methodology

3.3.2.1 Statistical characterization of profenofos biodegradation

The profenofos biodegradation kinetic rates (*k*) depending on three main parameters (pH, temperature, and profenofos concentration) were calculated by using Equation. (3.1). Twenty runs of the experiments were conducted and analyzed using CCD to evaluate the response (profenofos biodegradation kinetic rates) on the three main parameters (Table 2). From Figure 3.2, the accuracy of data was confirmed by the parity plot between normal percentage probability and internally standardized residuals. The data was in range of 95% confidence indicating that the profenofos biodegradation kinetic rates from the experiment were reliable.

$$Y = 0.091 + 0.007A + 0.007B + 0.14C - 0.004A^2 - 0.019B^2 - 0.005C^2 + 0.001AB - 0.006AC + 0.003BC \quad \text{Equation (3.3)}$$

where A, B, and C coded for pH, temperature (°C), and profenofos concentration (mg/L), respectively.

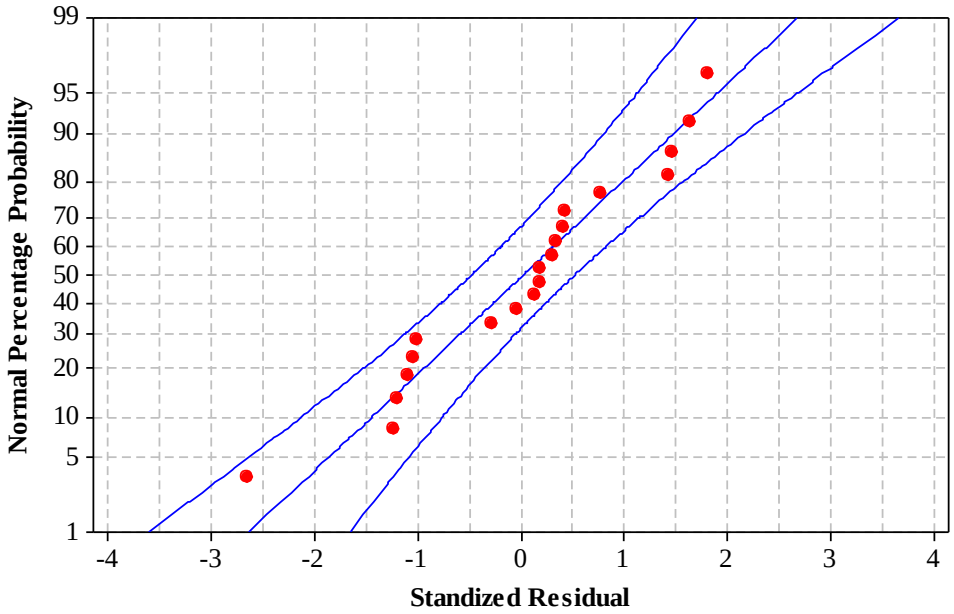


Figure 3.2 Normal probability of standardized residual for profenofos degradation kinetic rates

Based on Figure 3.3 which is graphical plot of profenofos degradation kinetic rates between experimental and predicted values, all the responses were accurate (Irie, 2008; Salunkhe et al., 2013). The model for profenofos biodegradation kinetic rates (*Y*) was shown in Equation (3.3). From Table 3.3, coefficients of the main parameter were positive and $p < 0.05$ indicating that all of the main parameters were significantly influenced the response. However, the quadratic coefficient of B^2 was the largest negative value comparing to the others. This indicated that there was an optimum temperature responsible for the highest response as seen in Figure 3.4. According to *p* value of quadratic term in Table 3.3, B^2 (temperature) was also the most significant parameters influencing on the response while the *p* values of A^2, C^2 and interaction of all parameters were insignificant ($p > 0.05$).

From Figure 3.3, R^2 indicates a goodness of the model which was 91.61%. This indicated that the profenofos biodegradation kinetic rates could be predicted from the model. From Table 4, the model showed that values of $p < 0.05$ (0.00), $F_{value} > F_{critical}$ (F_{value}=12.21 and $F_{critical}$=10.16), and lack of fit > 0.05 (0.06). This could say that the regression model was accepted with high precision (John & Shaike, 2015; Talwar & Ninnekar, 2015). This revealed that the model was useful to identify the significant parameters affecting response (pH, temperature, and profenofos concentration). The terms of A, B, C, B^2 were the significant terms and influenced response ($p < 0.05$). The result well correlated to profenofos biodegradation by microbial consortium (Jabeen et al., 2015). The previous work found the significant influence of pH, temperature, and inoculum size on the profenofos removal percentage.

Table 3.3 Regression coefficient for profenofos biodegradation by PF1

Terms	Coefficient	*p* value
Constant	0.091	0.00
A	0.007	0.02
B	0.007	0.02
C	0.140	0.00
A^2	-0.004	0.18
B^2	-0.019	0.00
C^2	-0.005	0.08
AB	0.001	0.83
AC	-0.006	0.09
BC	0.003	0.35

Table 3.4 Analysis of variance for profenofos degradation by PF1

Source	Degree of Freedom	*F* value	*P* value	Significant
Regression	9	12.21	0.00	Significant
Linear	3	15.23	0.00	Significant
A	1	7.70	0.02	Significant
B	1	7.75	0.02	Significant
C	1	30.24	0.00	Significant
Square	3	19.88	0.00	Significant
A^2	1	2.13	0.18	Insignificant
B^2	1	58.06	0.00	Significant
C^2	1	3.84	0.08	Insignificant
Interaction	3	1.51	0.27	Insignificant
AB	1	0.05	0.83	Insignificant
AC	1	3.52	0.09	Insignificant
BC	1	0.95	0.35	Insignificant
Residual Error	10			
Lack-of-Fit	5	4.72	0.06	Insignificant
Pure Error	5			
Total	19			

R^2 = 91.66%, $R^2_{(adj)}$ = 84.15%

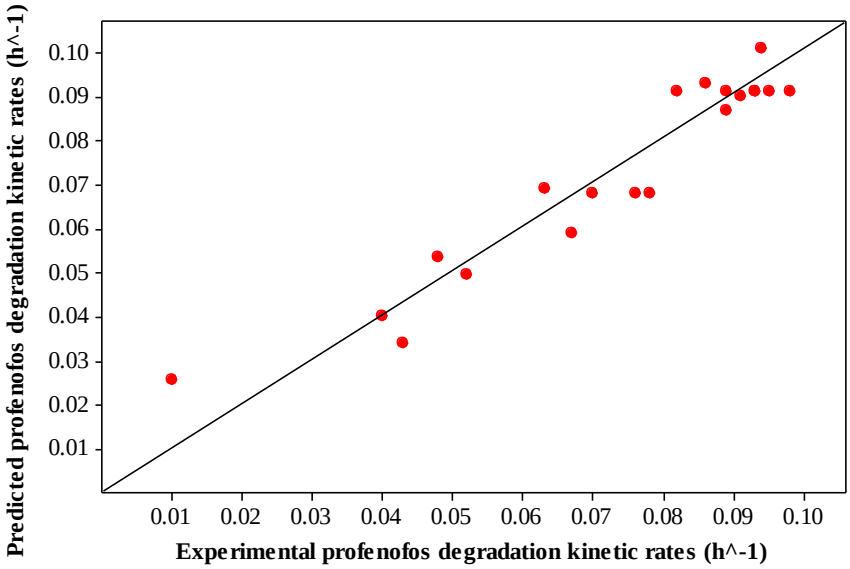


Figure 3.3 Graphical plots of profenofos degradation kinetic rates (h^{-1}) between experimental and predicted values

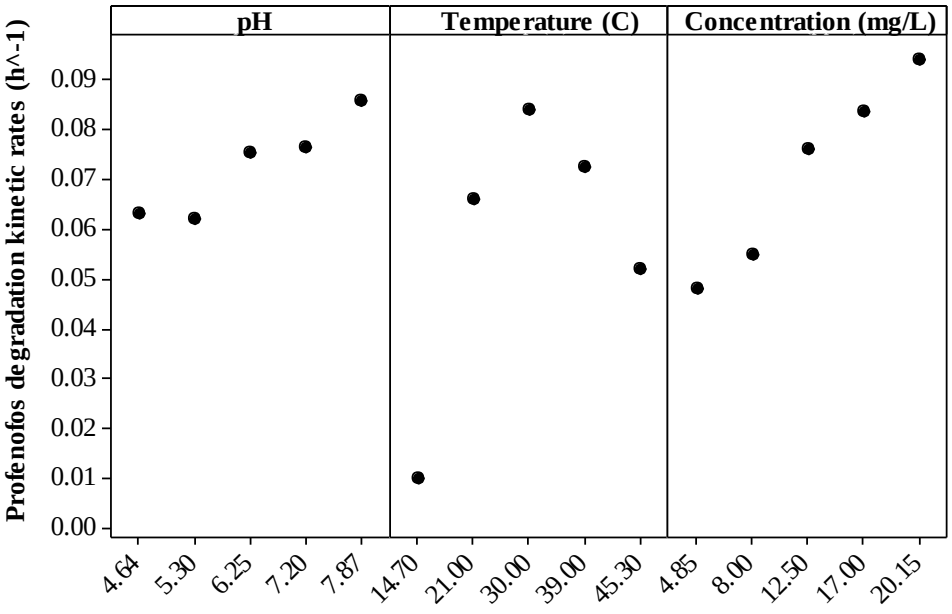


Figure 3.4 Main effects for profenofos biodegradation kinetic rates

3.3.2.2 Effects of pHs, temperatures, and profenofos concentrations on profenofos biodegradation

The main effects of profenofos degradation kinetics on pH, temperature, profenofos concentration were illustrated in Figrue 3.4. Trends of pH and concentration on profenfos degradation kinetics were similar. The kinetic rates increased with rising of pHs and profenofos concentrations. It has been known that profenofos easily breakdowns at alkaline condition leading to increase of the degradation kinetic rates (USEPA, 2006). The optimum pH for profenofos biodegradation was in range of 6.25-7.20. For profenofos concentration, the biodegradation kinetic rates increased along with concentrations followed the first order kinetic model as discussed earlier. This indicated that PF1 is the efficient bacterial strain for profenofos degradation at a wide range of up to 20 mg/L (water solubility value). This could state that PF1 possibly applied for agricultural, industrial, or spill contaminated sites. Additionally, according to Siripattanakul-Ratpukdi et al. (2014), PF1 well degraded other organophosphorus pesticides including chlorpyrifos and dicrotophos with up to 70% removal (Siripattanakul-Ratpukdi et al., 2014). In practice, it is likely that there were numerous types of pesticides contaminated in the same agricultural and agro-industrial contaminated area; therefore, PF1 is promising for remediating the contaminated site in the future.

The temperature as a main effect provided the different result from the other effects. The optimum temperature was approximately 30°C (Figure 3.4). The reason for this situation could be explained by the activity of enzyme that was responsible for profenofos biodegradation. The organophosphorus hydrolase lost its catalytic activity in the environment at inappropriate temperature (R. Li et al., 2009; Malghani et al., 2009). Too high temperature caused protein oligomerization while too low temperature resulted in substrate specificity of enzyme (Karpouzas & Singh, 2006).

In overall of the effect of environmental conditions including pH, temperature, and profenofos concentration, the range of each parameter was selected based on environmental values. High profenofos degradation rates at wide range of tested conditions indicated that PF1 well degraded profenofos and was applicable for real site remediation practice.

3.3.2.3 Interactions between pH, temperature, and profenofos concentration

The interaction between main effects and response (profenofos biodegradation kinetic rates) were demonstrated by the contour plots (Figure 3.5 (a)-(c)). The figures were based on the regression model from Equation (3.3) with one variable held constant at its optimum level and varied the other two variables. From Figure 3.5 (a), the interaction between pH and profenofos concentration on the response was investigated by keeping temperature constant at 30 °C. The result showed the elongated diagonal pattern. Figure 3.5 (a) suggested that the interaction between pH and profenofos concentration was significant for profenofos biodegradation kinetic rates. Even though the F_{value} (3.52) and $p > 0.05$ from ANOVA was insignificant, F_{value} was just slight different from $F_{critical}$ (3.62). Based on the results, the interaction of pH and profenofos concentration should be included.

From Figure 3.5(b) and 3.5(c), the interaction between temperature and profenofos concentration (kept pH constant at 6.25) and the interaction between pH and temperature (held profenofos concentration constant at 12.5 mg/L) suggested that there were less significant interactions on the response. The optimum levels from the experimental data were attained: pH 5.89, temperature 32.94°C, and profenofos concentration 20.15 mg/L. Comparing to the study by Jabeen et al. (2015), the consortium from the previous work and PF1 worked well in similar range of temperatures (32-35°C) which was typical optimum temperature range for mesophilic microbial cultures.

PF1 worked well in all tested pHs (the profenofos degradation kinetic rates of 0.06-0.09 h^{-1}). Interestingly, based on the optimum pH found, PF1 preferred slight acidic pH while the previous consortium worked well at approximately neutral pH (6.8). This could be because PF1 was originated from soil in the north-eastern region of Thailand. The soil pHs from most area in this region are in slight acidic range of 4.5-6.5. Therefore, the isolated microbial culture favored the slight acidic pH. This is the first report determining the bacterial culture which could successfully degrade profenofos at acidic pH. For future application, PF1 would be the best candidate for remediating the contaminated area with broad range of pH (acidic to neutral pHs).

Since there were no significant interactions between temperature versus pH and temperature versus concentration to the response, the interaction between pH and

concentration to response was focused. At low pH and low concentration of profenofos, the profenofos biodegradation kinetic rates were relatively lower compared to those from other conditions. Profenofos is likely to exist longer at acidic condition which introduced less abiotic degradation (Verma, Jaiswal, & Sagar, 2014). In addition, there was less biotic activity at low concentration of profenofos due to lower substrate (profenofos) for the bacterium. For the environment with neutral and basic pH, abiotic profenofos hydrolysis may occur (Patel & Kumar, 2016). It also increased bioavailability of organophosphorus pesticides and survival of microorganisms leading to higher profenofos biodegradation activity (Suwannaruang & Wantala, 2016).

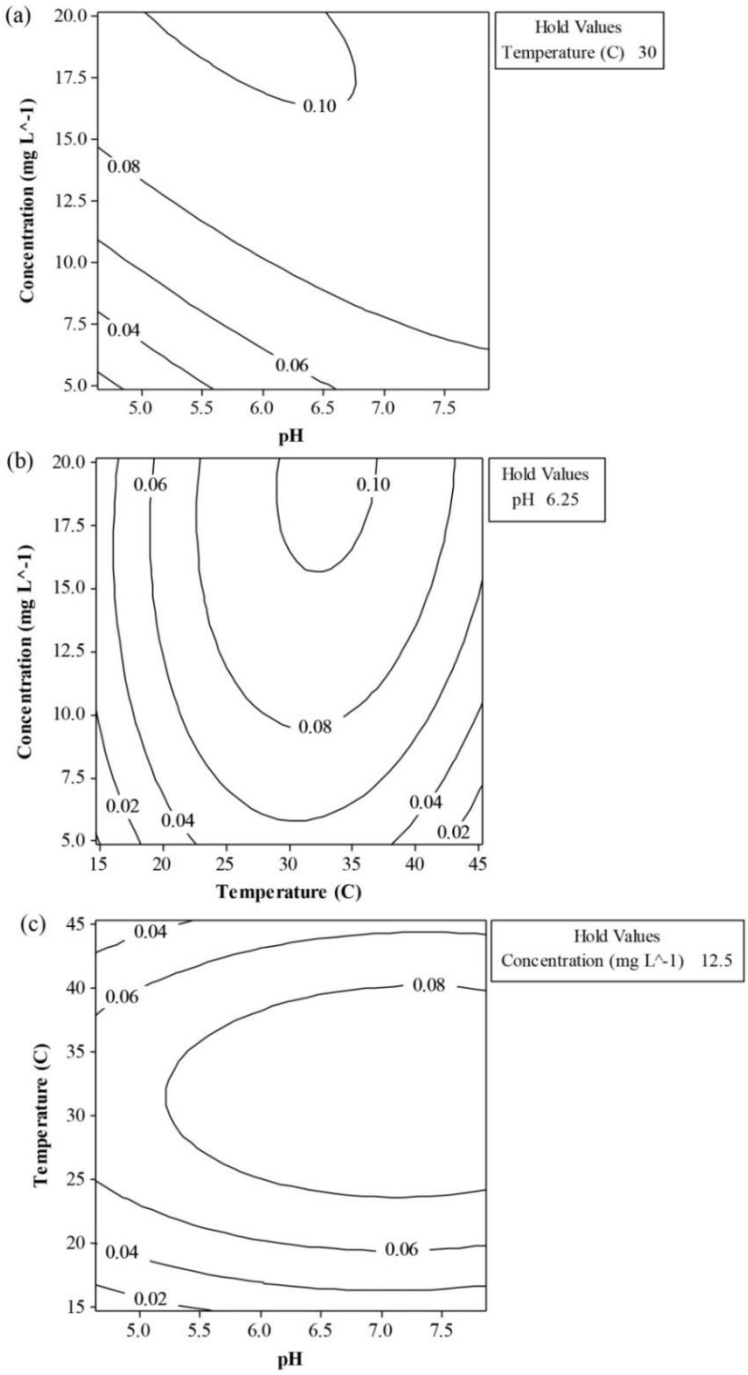


Figure 3.5 Contour plot for profenofos degradation kinetic rates (h^{-1}) resulting from interaction of initial profenofos concentration (mg/L), pH, and temperature (°C): (a) interaction between pH and initial profenofos concentration, (b) interaction between temperature and initial profenofos concentration, and (c) interaction between pH and temperature

3.4 Summary

This study aimed to characterize profenofos degradation by PF1 under influence of pH, temperature, and initial profenofos concentration. It was found that PF1 was an efficient profenofos-degrading microorganism. The profenofos removal by PF1 was up to 90%. The culture also well degraded BCP which was known as a toxic intermediate. Result from RSM analysis showed that pH, temperature, and profenofos concentration significantly affected profenfos degradation kinetic rates. The pH of 5.89, temperature of 32.94°C, and profenofos concentration of 20.15 mg/L were optimum levels for profenfos degradation. Interaction between pH and concentration influenced the profenofos biodegradation kinetic rate. Since PF1 well performed profenofos degradation in most tested condition, PF1 was promising for site remediation practice in the future. Effect of other environmental factors, such as organic carbon and nutrient contents should be performed for further characterization.

CHAPTER 4

Profenofos Removal by *Acinetobacter baylyi* Strain GFJ2: Biodegradation Kinetics and Influence of Environmental Conditions

4.1 Introduction

Profenofos ((O-4-bromo-2-chlorophenyl)-O-ethyl-S-propylphosphorothioate) is one of the organophosphorus insecticides broadly used for cotton, fruit, and vegetable production. This led to the contamination of profenofos (up to 20 µg/L) in environment over allowable concentration (0.3 µg/L) of drinking water standard (Hamilton et al., 2003; Berhan M. Teklu, Hailu, Wiegant, Scholten, & Van den Brink, 2016). Profenofos is toxic to human and animals leading to damage nervous system (Bajet, Kumar, Calingacion, & Narvacan, 2012; Lewis, Tzilivakis, Warner, & Green, 2016). Therefore, the technique to lessen this contamination problem is required.

Microbial degradation technology for site remediation is one of the successful techniques. Earlier, profenofos-degrading cultures, such as species in *Pseudomonas* genus were isolated (Siripattanakul-Ratpukdi et al., 2014). Although the cultures well degraded profenofos, the cultures were applied only for profenofos and other organophosphorus insecticide degradation. In practice, farmers use many types of pesticides including insecticides and herbicides resulting in numerous chemical residues concurrently contaminated in the agricultural area. Consequently, microbial cultures which are able to degrade various pollutants, is needed.

Among effective microbial cultures for the remediation applications, *Acinetobacter baylyi* strain GFJ2 (GFJ2), a previously isolated bacterium, could degrade various chemicals including 4-chloroaniline, 3,4-dichloroaniline, monohalogenated anilines, and other dichloroanilines with efficiencies of more than 90% (Hongsawat & Vangnai, 2010). These chemicals were in many industrial products, such as pesticides, polymers, and pharmaceuticals. Since the structure of these halogenated anilines are closed to profenofos, GFJ2 sounds potential for removing profenofos along with other compounds.

To date, no published work on the organophosphorus insecticide degradation by GFJ2. This work aims to examine the profenofos degradation performance by GFJ2. The profenofos removal kinetics and profenofos degradation production (4-bromo-2-chlorophenol, BCP) monitoring were emphasized. The optimization of profenofos biodegradation using surface methodology (RSM) with Central Composite Design (CCD) was performed. The effects of key environmental parameters including pH, temperature, and initial profenofos concentration on degradation performance were tested. The ranges of tested parameters (pHs of 5-8, temperatures of 15-45°C, and the profenofos concentrations of 5-20 mg/L) were chosen based on environmental values. The result from this study is the first report on the use of GFJ2 to treat profenofos pesticide. This information will be helpful data for future co-contaminant remediation.

4.2 Materials and methods

4.2.1 Chemicals

Commercial profenofos (50%, w/v, EC, Syngenta Crop Protection Co., Thailand) supplying from a local distributer was applied for all experiment. For chemical analysis, the standard grade of profenofos and BCP were obtained from Dr. Ehrenstorfer GmbH, LGC Standards, UK. Other chemical and solvent were acquired from Himedia (India), Ajax (Australia), and RCI Labscan (Australia).

4.2.2 Microorganism and enrichment

The isolated bacterium from herbicide-contaminated soil named *Acinetobacter baylyi* strain GFJ2 (GenBank number: HQ612277) was applied for this experiment (Hongsawat & Vangnai, 2011). The strain from stock culture (-80 °C) was transferred into Luria-Bertani (LB) broth and kept on shaking incubator at 30 °C with speed of 200 rpm for overnight. The cultivation of GFJ2 started with the addition of active GFJ2 at 1% (by volume) into a liter of minimal salt medium supplementing with 4 mM of succinic acid, 1 mM of ammonium chloride, and 0.1% (w/v) of yeast extract (called MMSAY medium). The mixture was incubated at 30°C with speed of 150 rpm for 12 h. The enriched suspension (OD_{600} = 1) was harvested and twice washed using

a 0.85% NaCl solution. The washed cell was re-suspended by minimal salt medium without yeast extract (Siripattanakul-Ratpukdi et al., 2014). The starting cell number was approximately 10^{12} CFU/mL.

4.2.3 Profenofos biodegradation kinetic test

The washed suspended cell of 3 mL was transferred into 27 mL of a bacterial medium (named MSM) in a 125-mL serum bottle. Formulation of MSM included $Na_2HPO_4 \cdot 2H_2O$ 6.82 g, KH_2PO_4 3 g, NaCl 0.5 g, NH_4Cl 2 g, and $MgSO_4 \cdot 7H_2O$ 0.51 g in 1000 m/L of phosphate buffer at pH 6.80 ($NaH_2PO_4 \cdot 2H_2O$ 0.083% (w/v) and $Na_2HPO_4 \cdot 2H_2O$ 0.17% (w/v)) (Siripattanakul-Ratpukdi et al., 2014). The sterilized filtered profenofos was then added to obtain the final concentration of 20-200 mg/L in the serum bottle. TSM without the cell suspension was used as control. The serum bottles were incubated on a rotary shaker at 150 rpm and 30°C for 4 days. The concentration of profenofos and BCP were monitored consecutively. The kinetic data was calculated according to the Michaelis-Menten model as shown in Equation (4.1) (Maya, Singh, Upadhyay, & Dubey, 2011).

$$V_0 = -V_{max} \frac{S}{S + K_s} \qquad \text{Equation (4.1)}$$

where V_0 is the initial biodegradation rate (mg/L/h), V_{max} is the maximum rate (mg/L/h), S is profenofos concentration (mg/L) and K_s is the half-saturation concentration (mg/L). The reciprocal of Equation (4.1) was simplified and called the Lineweaver-Burk equation as can be seen from Equation (4.2)

$$\frac{1}{V_0} = \frac{K_S}{V_{max} S} + \frac{1}{V_{max}} \qquad \text{Equation (4.2)}$$

4.2.4 Response Surface Methodology (RSM)

According to literatures, the environmental conditions influencing on profenofos biodegradation were pHs (6.50-8.50), temperatures (20.00-40.00 °C), and profenfos concentrations (0.32-1.00 mg/L) from surface water of agricultural area (Chandrasekar et al., 2014; Singaraja et al., 2014; B. M. Teklu, Adriaanse, & Van den

Brink, 2016). Profenofos biodegradation kinetic rates were calculated using Equation (4.3):

$$C_t = C_0 \times e^{-kt} \quad \text{Equation (4.3)}$$

where C_0 and C_t are the profenofos concentration at the initial and final times (mg/L). The k value is the profenfos biodegradation kinetic rates (h^{-1}) whereas t is time (h). The interaction between each parameter was generated using central composite design (CCD) at 5 different levels by Minitab 16 statistical software (Minitab, Inc., Pennsylvania, USA) (Table 4.1).

Table 4.1 Parameters and factors applied in the experimental design of profenofos biodegradation by GFJ2 (α = 1.5)

Parameters	**Factors**	**Level**				
		-α	**-1**	**0**	**+1**	**+α**
pH	A	4.64	5.30	6.25	7.20	7.87
Temperature (°C)	B	14.70	21.00	30.00	39.00	45.30
Profenofos (mg/L)	C	4.85	8.00	12.50	17.00	20.15

Profenfos biodegradation kinetic rates was utilized in the model generates by the full quadratic equation (Equation (4.4)). Analysis of variance (ANOVA) was used for analysis of Fisher test (F), its supplementary probability (p) and the coefficient of determination (R^2) in the regression model. The contour plots was conducted by fitting to the quadratic equation from regression analysis, holding one parameter at the optimum level, varying the other two parameters. The interaction between the significant parameters was assumed from the plots

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{i \neq j}^{3} \beta_{ij} x_{ij} \quad \text{Equation (4.4)}$$

Y is the profenfos biodegradation kinetic rates. x_i and x_j are the parameters including pH, temperature, and profenofos concentration. β is the regression coefficient values of the model.

4.2.5 Analytical methods

The analysis of profenofos remaining and BCP increasing concentration were investigated by gas chromatography (GC) with electron capture deteactor (Agilent 4890, Agilent, USA) with liquid/liquid extraction technique. N-hexane supplemented with 0.01% acetic acid was used as the solvent for extraction with ratio of 1:1 (solvent and sample). The sample was vigorously mixed with the solvent for 10 min and centrifuged at 10,000 rpm for 5 min. The organic phase was filtered by 0.22 µm filter nylon.

One µL of the filtered sample was injected into the GC with a HP-5 column (30-m length, 0.25-mm i.d., and 0.25-µm film thickness). The GC condition was splitless, injection temperature of 240 °C, and helium gas flow of 1.5 mL min^{-1}. The GC temperature program was 10 min period. The temperature started at 180 °C and hold for 2.00 min, increased to 250 °C with the rate of 40 °C min^{-1} and hold for 6.25 min. Profenfos and BCP peaks came out at 8.78 min and 3.08 min, respectively.

4.3 Results and discussion

4.3.1 Profenofos biodegradation kinetics

Biodegradation of profenofos on GFJ2 was conducted at the different concentration (5, 10, and 20 mg/L). The results of all tests were demonstrated in the same trends (Figure 4.1). The biodegradation rapidly occurred at the first 10 h and remained steady thereafter with the utilization rates of 0.51 to 4.26 mg/L/h. The profenofos biodegradation removal efficiencies were 10-80% as can be seen in Table 4.2. The experiments fit with the first order kinetic model (R^2>0.83). The accumulation of intermediate as BCP was detected along profenofos biodegradation (0.35-4.10 mg/L). This indicated that GFJ2 contained esterase (hydrolase) enzyme responsible for hydrolyzing ester bond linkage of profenofos. Profenofos can be used as a carbon source for the catabolic activity of GFJ2. It was reported that BCP is a major primary intermediate of the profenofos biodegradation by microbial cultures (Malghani et al., 2009; Siripattanakul-Ratpukdi et al., 2014; Jabeen et al., 2015). This is the first published report on the profenofos biodegradation by a bacterial strain in *Acinetobacter* genus.

Previously, GFJ2 was tested for degrading numerous contaminants, such as halogenated anilines. Hongsawat and Vangnai (2011) found that GFJ2 degraded chloroaniline to be catechol *via* dioygenase enzyme. The result from prior and this studies showed that GFJ2 contained several enzymes related to contaminant biodegradation. In future practice, GFJ2 is potential for remediating agricultural sites which likely are contaminated by several pesticides. However, the co-contamination of pesticides should be further studied before application.

According to modified Michaelis-Menten and Lineweaver-Burk kinetic models (Equation (4.1)-(4.2)), the kinetic constants K_S, V_{max}, and V_{max}/K_S were calculated as 186.66 mg/L, 2.42 mg/L/h, and 13.00×10^{-3} 1/h, respectively (Figure 4.2). The high affinity of profenofos biodegradation depends on lower values of K_S and higher value of V_{max}. Comparing to the kinetic parameters from Hongsawat and Vangnai (2011), the biodegradation of chloroaniline by GFJ2 was conducted. The study showed K_S and V_{max}/K_S values were 100.78 mg/L and 0.22×10^{-3}1/h, respectively. The much higher V_{max}/K_S found in this study could imply that hydrolase from GFJ2 may be more active compared to dioygenase resulting in better profenofos degradation. The complete enzymatic degradation should be tested to confirmed enzyme activities and enzymatic degradation kinetics.

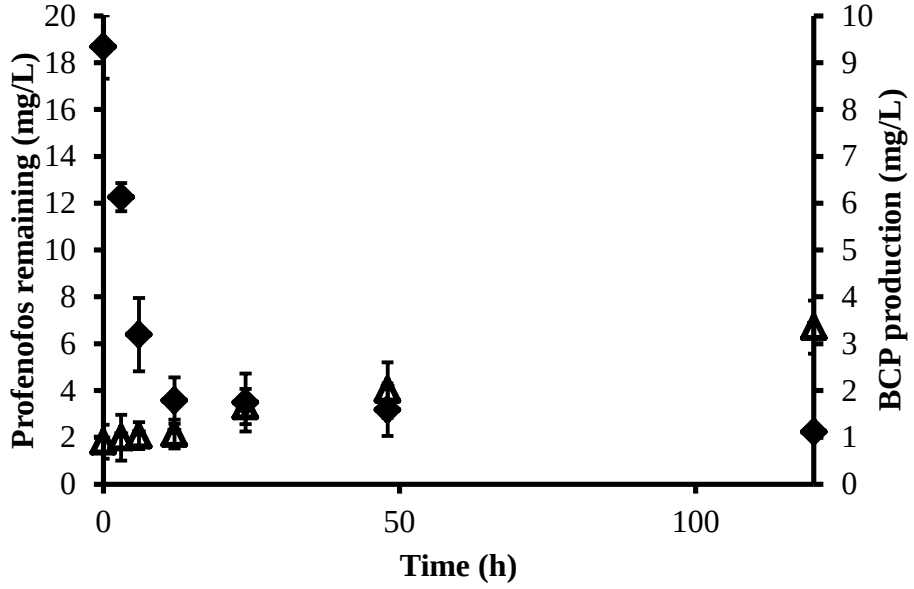


Figure 4.1 Profenofos biodegradation at initial profenofos concentration of 20 mg/L (◆) and BCP production (Δ)

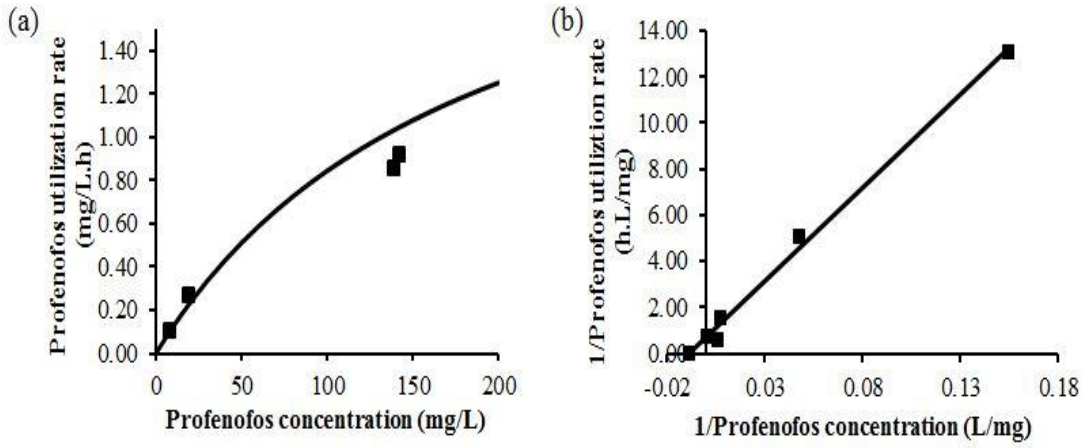


Figure 4.2 Profenofos biodegradation kinetics by GFJ2: (a) Michaelis-Menten plot and (b) Lineweaver-Burk plot. ■ represents experimental values while line plot (-) shows estimated values.

Table 4.2 Kinetic rates and removal efficiencies of profenofos biodegradation

Run	pH	Temperature	Profenofos concentration (mg/L)	*k* (h^{-1} x 10^{-2})	Profenofos removal efficiency (%)	Profenfos utilization rate (mg/L/h)
1	7.20	39.00	17.00	6.60	67.58	0.62
2	6.25	30.00	12.50	6.60	69.47	0.63
3	6.25	14.70	12.50	8.50	77.10	0.44
4	7.20	21.00	8.00	5.80	65.30	0.26
5	5.30	21.00	8.00	5.40	65.41	0.24
6	5.30	39.00	8.00	1.30	26.49	0.08
7	4.64	30.00	12.50	5.30	65.28	0.33
8	7.87	30.00	12.50	7.00	75.29	0.44
9	6.25	30.00	12.50	5.90	68.67	0.63
10	7.20	39.00	8.00	2.70	33.38	0.17
11	6.25	30.00	12.50	5.20	64.34	0.53
12	6.25	30.00	12.50	5.60	66.39	0.58
13	6.25	30.00	20.15	9.50	80.88	0.80
14	7.20	21.00	17.00	8.40	77.11	0.71
15	6.25	30.00	12.50	7.50	71.87	0.72
16	6.25	30.00	12.50	6.20	67.21	0.59
17	6.25	30.00	4.85	1.00	13.54	0.05
18	6.25	45.30	12.50	4.20	52.95	0.30
19	5.30	39.00	17.00	7.90	61.13	1.13
20	5.30	21.00	17.00	9.00	78.66	0.67

4.3.2 Response surface methodology

4.3.2.1 Characterization of profenofos biodegradation

According to the previous studies, the main parameters influencing on profenofos biodegradation kinetic rates (*k*) were pHs, temperature, initial concentration of profenofos (Anwar, Liaquat, Khan, Khalid, & Iqbal, 2009; Xu et al., 2008). The characterization of these three parameters on profenofos biodegradation performance was conducted and evaluated with CCD (Equation (4.3), Table 4.2). The equation model applying for this experiment was presented in Equation (4.5). The normal percentage probability plot was along the straight line indicating the accuracy and acceptable of data from the experiment with in the range of 95% confidence.

$$Y = 0.062 + 0.002A - 0.013B + 0.023C + 0.001B^2 - 0.003C^2 - 0.005AC + 0.005BC \quad \text{Equation (4.5)}$$

where A, B, and C coded for pH, temperature (°C), and profenofos concentration (mg/L), respectively.

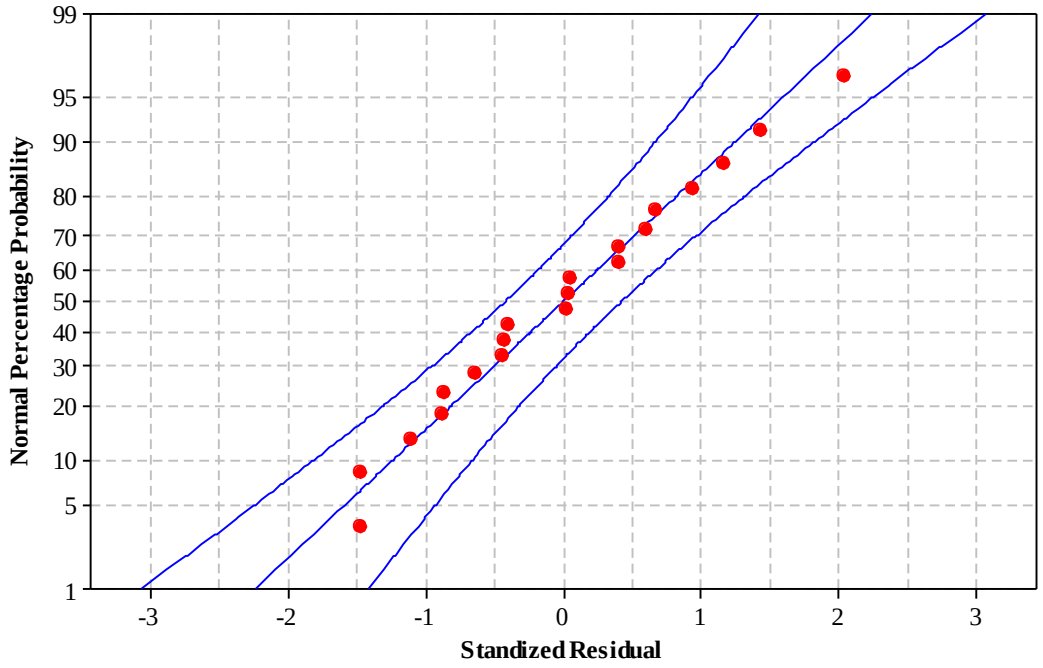


Figure 4.3 Normal percentage probability plot for profenofos biodegradation kinetic rates

The accuracy of the profenofos biodegradation kinetic rates response was confirmed by the graphical plot between experimental and predicted values (Figure 4.4). The *p* values of two parameters (temperature and profenofos concentration) were less than 0.05 indicating that these two parameters influenced on the response. While the other parameters ($p > 0.05$) were classified as the insignicant factor on profenofos biodegradation response.

According to Figure 4.4, the correlation between experimental and predicted value of profenofos biodegradation kinetic rates was nearly to 1. In addition, the goodness of the model (R^2) was 95.08%. These two results indicated that the model can be applied for the prediction of profenofos biodegradation kinetic rates (Table 4.4). ANOVA results were demonstrated that *P* value of the model was less than 0.05 (0.00), $F_{value} > F_{critical}$ (F_{value} = 21.47 and $F_{critical}$ = 10.16), and lack of fit > 0.05 (0.736). This can be confirmed that the regression model was acceptable with high precision (Mekuto, Ntwampe, & Jackson, 2015). According to *F* and *P* values, the model can be applied to explain the interaction between parameters and the significant parameters influencing on the response (pH, temperature, and profenofos concentration). B (temperature) and C (profenofos concentration) was a significant impact on the response ($p < 0.05$).

Table 4.3 Regression coefficient for profenofos biodegradation by GFJ2

Terms	**Coefficients**	***p* value**
Constant	0.062	0.000
A	0.002	0.318
B	-0.013	0.000
C	0.023	0.000
A^2	0.000	0.959
B^2	0.001	0.755
C^2	-0.003	0.114
AB	0.000	0.885
AC	-0.005	0.097
BC	0.005	0.059

Table 4.4 Analysis of variance for profenofos degradation by GFJ2

Source	Degree of Freedom	*F* value	*p* value	Significant
Regression	9	21.470	0.000	Significant
Linear	3	60.670	0.000	Significant
A	1	1.110	0.318	Insignificant
B	1	43.060	0.000	Significant
C	1	137.850	0.000	Significant
Square	3	1.090	0.397	Insignificant
A^2	1	0.000	0.959	Insignificant
B^2	1	0.100	0.755	Insignificant
C^2	1	3.010	0.114	Insignificant
Interaction	3	2.630	0.108	Insignificant
AB	1	0.020	0.885	Insignificant
AC	1	3.350	0.097	Insignificant
BC	1	4.520	0.059	Insignificant
Residual Error	10			
Lack-of-Fit	5	0.550	0.736	Insignificant
Pure Error	5			
Total	19			

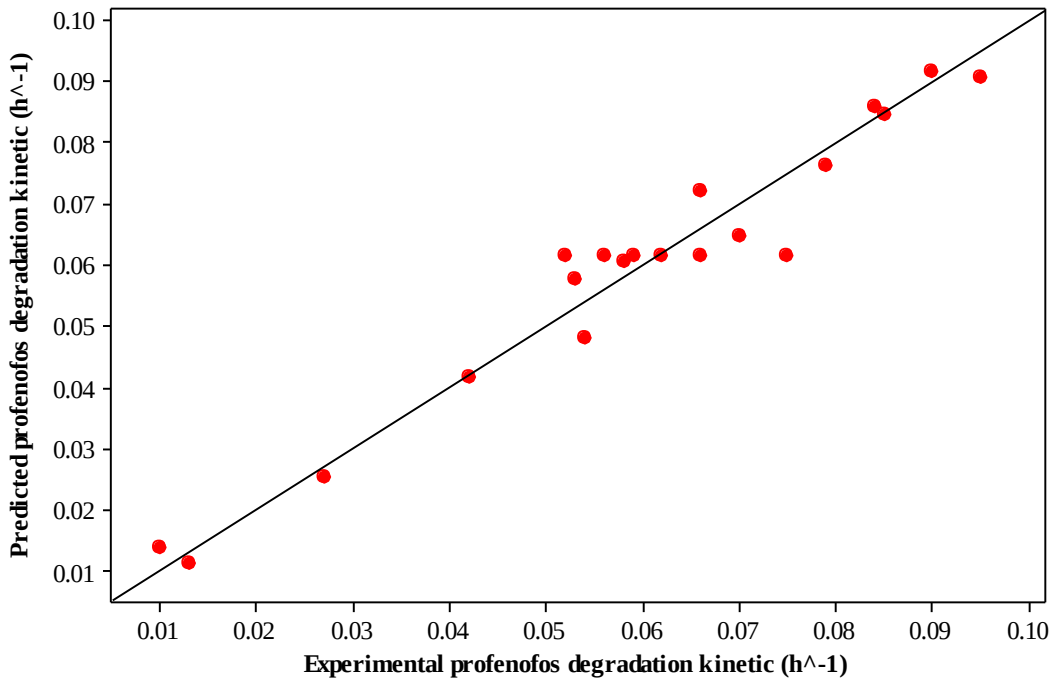


Figure 4.4 Parity plot of profenofos degradation kinetic rates (h^{-1}) between experimental and predicted values

4.3.2.2 Influence of pHs, temperatures, and initial profenofos concentrations on profenofos biodegradation

The main effects of profenofos biodegradation kinetic rates on pH, temperature, profenfos concentration were demonstrated in Figure 4.5. The increasing of pHs and profenofos concentrations resulted in rising of the kinetic rates. From the literature review, natural hydrolysis of profenofos occurred at alkaline condition contributing to increase of the degradation kinetic rates (Irie, 2008). The optimum pH for profenofos biodegradation was in range of 5.30-7.87. The increasing of the kinetic rates along with the increasing profenofos concentration indicated that GFJ2 is the potential strain to degrade profenofos at the wide range of environmental concentrations. This indicated that GFJ2 can be applied to contaminated agricultural fields, wastewater treatment plants, or spill contaminated sites.

The temperature profile illustrated that GFJ2 was able to degrade profenofos in wide range of temperatures (15-40 °C) (Figure 4.5). This could be explained by the activity of organophosphorus hydrolase. According to Grimsley et al. (2005), the hydrolase enzyme isolated from *Brevundimonas diminuta* was able to degrade paraoxon and chemical warfare agents within wide range of temperatures from 20 to 45 °C. In overall result from the influence of pHs, temperatures, and profenofos concentrations indicated that GFJ2 well degraded profenofos in the wide range of environmental parameters and could be applied for real practice (Grimsley, Calamini, Wild, & Mesecar, 2005).

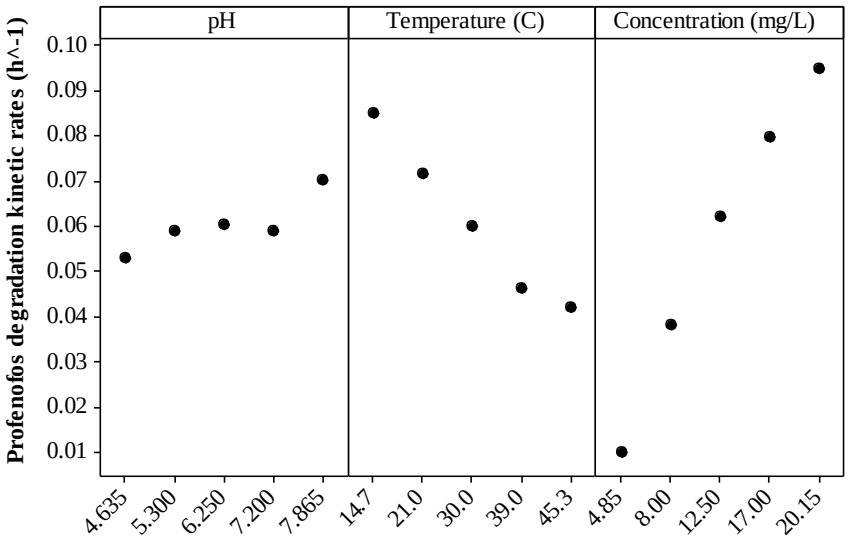


Figure 4.5 Main effects for profenofos biodegradation kinetic rates

4.3.2.3 Interactions of pHs, temperatures, and initial profenofos concentrations

The contour plots from Figure 4.6 (a)-(c) showed the interaction between main effects on profenofos biodegradation kinetic rates. The regression model from Equation (4.4) with one variable held constant at it optimum level and differentiated the other two was illustrated. From Figure 4.6 (a)-(c), the interaction between pH and profenofos concentration (held temperature at 30 °C), temperature and profenofos concentration (held pH at 6.25), and temperature and pH (kept profenofos concentration constant at 12.5 mg/L) with the responses was examined.

The results were similar. Based on ANOVA result shown in Table 4.4, there was not significant interaction among parameters. All *p* values of the interactions between pH and concentration, temperature and concentration, and temperature and pH were higher than 0.05. It could be indicated that there were insignificant interaction among the main parameters on profenofos biodegradation kinetic rates.

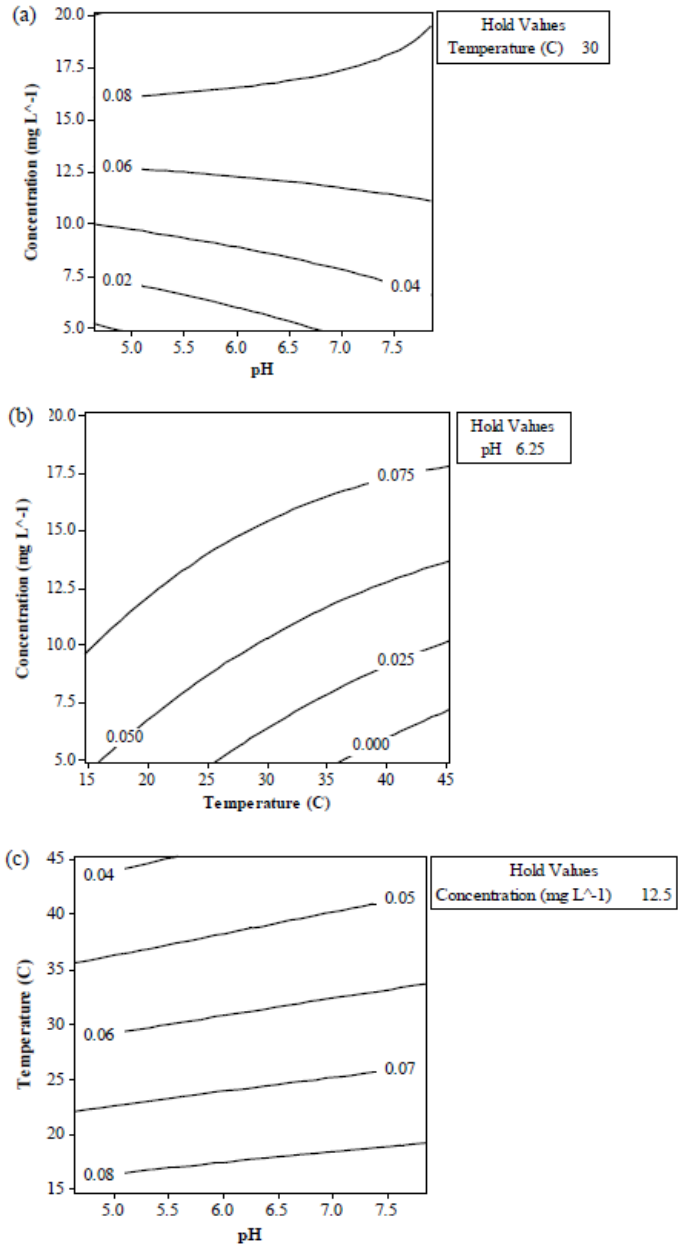


Figure 4.6 Contour plots for profenofos biodegradation kinetic rates (h^{-1}) resulting from interaction of initial profenofos concentration (mg/L), pH and temperature (°C): (a) pH and intial profenofos concentration, (b) temperature and initial profenofos concentration and (c) pH and temperature

The tested values for all effects (pH, temperature, and initial profenofos concentration) were selected based on environmental values. The interaction results from this study confirmed that GFJ2 is really potential to apply for site remediation since the main environmental factors did not influence the biodegradation performance.

4.4 Summary

The aim of this study was to characterize profenofos biodegradation by GFJ2 at different environmental parameters including pH, temperature, and initial profenofos concentration. From the preliminary test found that GFJ2 which was known as a chlorinated compound-degrading culture was able to degrade profenofos. GFJ2 well degraded profenofos removal (approximately 60%) with in a day. The RSM analysis also showed that the main parameters significantly influencing profenofos biodegradation kinetic rates were temperature and initial profenofos concentration. The optimum levels of pHs, temperatures, and profenofos concentrations were 5.30-7.87, 20-45 °C and 20.15 mg/L. The interactions among parameters on the profenofos biodegradation kinetic rates were insignificant. In overall, GFJ2 is potential for further site remediation since the culture efficiently degrades various contaminants at wide range of environmental conditions. According to the previous result of PF1 and GFJ2, it can be concluded that both cultures well performed in broad ranges of environmental conditions, the strains were able to utilize for both on-site and off-site applications including water and soil treatment systems and in-situ agricultural infiltrate, groundwater, and soil remediation systems.

CHAPTER 5

Profenofos Removal by Immobilized *Pseudomonas plecoglossicida* strain PF1 and *Acinetobacter baylyi* strain GFJ2 and Their Cell Retention Ability: Influence of Environmental Condition and Immobilized Cell Characteristics

5.1 Introduction

Profenofos ($C_{11}H_{15}BrClO_3PS$), O-4-bromo-2-chlorophenyl O-ethyl S-propyl phophorothioate is one of the most widely used organophosphorus insecticides in many countries such as Thailand (3,892 t/y), Malaysia (2,826 t/y), Myanmar (476 t/y), and India (16,922 t/y) (FAOSTAT, 2013). Profenofos was utilized to control pest in cotton, vegetable, tobacco, and crop production (Irie, 2008 ; USEPA, 2006). This situation led to profenofos contamination in environment and agricultural products. For example, Harnpicharnchai et al. (2013) reported the profenofos-contaminated concentrations in water and soil during summer of up to 0.95 mg/L and 41.81 mg/kg, respectively. Another example is profenofos of 0.5-183,000 µg/L contaminating in Chinese kale (Maximum residue limits by Codex Alimentarius Commission of 50 µg/L) (Sapbamrer & Hongsibsong, 2014). This compound has been known as acetylcholinesterase inhibitors and toxic to organisms (Santos da Silva, Chiavone-Filho, de Barros Neto, Foletto, & Mota, 2013). Based on this information, profenofos remediation technology is required.

Microbial degradation is an effective process to remediate contaminated sites. There were numerous successful applications of microbial degradation including organochlorine and organophosphorus pesticides (Chishti & Arshad, 2013; John & Shaike, 2015; B. K. Singh, 2009; B. K. Singh & Walker, 2006; Van Dyk & Pletschke, 2011). It was reported environmental factors influencing the microbial degradation in the contaminated sites, such as pH, temperature, inorganic salts (Agudelo, Peñuela, Aguirre, Morató, & Jaramillo, 2010; Karpouzas & Walker, 2000; B. K. Singh, Walker, & Wright, 2006). Previously, it was found that bacterial species including Pseudomonas plecoglossicida strain PF1 (PF1) and Acinetobacter baylyi strain GFJ2

(GFJ2) successfully degraded profenofos (Siripattanakul-Ratpukdi et al., 2014). Prior works focused on effects of the initial profenofos concentrations and organic carbon supplement. So far, there was no published report on influence of inorganic salts which typically found in groundwater on profenofos biodegradation.

Cell immobilization technique by entrapping microbial cells in polymeric materials could lessen the problem described previously. Advantages of the cell immobilization were the cell protection from toxic substances and environmental stresses, high cell density and retention, and easy for cell preparation and reuse (Ma et al., 2016; Siripattanakul & Khan, 2010). The biodegradation performance by immobilized cells mainly depended on bead characteristics, such as bead size and porosity (Ahmad et al., 2012; Bergero & Lucchesi, 2013; Ibrahim et al., 2016; Kathiravan, Praveen, Gim, Han, & Kim, 2014).

The key success factor of the immobilized cells is to retain large amount of active cells inside of the polymeric beads. The information on how well of the retained cell in the immobilization matrices was limited. Previous studies on the immobilized cells mostly focused on the cell viability (Alonso et al., 2015; Siripattanakul et al., 2008). For bioremediation by the free cells, cell motility including general transport and chemotaxis is an important factor (R. Singh & Olson, 2008). Bacterial cells may generally move or chemically attract by contaminants (Filloux & Ramos, 2014). This motility especially chemotactic behavior exhibited the increasing or decreasing of toxic substance biodegradation (Barrionuevo & Vullo, 2012). Till now, there has been no study on cell motility related to biodegradation performance by the immobilized cells.

This study aimed to investigate profenofos biodegradation by the immobilized PF1 and GFJ2 under different immobilized cell characteristics (bead sizes) and contaminant and inorganic salt concentrations. The bacterial cells immobilized in a widely used immobilization material, calcium alginate. It is noted that the types and concentrations of inorganic salts (NaCl, $MgSO_4$, and $CaCO_3$ at concentrations of 100-1000 mg/L) were chosen based on typical salt types and concentrations present in groundwater. This is the first report on cell motility (based on swimming, swarming, twitching, and chemotaxis assays) related to profenofos biodegradation by the immobilized cells. The study by the free cells was performed along with the test by

the immobilized cells for comparative purpose. The information from this study could be used as fundamental knowledge for the profenofos bioremediation by the immobilized cells.

5.2 Materials and Methods

5.2.1 Chemicals

Commercial profenofos emulsifiable concentrate (50% w/v) applied in the experiment was purchased from a local distributer (Syngenta Crop Protection Co., Thailand). The chemicals used for bacterial medium and chemical analysis were laboratory and analytical grades obtained from Himedia (India), Ajax (Australia), and RCI Labscan (Australia). Alginic acid sodium salt (ACROS Organics, Singapore) was used for cell immobilization.

5.2.2 Bacterial cultivation and growth conditions

Pseudomonas plecoglossicida strain PF1 (GenBank accession number KJ620776) was previously isolated from profenofos-contaminated chili farm soil (Siripattanakul-Ratpukdi et al., 2014). The strain was subcultured in minimal salt medium (MSM) in every 4 days for 2 weeks before used. The formulation of MSM (pH of 7.0) was followed Siripattanakul-Ratpukdi et al. (2014). For biodegradation assay, the activated culture (10%) was enriched by transferring into 1 liter of MSM medium with 0.1% yeast extract (MSMY) with incubation time for 15 h at shaking condition of 120 rpm and 30 °C. The PF1 suspended cells were used to prepare the immobilized cells.

Acinetobacter baylyi strain GFJ2 (GenBank accession number HQ612277) was isolated from herbicide-contaminated soils (Hongsawat & Vangnai, 2011). The GFJ2 cells from Luria-Bertani (LB) agar plate was inoculated into LB broth with shaking condition at 200 rpm and 30 °C for overnight. To enrich GFJ2 for the experiment, the active GFJ2 culture of 1% was transferred into 1 liter of minimal medium (called MMSAY) with incubation time for 12 h at 30 °C and 120 rpm. Formulation of MMSAY was modified from Hongsawat and Vangnai (2011) by

adding 4 mM of succinic acid, 1 mM of ammonium sulfate and 0.1% (w/v) of yeast extract.

The filtered sterile profenofos (the concentration of 20 mg/L in bacterial medium) was applied as the main carbon source on the enrichment process for both strains. After enrichment, the strains were harvested by centrifugation (5,000 rpm, 30 min, and 4 °C). The pellets were washed and resuspended with 0.85% NaCl. The cells of approximately 1.5×10^{12} CFU/mL were used for the profenofos biodegradation assay by the free and immobilized cells.

5.2.3 Cell immobilization procedure

The suspension of PF1 and GFJ2 were separately added and mixed into 90 mL of a sterile sodium alginate solution (3% (w/v)). The cell-alginate mixture was dropped by a peristaltic pump into a $CaCl_2$ solution of 3.5% (w/v). For the experiment on influence of the bead sizes, the immobilized cell bead sizes were varied at 2, 4, and 6 mm in diameter. For other experiments, the 4 mm-immobilized PF1 and GFJ2 (based on biodegradation performance described later) were selected. The beads were hardened in the $CaCl_2$ solution for 2 h with gentle agitation. The hardened beads were washed before used. The washing solution containing Tris-HCl (10 mM, pH 7.0) (designated as TSM) was applied to avoid de-entrapment of the immobilized cells.

5.2.4 Profenofos biodegradation and immobilized cell leaching assay

Profenofos biodegradation assay divided into 3 subsections including 1) the influence of immobilized cell bead sizes (2, 4, and 6 mm), 2) initial profenofos concentrations (20-200 mg/L), and 3) inorganic salt types and concentrations (NaCl, $MgSO_4$, and $CaCO_3$ at 100-1000 mg/L). The experiments by the free PF1 and GFJ2 cells were run along with ones by the immobilized cells. It is noted that the tests with different bead sizes were control the same cell number and sodium alginate volume for comparative purpose. All experiments were carried out in duplicate.

The cells (PF1 and GFJ2) of 3 mL and 10 g for the free and immobilized cells, respectively was inoculated into 27 mL of TSM in 125 mL serum bottles. The filtered sterile profenofos was added to obtain the initial concentration of 20 mg/L in the serum bottle (except the tests with variation of profenofos concentrations). The batch

tests were conducted on rotary shaker at 150 rpm and 30 °C for 4 days. The profenofos concentration remaining in the reactors was monitored continuously.

Some tested conditions (bead size and contaminant concentration) were chosen. The cells leaching from the immobilized cell beads to the medium were monitored for 4 days along with the biodegradation tests. This test was performed to preliminarily investigate the movement of the immobilized cells related chemical attractant. The leaching of the cells was enumerated by spread plate counting technique.

To confirm the cell leaching, the immobilized PF1 and GFJ2 cells was prepared as double layers (inner layer with the bacterial cells and outer layer without the bacterial cell) as shown in schematic picture (Figure 5.1). The typical immobilized cells (inner layer) was dipped into the sodium alginate solution and soaked in the $CaCl_2$ solution to form the second layer (without bacterial cells). The morphological observation of the immobilized cells after the selected experiment with profenofos was examined by scaning electron microscope (SEM).

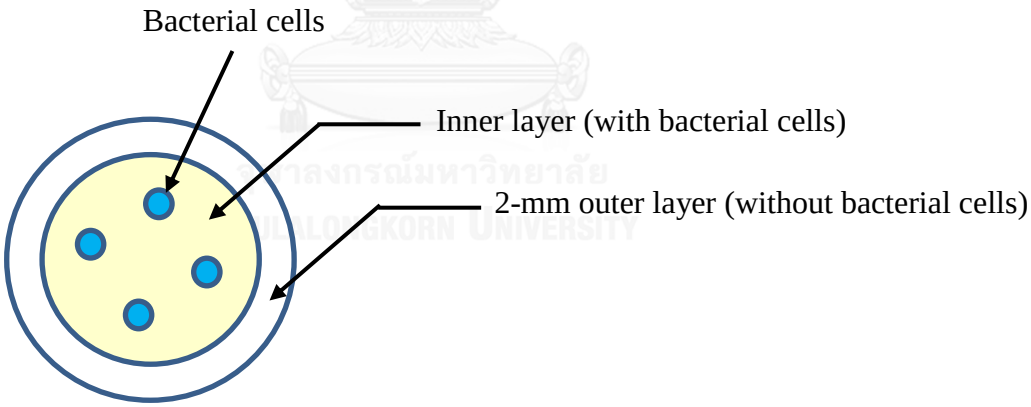


Figure 5.1 Schematic picture of double layer-immobilized cell

5.2.5 Motility assay

For the motility assays, the tests divided into 2 tasks including 1) plate assay and capillary assay. The plate assay involved swimming, swarming, and twitching assays. The media used for the plate assay were LB, LB with profenofos of 20 or 100 mg/L, and NaCl of 0.5% with profenofos of 20 or 100 mg/L. Different agar concentrations were varied according to different motility tests (0.3% for swimming,

0.5% for swarming, and 1.0% for twitching). Swimming assay showed the ability of flagellum-dependent movement in liquid condition. Swarming assay indicated flagellum-dependent movement across a semisolid surface. Twitching assay was the ability of the cell to translocate under humid conditions with type IV pili.

The freshly activated cultures were applied. For swimming test, the inoculum was dipped perpendicular into the center of agar plate. For swarming test, the 2.5 µL inoculation was dropped on the center of the plate. The incubation conditions were 35°C for 24 h (swimming and swarming). The diameter of bacterial travelling zone from the inoculum point was measured in both assays. For twitching test, the inoculum was stabbed perpendicular to the bottom of the plate. The incubation condition was 35°C for 48 h. The calculation on the surface area of the interstitial colony was analyzed. All plates after the cultivation were photographed.

The capillary chemotaxis assay was conducted by computer-assisted capillary (Nikata, Sumida, Kato, & Ohtake, 1992; Vangnai et al., 2013). The 20 µL of freshly inoculum (PF1 and GFJ2) was placed into a coverslip and recorded under an inverted microscope. The substrates (the medium with profenofos of 20 or 100 mg/L) at the mouth of the capillary tubes were solidified with 1% (w/v) agarose. Yeast extract at 2% (w/v) was used as positive control. The digital images were captured and counted in each frame to amount of bacterial cells within 2 min after starting under the microscopic observation. The ratio of the amount of cell at each time point and starting per frame was represented of the chemotactic response to the substrate.

5.2.6 Analytical methods

To analyze profenofos remaining, the sample was extracted using liquid/liquid extraction technique. The samples from the batch reactors were added (ratio 1:1) with n-hexane plus 0.01% acetic acid. The mixtures was vigorously mixed by vortex mixer for 20 min and centrifuged at 10,000 rpm for 5 min. The organic phase (the extract) was filtered with nylon filter (0.22 µm). A gas chromatograph (GC) with electron captures detector (model GC-2014, Shimazu) with a DB-5 column (30-m length, 0.32-mm i.d., and 0.25-µm film thickness). A microliter of the filtered extract was injected into GC with conditions of splitless injection, injection temperature of 240°C, and helium gas flow of 37.4 mL/min. The GC temperature program started at 180 °C

and hold for 3 min, increased to 240 °C with the rate of 30 °C/min and hold for 4 min. The total run time was 9 min. The peak retention time of profenofos was 7.9 min.

For the SEM observation, the immobilized cells were fixed with 0.1% OsO4 solution for 30 min. The fixed beads were washed for 4 times with distilled water. The fixed beads were cut into two parts by cryocut microtome (Leica, CM3000, Germany). After that, the beads were dehydrated with 30% ethanol and 0.07 M CaCl2 solution, 50% ethanol and 0.05 M $CaCl_2$ solution, 70% ethanol and 0.03 M $CaCl_2$ solution, 90% ethanol and DI, and 100% ethanol, respectively. Then, the absolute ethanol was applied for 3 times for 10 min each as the last step of dehydration. The dehydrated beads were critical point dried using a critical point dryer (Balzers, CPD 020, Liechtenstein). Then, the beads were attached to the stub by glue, coated with gold using an ion sputter (Balzers, SCD 040, Liechtenstein). The dried beads were observed using SEM with EDS Attachment (SEM-EDS) (JEOL, JSM-6610LV, Tokyo, Japan). The surface and cross-sectional views of the beads (inner and outer layers) were focused.

5.3 Results and discussion

5.3.1 Profenofos biodegradation assay

5.3.1.1 Influence of immobilized cell bead sizes on profenofos biodegradation

The profenofos biodegradation by PF1 and GFJ2 with the different sizes of the beads (2, 4, and 6 mm) were shown in Figure 5.2 and 5.3. Trend of the biodegradation by both strains was similar. The biodegradation of the free and immobilized PF1 and GFJ2 obviously occurred within the first 40 h and slightly decreased or reached plateau thereafter. The result in Table 5.1 showed that the biodegradation percentages by PF1 and GFJ2 were 50-88% with the utilization rates of 0.08-0.38 mg/L/h. The degradation kinetics followed the first order kinetic reaction with the rates of 0.0017-0.0516 1/h.

The biodegradations by the immobilized cells were better than ones by the free cells (Table 5.1). It was clear that the immobilization technique improved the biodegradation by PF1 which resulted in higher removal percentage compared to the efficiency of the free PF1. On the other hand, the biodegradation by the immobilized

GFJ2 was not apparently different from the free cells (Table 5.1). This could be from the difference of cell physiology and behavior of PF1 and GFJ2. Previously, it was reported the limitation of mass (substrate) transfer inside the immobilized cells related to biodegradation performance (Bergero & Lucchesi, 2013; Siripattanakul et al., 2008). In this study, there was no limitation leading to higher profenofos removal.

Based on the result presented in Table 5.1, the microorganisms in the immobilization matrices at different bead sizes performed the different biodegradation efficiencies. The small bead (2 mm) may have less diffusion resistance but could increase toxic to the cells (Kathiravan et al., 2014). The result from this study showed that 4-mm bead was the suitable size for profenofos biodegradation.

Table 5.1 Profenofos biodegradation kinetic rates at the different sizes of the immobilized beads

Strains	Sizes (mm)	Profenofos degradation kinetic		Profenofos utilization rate (mg/L/h)	Profenofos removal (%)
		Equation	R^2		
PF1	Free cell	y=-0.0159x+1.8448	0.97	0.08	49.90
	2	y=-0.0017x+2.7704	0.92	0.20	72.75
	4	y=-0.0297x+1.8993	0.71	0.15	87.94
	6	y=-0.0156x+2.9422	0.94	0.17	76.24
GFJ2	Free cell	y=-0.0202x+1.9095	0.81	0.10	60.36
	2	y=-0.0290x+2.8767	0.88	0.38	66.51
	4	y=-0.0516x+1.9539	0.84	0.27	68.33
	6	y=-0.0193x+2.8220	0.84	0.25	68.74

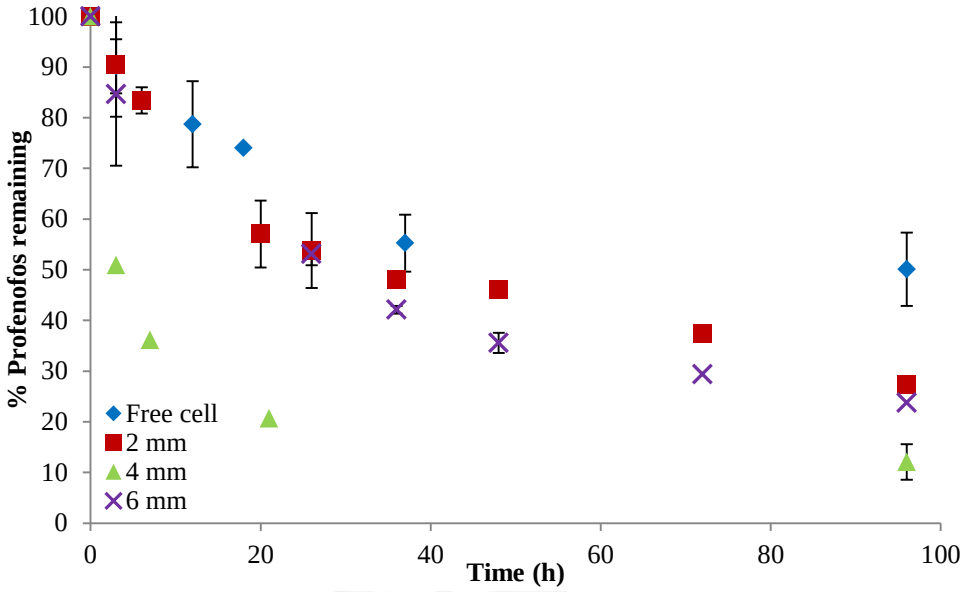


Figure 5.2 Profenofos biodegradation of PF1 at different sizes of immobilized beads (Free cell (◆), 2-mm bead (▪), 4-mm bead (▲), and 6-mm bead (x))

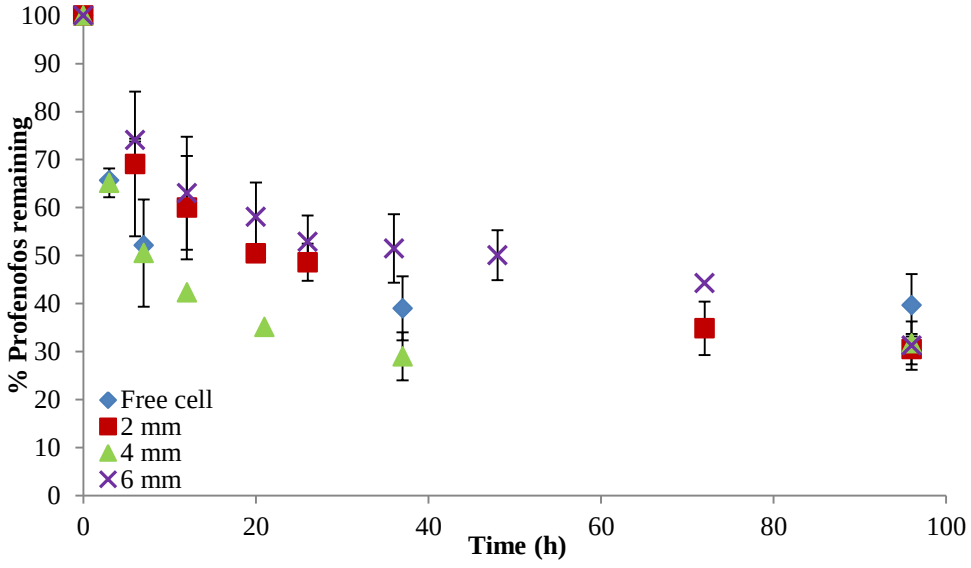


Figure 5.3 Profenofos biodegradation of GFJ2 at different sizes of immobilized beads (Free cell (◆), 2-mm bead (▪), 4-mm bead (▲), and 6-mm bead (x))

5.3.1.2 Influence of profenofos concentrations on profenofos biodegradation

Profenofos biodegradation of the free and immobilized PF1 and GFJ2 were conducted at the different concentrations from 20 to 200 mg/L in MSM medium. According to Figure 5.4, the biodegradation of the free and immobilized PF1 was rapidly occurred with 40 h with removal range from 35-90%. The immobilized cells (50-90%) with profenofos concentrations at 20-100 mg/L improved the degradation almost 2 higher than the free cells (35-50%). Based on results in Figure 5.4, the degradation by the free PF1 at different initial profenofos concentrations was quite stable. The concentration did not noticeably affect the profenofos removal. This is because the culture was acclimated in the environment containing profenofos for a long time. The self-substrate inhibition did not occur.

Theoretically, this substrate diffusion into the immobilized bead can cause profenofos adsorption at pores inside the matrices. From abiotic (control) test in this study, the removal of profenofos by adsorption was less than 20% (data not shown). Previously, it was reported that the immobilization matrices could provide better environment for the microbial cells (Siripattanakul et al., 2008). Although the immobilization materials could retain the microbial cells, porosity of the materials (number and size of pores) was likely satisfactory for biodegradation ability and microbial cell movement to the substrate. However, this implication on the cell movement inside the immobilized cells was needed to be confirmed (the result was shown in the next subsection). It is found that the initial profenofos concentrations clearly influenced the profenofos removal by the immobilized PF1. At low initial profenofos concentrations (less than 100 mg/L), PF1 in immobilized form performed better because of better environment as discussed earlier. At high concentration (200 mg/L), the efficiency by the immobilized cells was similar to the free cells. The reduced ability could be from toxicity of the profenofos pesticide (Chen, 2007; J. M. Saez, Álvarez, Benimeli, & Amoroso, 2014).

Profenofos biodegradation performances by the free and immobilized GFJ2 were different from the result by PF1 (Figure 5.6). At low concentrations (20-50 mg/L), profenofos removal of immobilized cells was 10% higher than free cells. In contrast, the biodegradation from the free cells was higher than immobilized cell at 100 mg/L. At the higher concentration (200 mg/L), the free and immobilized GFJ2

activities were inhibited. Both microbial cells in this study (PF1 and GFJ2) were immobilized in the same material; therefore, the different trend of profenofos removal should be from microbial cell behavior (cell activity and movement).

Profenofos biodegradation kinetics from the free and immobilized PF1 and GFJ was reported in Table 5.2. According to modified Michaelis-Menten kinetics model from (Maya et al., 2011), K_m was replaced to K_s as the activity of cell are monitored by applying whole cells instead of purified enzymes as can be seen from Equation (5.1).

$$V_0 = -V_{max} \frac{S}{S + K_s} \qquad \text{Equation (5.1)}$$

where V_0 is the initial rate (mg/L/h), V_{max} is the maximum biodegradation rate (mg/L/h), S is profenofos concentration (mg/L) and K_s is the half-saturation concentration (mg/L). The reciprocal of Equation (5.1) was simplifies and called the Lineweaver-Burk equation as can be seen from Equation (5.2).

$$\frac{1}{V_0} = \frac{K_S}{V_{max} S} + \frac{1}{V_{max}} \qquad \text{Equation (5.2)}$$

The high affinity of microorganisms to degrade profenofos depended on lower values of K_s and higher values of V_{max}. From the result showed that immobilization technique led to the lower values of K_s in the both strains comparing to the free cells while V_{max} values was reduced in the immobilized cell (Figure 5.4 to 5.11). The V_{max}/K_s values from the Lineweaver-Burk equation was initiated for comparing the efficiency of profenofos biodegradation in free and immobilized cell (Equation (5.2)). The higher ratio of V_{max}/K_s indicates the higher ability to degrade profenofos. The immobilized PF1 provided the highest value of V_{max}/K_s comparing with free cell. The V_{max}/K_s ratios of free and immobilized GFJ2 were not significantly different. The immobilization technique is suitable for PF1 than GFJ2 and supported PF1 to tolerate to higher concentration of profenofos (Basak, Bhunia, & Dey, 2014; Sedighi, Zamir, & Vahabzadeh, 2016; Zheng et al., 2009). The Michaelis-Menten kinetic graph was plotted from 4 different concentrations of profenofos. The present results only gave the trends of profenofos biodegradation kinetics by the free and immobilized cells. The continued work at higher profenofos concentrations was needed for more precise kinetic information.

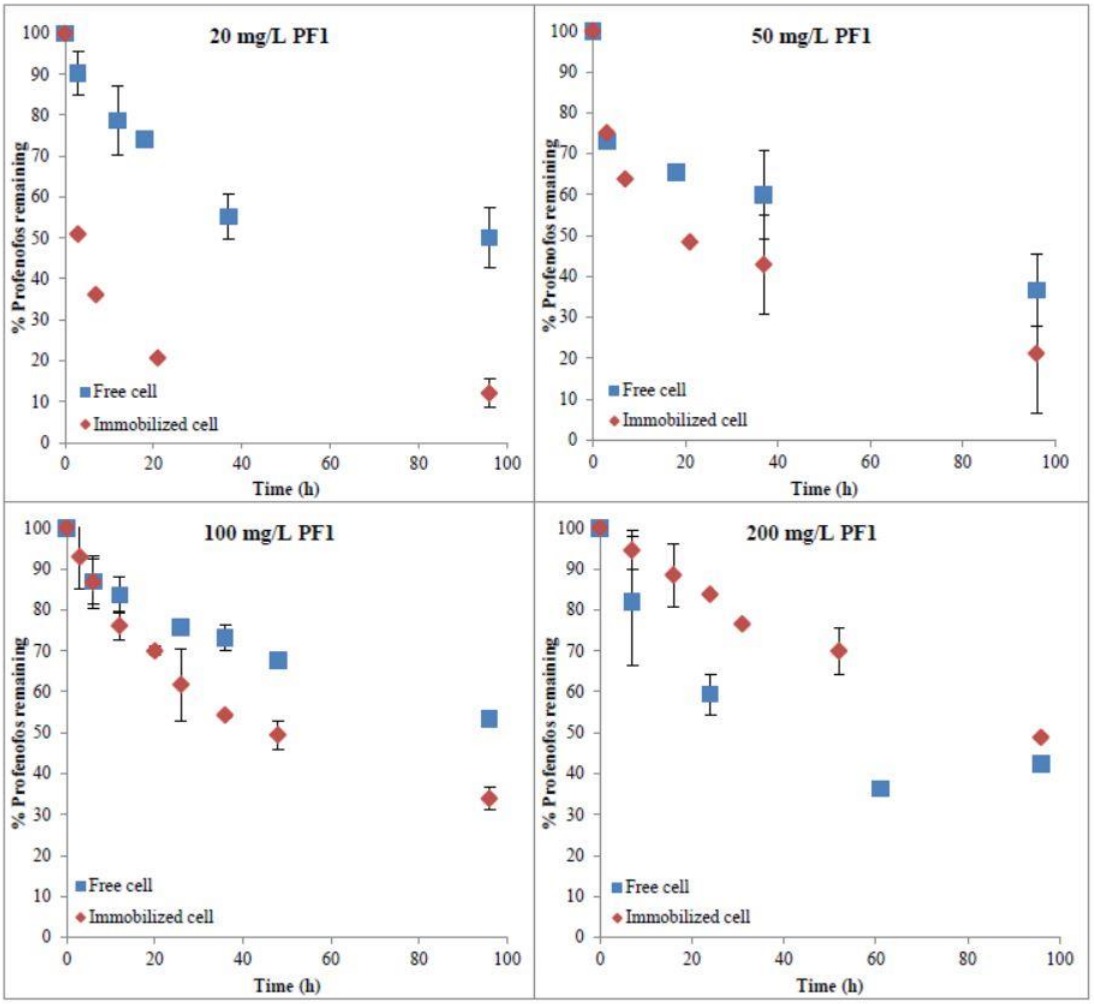


Figure 5.4 Profenofos biodegradation of PF1 at the different concentrations

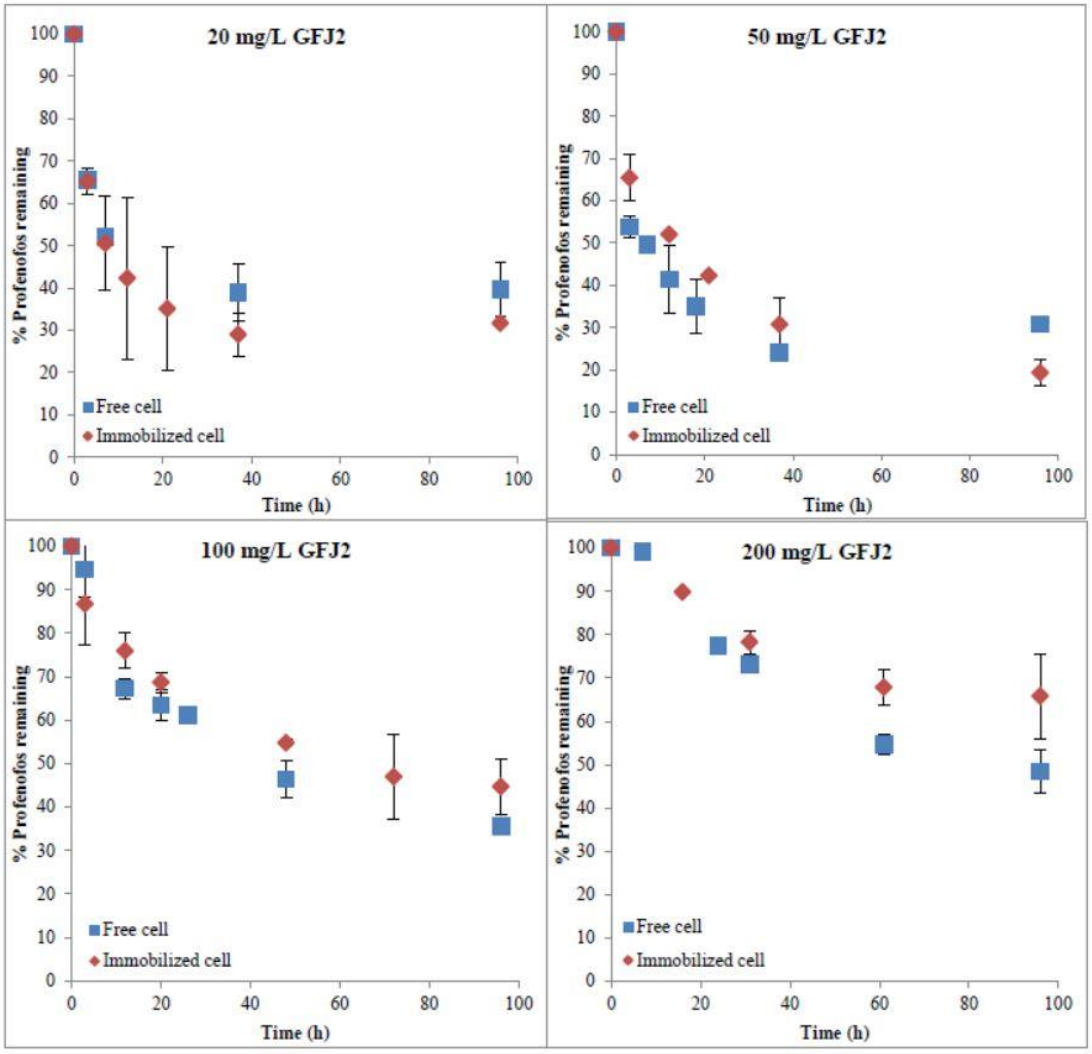


Figure 5.5 Profenofos biodegradation of GFJ2 at the different concentrations

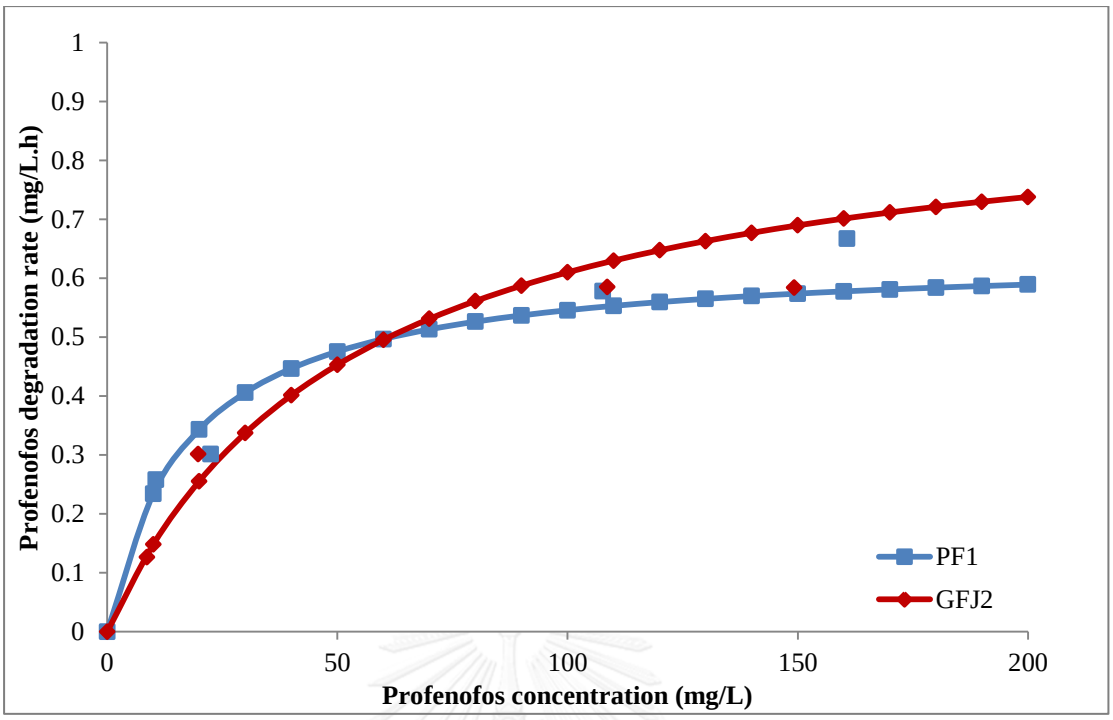


Figure 5.6 Profenofos degradation of immobilized PF1 and GFJ2 followed Michaelis-Menten equation

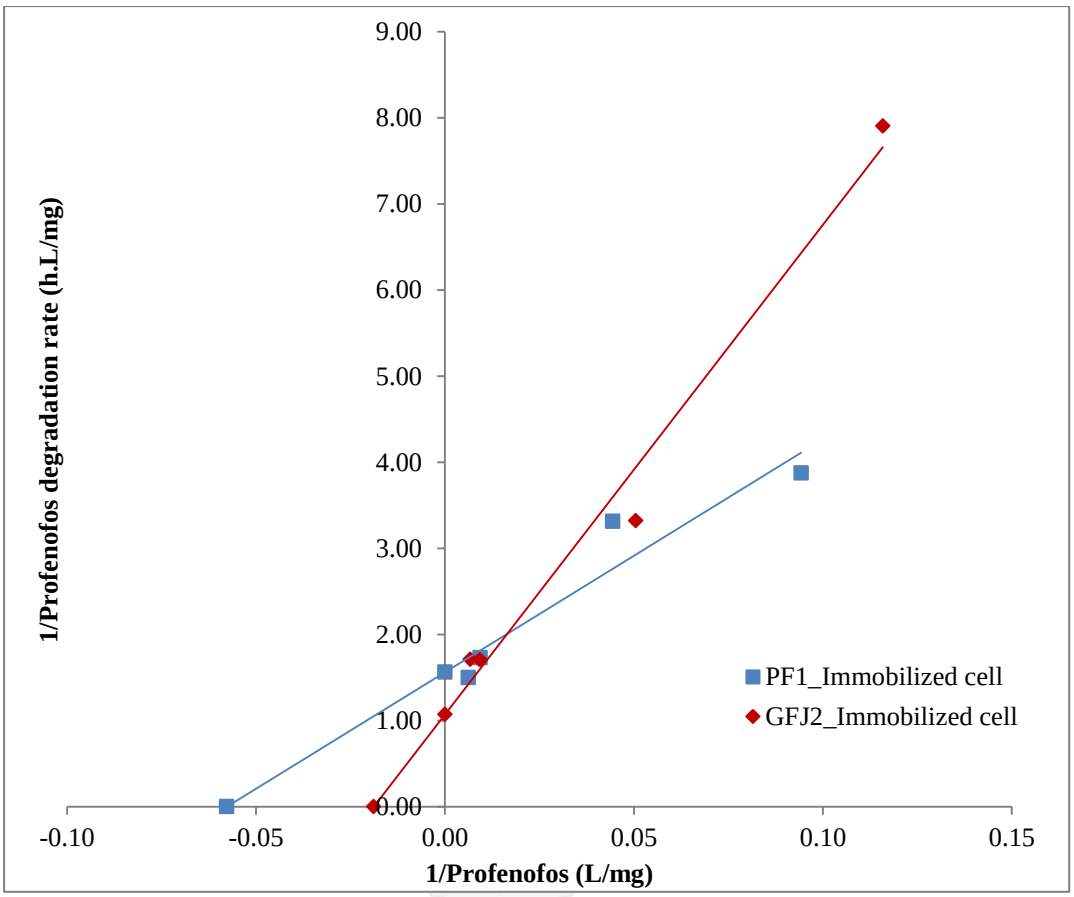


Figure 5.7 Profenofos degradation of immobilized PF1 and GFJ2 followed Lineweaver-Burk equation

Table 5.2 Profenofos biodegradation kinetic information

Type of cell	Strains	Kinetic constants		
		K_S (mg/L)	V_{max} (mg/L/h)	V_{max}/K_S (1/h)
Free cell	PF1	110.01	1.36	0.0124
	GFJ2	186.66	2.42	0.0130
Immobilized cell	PF1	17.32	0.64	0.0370
	GFJ2	53.07	0.93	0.0176

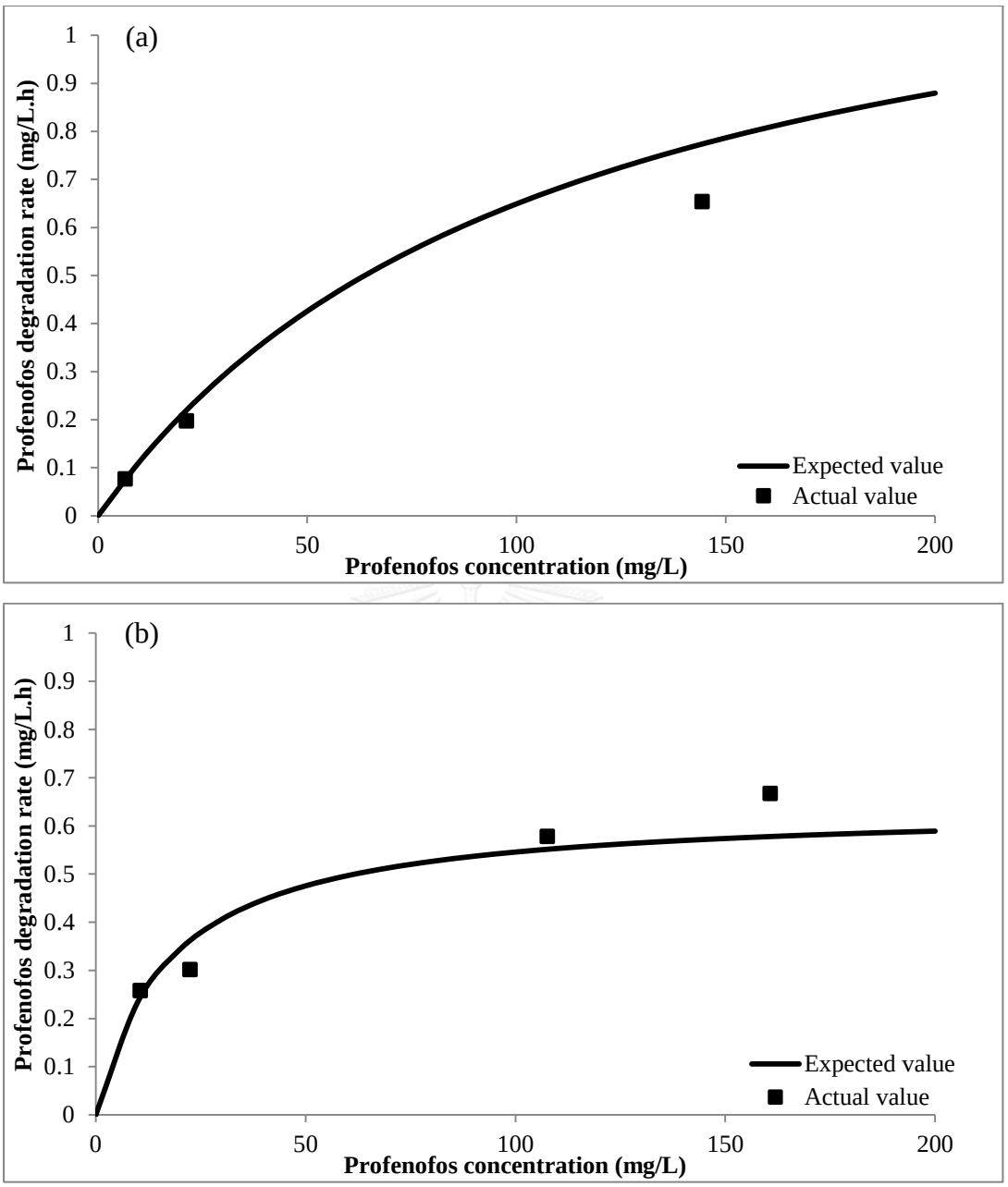


Figure 5.8 Profenofos degradation of PF1 followed Michaelis-Menten equation: a) Free cell and b) Immobilized cell

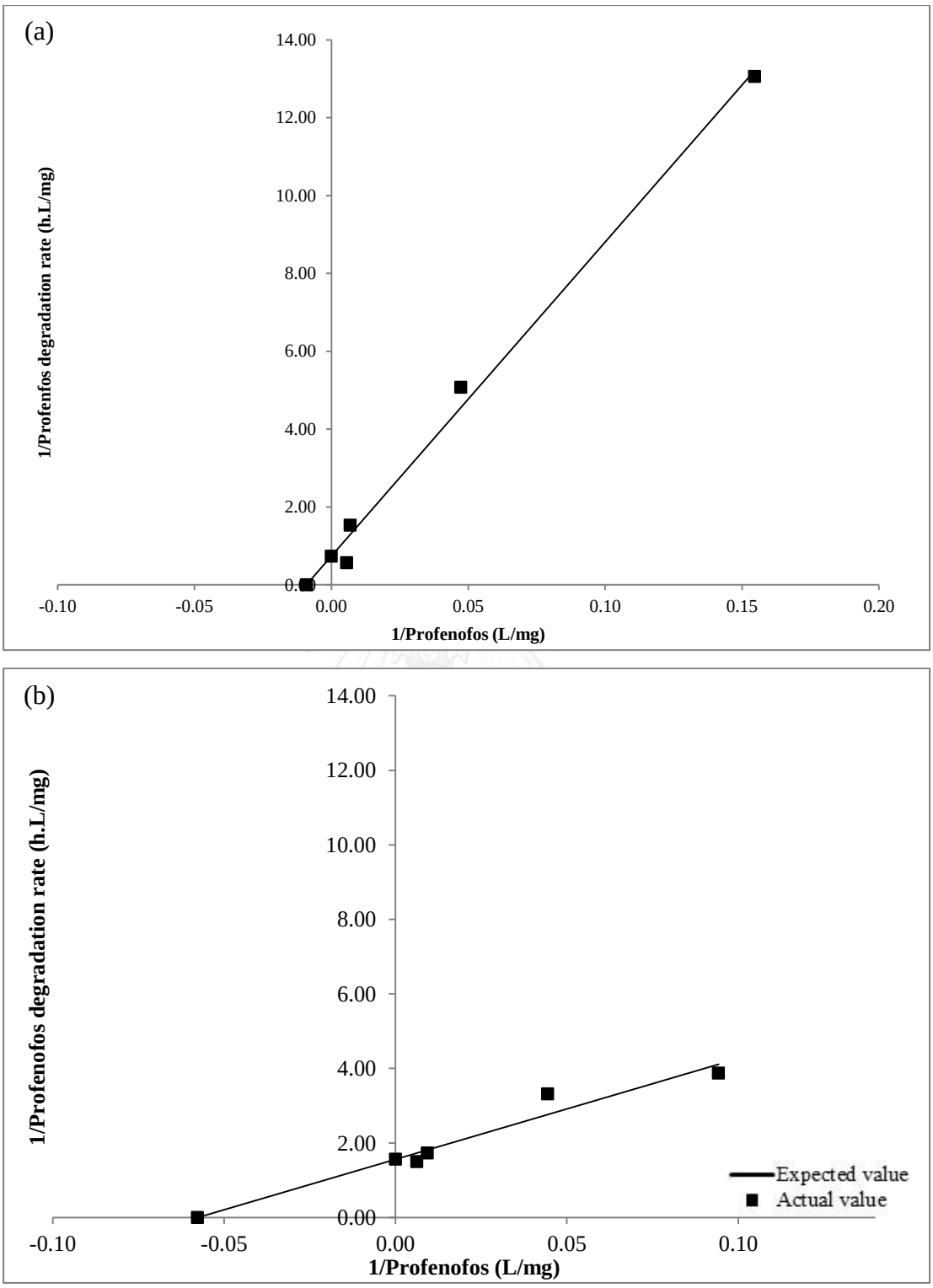


Figure 5.9 Profenofos degradation of PF1 followed Lineweaver-Burk equation: a) Free cell and b) Immobilized cell

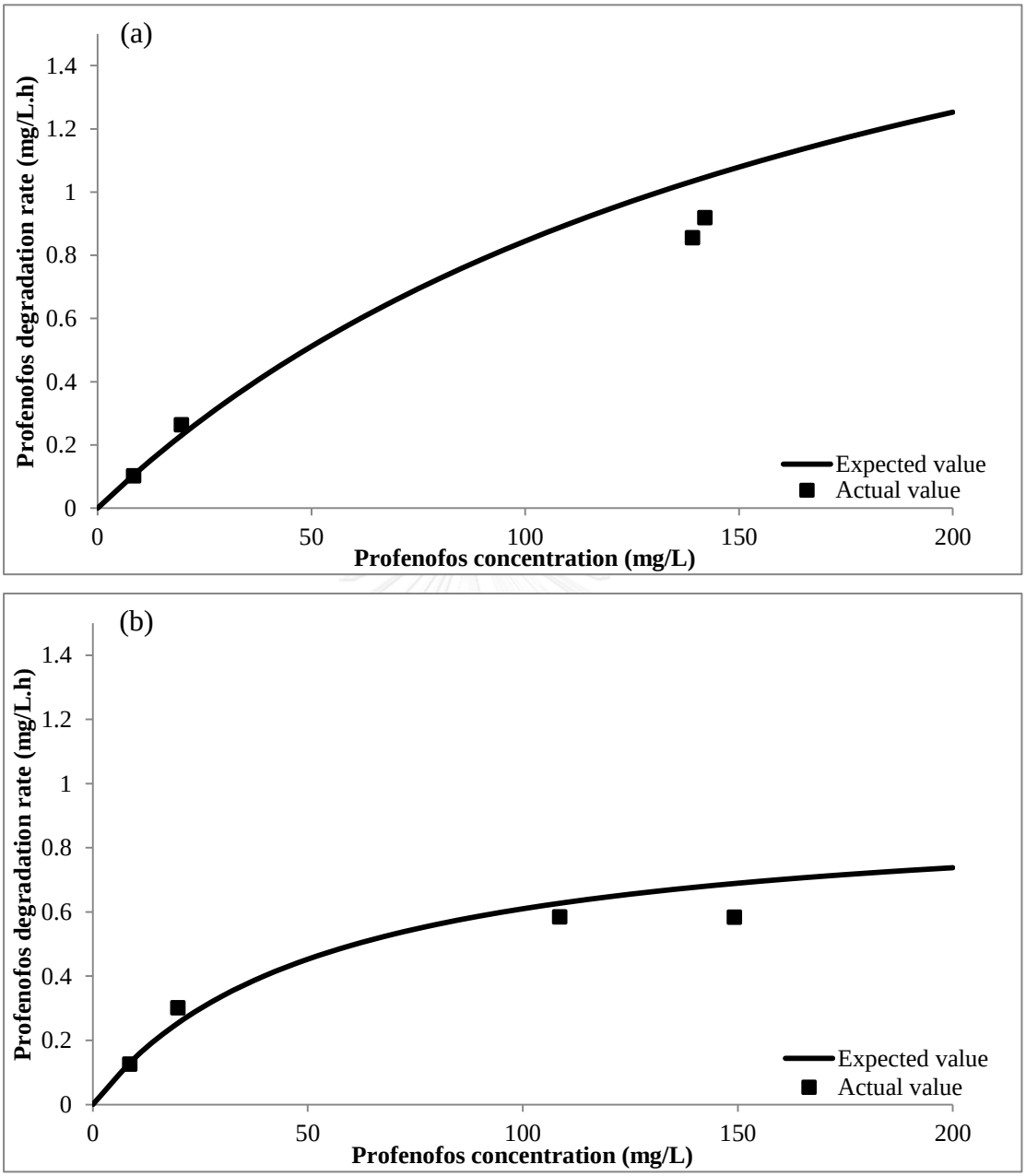


Figure 5.10 Profenofos degradation of GFJ2 followed Michaelis-Menten equation: a) Free cell and b) Immobilized cell

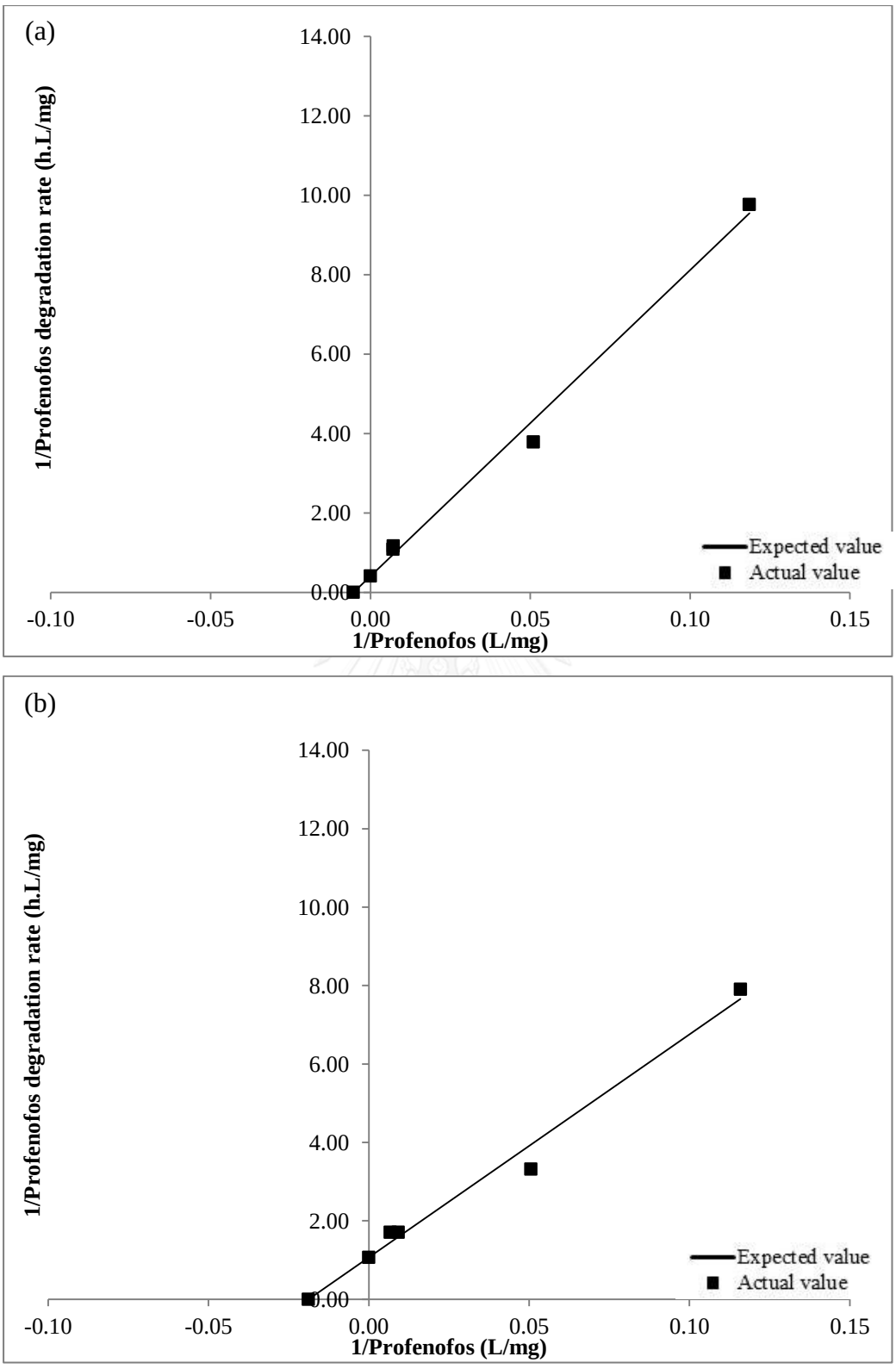


Figure 5.11 Profenofos degradation of GFJ2 followed Lineweaver-Burk equation: a) Free cell and b) Immobilized cell

5.3.1.3 Influence of inorganic salt types and concentrations on profenofos biodegradation

Inorganic salts as NaCl, $MgSO_4$, and $CaCO_3$ in range of 0 to 3,000 mg/L are mostly found in groundwater and surface water on agricultural area (Chandrasekar et al., 2014; Chung, Venkatramanan, Kim, Kim, & Ramkumar, 2015; Singaraja et al., 2015). The existence of these inorganic salts might influence on profenofos degradation. In this study, types (NaCl, $MgSO_4$, and $CaCO_3$) and concentrations (0, 100, and 1,000 mg/L) of inorganic salt were varied. The result can be seen from Table 5.3 and 5.4.

The biodegradation performance of the free PF1 under presence of low salt concentration (100 mg/L) was slight better (18-28%) than the test without salt. It indicated that the adaptation of the cell occurred under the low concentration of inorganics (Ebrahimi et al., 2015). For the tests with salts at 1,000 mg/L, the biodegradation performance was depended on salt types. The tests under presence of high $MgSO_4$ and $CaCO_3$ still achieved good biodegradation performance. This could be because PF1 was used to the environment with these salts. The culture originated from north-eastern area of Thailand which was normally reported about high hardness in groundwater.

From Table 5.3, the different types of inorganic salt influenced on the biodegradation of free GFJ2 in similar manner. Profenofos removal from the tests with salts (both low and high concentrations) was lower (up to 19%). The result showed different trend of influence by salts for PF1 and GFJ2. This may be because GFJ2 which was isolated from soil in central of Thailand. Most location in the area did not report about hardness and salt in groundwater. The culture may not be able or take longer time for adaptation led to lower biodegradation performance.

For the immobilized cell, trend of the biodegradation by PF1 and GFJ2 were similar. Under absence of salt, the immobilization material could accelerate biodegradation ability. It was found that the free PF1 and GFJ2 removed profenofos for 50 and 60% while profenofos of 88 and 71% was reduced by the immobilized PF1 and GFJ2, respectively. It could say that the immobilization material provided better environment (slow releasing toxic substance) leading to higher biodegradation ability. However, under presence of salt, the biodegradation of the immobilized cells was

much lower in all tests. This may be about the substrate diffusion. However, the clear mechanism of salt influence was inconclusive. The continued work on substrate diffusion in porous material under presence of salt should be performed.

Table 5.3 Profenofos biodegradation kinetic rates with inorganic salt types and concentration from the free cells

Strains	**Inorganic salts**	**Concentration of salt (mg/L)**	**Profenofos degradation kinetics**		**Profenofos utilization rate (mg/L/h)**	**Profenofos removal (%)**
			Equation	R^2		
PF1	No salt		y=-0.0159x+1.8448	0.97	0.08	49.90
	NaCl	100	y=-0.0171x+2.5067	0.95	0.15	61.10
		1,000	y=-0.0072x+2.2378	0.98	0.06	36.53
	$MgSO_4$	100	y=-0.0143x+2.5251	0.90	0.12	68.92
		1,000	y=-0.0163x+2.1522	0.87	0.10	58.58
	$CaCO_3$	100	y=-0.0139x+2.2760	0.93	0.10	58.15
		1,000	y=-0.0146x+2.3303	0.91	0.11	59.43
GFJ2	No salt		y=-0.0202x+1.9095	0.81	0.10	60.36
	NaCl	100	y=-0.0138x+1.8403	0.93	0.07	41.96
		1,000	y=-0.0136x+1.8269	0.85	0.07	46.03
	$MgSO_4$	100	y=-0.0143x+1.9548	0.99	0.08	45.12
		1,000	y=-0.0125x+1.9036	0.94	0.07	56.78
	$CaCO_3$	100	y=-0.0136x+1.9608	0.92	0.08	51.57
		1,000	y=-0.0184x+2.2143	0.97	0.11	62.59

x = time (h) and y = ln (profenofos concentration)

Table 5.4 Profenofos biodegradation kinetic rates with inorganic salt types and concentration from the immobilized cells

Strains	Inorganic salts	Concentration of salt (mg/L)	Profenofos degradation kinetics		Profenofos utilization rate (mg/L/h)	Profenofos removal (%)
			Equation	R^2		
PF1	No salt		y=-0.0446x+2.0186	0.76	0.26	87.93
	NaCl	100	y=-0.0091x+1.8228	0.97	0.04	52.15
		1,000	y=-0.0136x+2.0100	0.92	0.06	68.21
	$MgSO_4$	100	y=-0.0247x+2.3224	0.97	0.15	73.61
		1,000	y=-0.0229x+2.0624	0.84	0.12	54.44
	$CaCO_3$	100	y=-0.0153x+2.0137	0.93	0.07	69.82
		1,000	y=-0.0156x+1.9947	0.85	0.07	74.51
GFJ2	No salt		y=-0.0271x+1.776	0.69	0.13	71.00
	NaCl	100	y=-0.0161x+1.9128	0.95	0.08	60.94
		1,000	y=-0.0113x+1.9303	0.95	0.06	57.76
	$MgSO_4$	100	y=-0.0237x+1.8656	0.90	0.12	63.59
		1,000	y=-0.0112x+1.7775	0.75	0.06	52.34
	$CaCO_3$	100	y=-0.0100x+1.8515	0.96	0.05	53.00
		1,000	y=-0.0099x+1.8188	0.87	0.05	50.06

x = time (h) and y = ln (profenofos concentration)

5.3.2 Motility assay

5.3.2.1 Immobilized cell leaching

Cell leaching in TSM (bacterial medium) at the different sizes of the beads were conducted to investigate cell retention by the immobilization material (Figure 5.12 and 5.13). At the beginning, PF1 and GFJ2 cells of 10^4 to 10^5 CFU/mL were detected in TSM. Later, PF1 and GFJ2 cell numbers (up to 10^{11} CFU/mL) increased along with the time (20-36 h). Finally, cell numbers in TSM dropped down to approximately 105-109 CFU/mL. Based on the result, it could say that the cell number detected at the beginning was the cells immobilized on the surface of the material. Calcium alginate was used for immobilized both PF1 and GFJ2 (109 CFU/mL); therefore, similar numbers of cells were found at 0 h (the beginning). By

subtracting the leached cell number, the bacterial cells of approximately 10^5 CFU/mL was left in the beads.

In later period (20-36 h), the increasing of leached cells was from the cell movement. The cell growth did not play a role in this study since the resting cell technique was used for cell preparation. The cell growth was also confirmed by detecting cell number of the free cell experiment along with one of the immobilized cells (data not shown). For final period, 96 h, profenofos was used up, so number of the cells decreased.

Comparison of PF1 and GFJ2 behavior, PF1 obviously moved out compared to GFJ2. This result was sensible. PF1 is Pseudomonas sp. which known as a motile bacterium and potentially had a chemoreceptor influencing on chemoattractant/repellant by using flagella or pili (Parales et al., 2015; Sampedro et al., 2015). On the other hands, GFJ2 is Acinobacter baylyi which is a non-motile bacterium. The movement of this species was governed only by pili (Harding et al., 2013; Jung & Park, 2015). The leaching result may imply that PF1 moved out to reach substrate (profenofos) via chemoattractant ability.

According to Figures 5.12 to 5.13, different sizes of the beads could retain number of cells differently. Number of leached cells from the 2-mm beads was higher than that of the 6-mm beads. The smaller beads retained the less cell number. It was noticed that the leached PF1cell numbers were not obviously different because PF1 well moved as discussed prevoisuly.

To confirm the cell leaching result as shown Figures 5.12 to 5.13, SEM observation for selected beads was performed. At the beginning, the inner layer had plenty of the cells as shown in Figure 5.14 whereas there was no cell presenting in the outer layer. After 48-h tests, the PF1 cells was found at the outer layer which confirmed that the PF1 cells had ability to move out while GFJ2 was not apparently found at the outer layer (Figures 5.15 and 5.16).

Initially, the immobilized cells were initiated to improve the retaining ability of the cells in the contaminated environment. Anyway, it frequently found that the cells in the immobilization beads got difficulty about substrate diffusion. In this study, it was proved that the immobilization material could retain some portion of the cells and no obstacle of substrate availability leading to higher profenofos degradation.

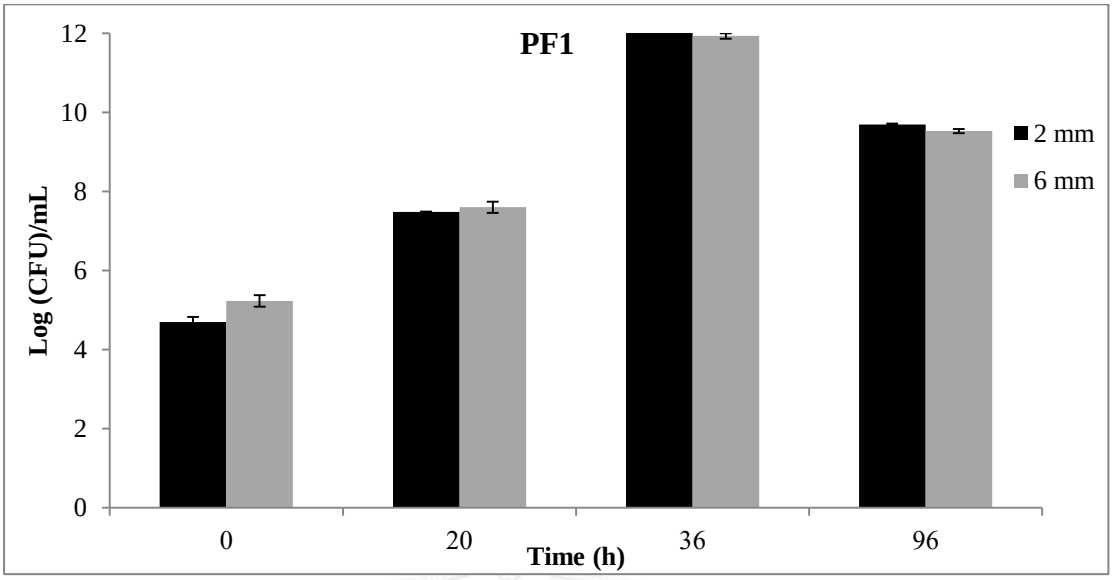


Figure 5.12 Leaching cells from the immobilized PF1 beads in TSM medium

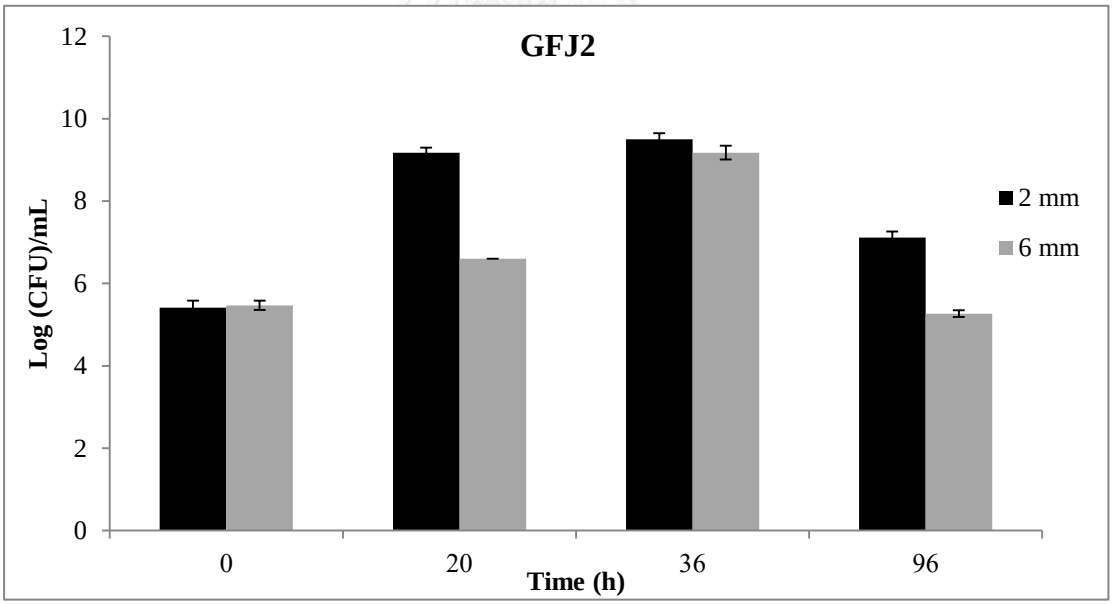


Figure 5.13 Leaching cells from the immobilized GFJ2 beads in TSM medium

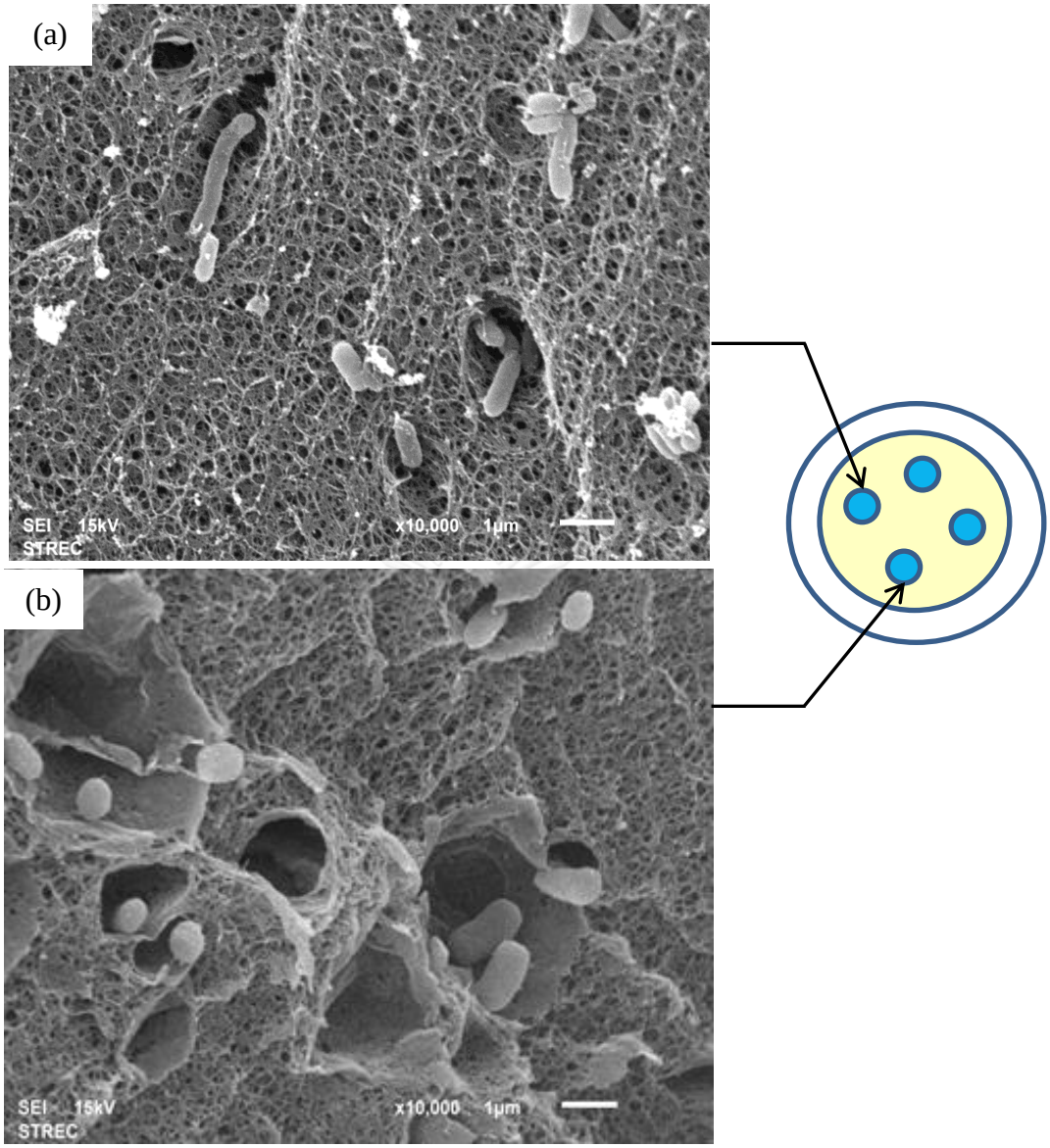


Figure 5.14 SEM observation of the immobilized cell at the inner layer: a) PF1 and b) GFJ2

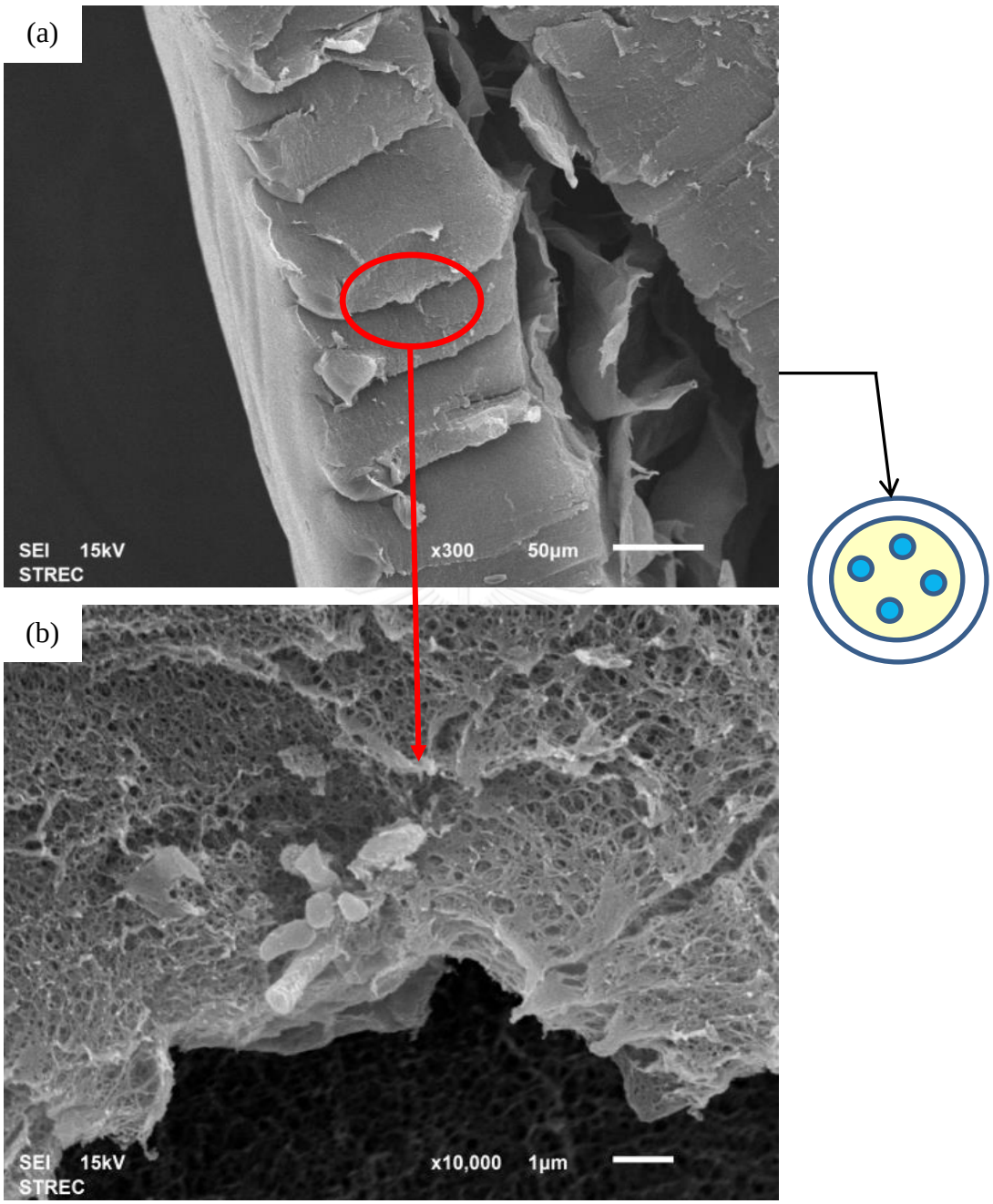


Figure 5.15 SEM observation on the outer layer of the beads of PF1 after treated with profenofos at 20 mg/L for 48 h: a) magnification at x300 and b) magnification at x10,000

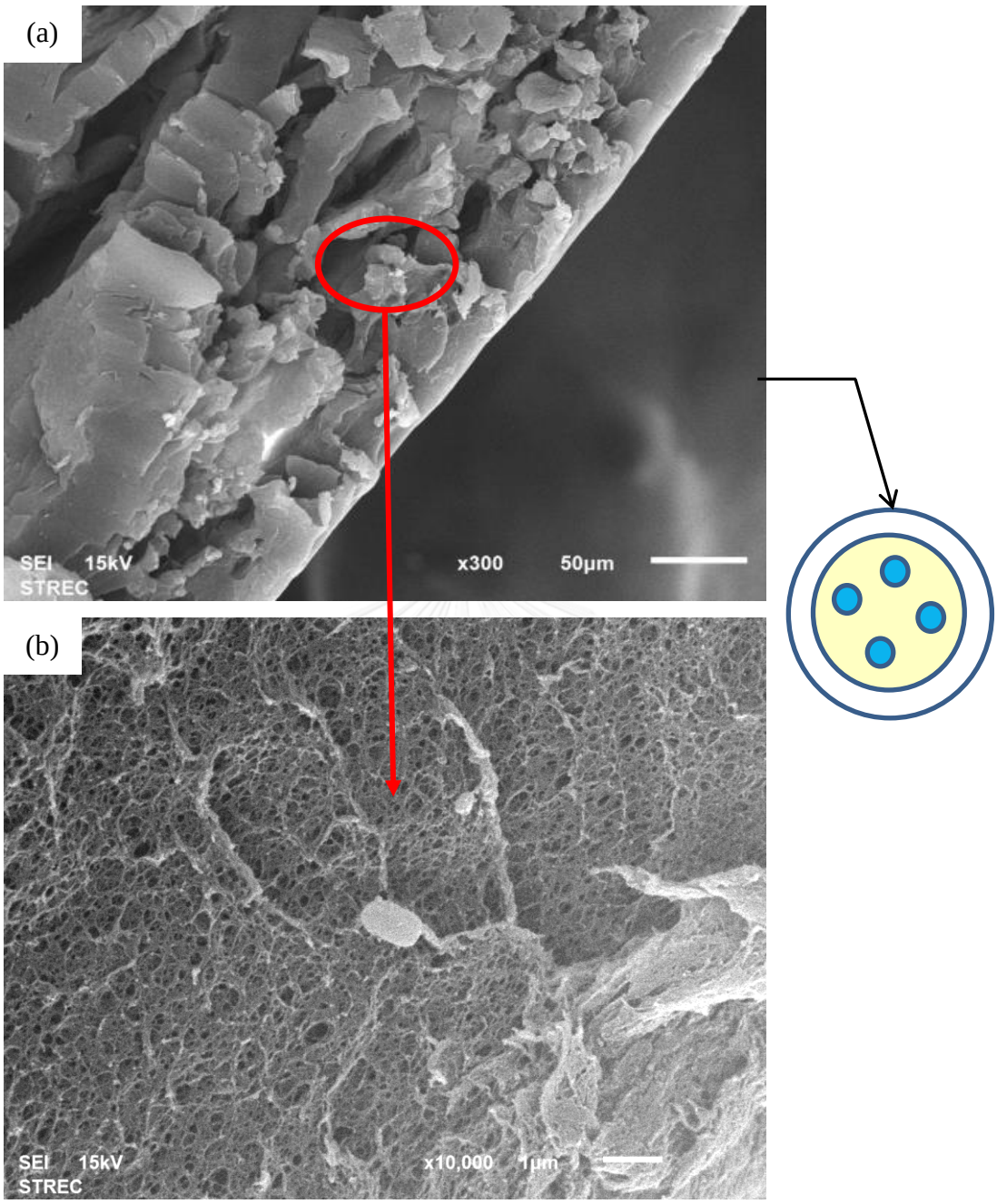


Figure 5.16 SEM observation on the outer layer of the beads of GFJ2 after treated with profenofos at 20 mg/L for 48 h: a) magnification at x300 and b) magnification at x10,000

5.3.2.2 Swimming, swarming, twitching assays

Previous section was proved that the PF1 and GFJ2 cells could move in the environment. In this section, the movement behavior was investigated based on swimming, swarming, twitching assays. The assays using rich medium (LB) and salt solution (NaCl) with and without profenofos (20 and 100 mg/L) was carried out. The images were shown in Tables 5.5 and 5.6

The movement of PF1 and GFJ2 in the rich medium (LB) was similar. The range of travelling zone from inoculum point of up to 8.5 cm was found (Tables 5.7 and 5.8). It is known that species in Acinetobacter genus was defined as non-motile bacteria. These species could move only by gliding or twitching on surface of medium agar using pili (Na et al., 2016). Hence, in case of GFJ2, it was confirmed that the movement found was function of pili but it was inconclusive for PF1.

The assays by PF1 and GFJ2 in the rich medium supplement with profenofos gave different results. In the rich medium with profenofos, PF1 well moved; higher profenofos concentration (100 mg/L) resulted in better movement. Conversely, GFJ2 got inhibition in the rich medium with high profenofos concentration (Tables 5.6 and 5.8). PF1 was isolated from profenofos-contaminated site; it may tolerate in environment with profenofos better than GFJ2. In addition, Pseudomonas species have been reported about polar flagellation which can lead to chemoattractant or chemorepellent in the environment (Kato et al., 2008; Shamim et al., 2014). In this case, PF1 may have chemoreceptors sensing for profenofos. The chemotactic assay was needed for clear explanation. For GFJ2, the movement was only depended on pili function which was more sensitive and required more suitable condition than PF1 (Harding et al., 2013; Na et al., 2016).

The movement of PF1 and GFJ2 in 0.5% NaCl agar was noticeably less than that in LB agar. The assays in 0.5% NaCl agar with high profenofos concentration had better cell movement (0.8 to 7.0 cm) (Tables 5.7 and 5.8). The result indicated that medium formulation obviously influenced cell movement. In 0.5% NaCl agar with profenofos, only profenofos was the main substrate; therefore, higher concentration of profenofos (substrate) led more movement. Likely, Kang and Park (2010) stated that the ability to swimming and swarming on the surface of semi-solid agar was reduced when substrate was limited (Kang & Park, 2010).

These motility assays were preliminarily showed ability of bacterial cells reaching to contaminant (profenofos). Comparing PF1 and GFJ2, free GFJ2 could reduce profenofos more than PF1 (Table 5.1). However, in practice, the use of immobilized cells sounds more applicable. Substrate accessibility became an important factor for removing contaminant. From the results of cell leaching and motility assays, it was found lower cell movement ability of GFJ2. Consequently, for site remediation application, PF1 may be more potential. GFJ2 likely to be suitable for wastewater treatment system with well mixing design since the system would not have problem on substrate accessibility. Anyway, influence of operational factors for each environmental treatment system (such as mixing speed and additional nutrient) is needed for further study before the real application.

Table 5.5 Motility assay of PF1 towards different profenofos concentrations

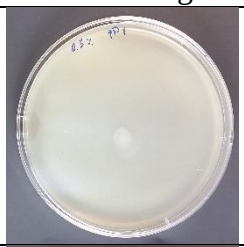
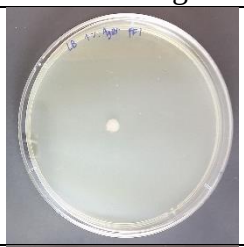
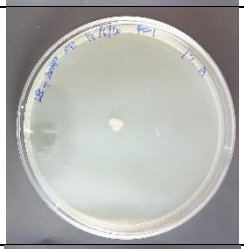
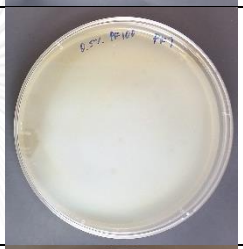
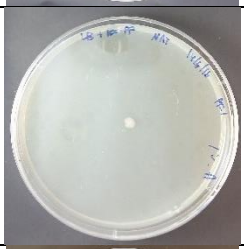
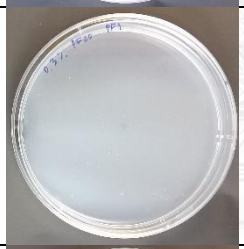
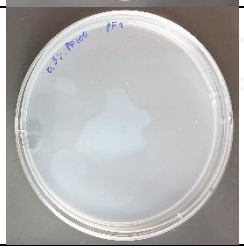
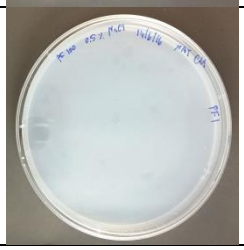
Type of plates	Swimming	Swarming	Twitching
LB			
LB + Profenofos 20 mg/L			
LB + Profenofos 100 mg/L			
0.5% NaCl + Profenofos 20 mg/L			
0.5% NaCl + Profenofos 100 mg/L			

Table 5.6 Motility assay of GFJ2 towards different profenofos concentrations

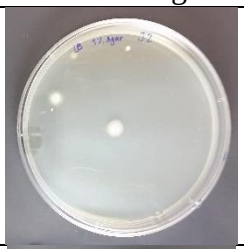
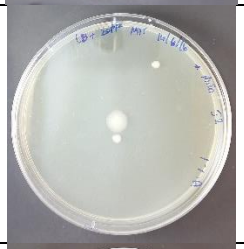
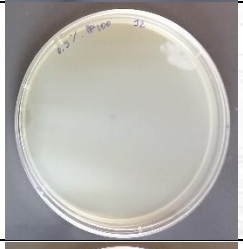
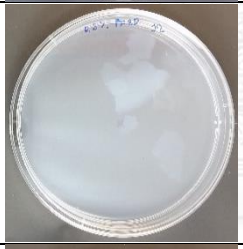
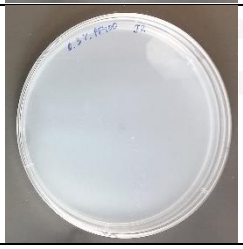
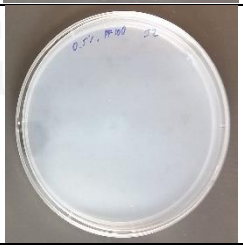
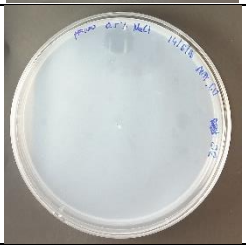
Type of plates	Swimming	Swarming	Twitching
LB			
LB + Profenofos 20 mg/L			
LB + Profenofos 100 mg/L			
0.5% NaCl + Profenofos 20 mg/L			
0.5% NaCl + Profenofos 100 mg/L			

Table 5.7 Motility assays of PF1 towards different profenofos concentrations

Type of plates	Zone of travelling from inoculum point (cm)		
	Swimming	Swarming	Twitching
LB	7.0	4.0	0.7
LB + Profenofos 20 mg/L	4.5	7.5	0.9
LB + Profenofos 100 mg/L	8.1	8.5	0.6
0.5% NaCl + Profenofos 20 mg/L	6.6	-	-
0.5% NaCl + Profenofos 100 mg/L	7.0	4.9	0.8

Table 5.8 Motility assays of GFJ2 towards different profenofos concentrations

Type of plates	Zone of travelling from inoculum point		
	Swimming	Swarming	Twitching
LB	8.5	6.0	1.2
LB + Profenofos 20 mg/L	8.5	8.5	1.5
LB + Profenofos 100 mg/L	-	-	0.9
0.5% NaCl + Profenofos 20 mg/L	-	-	-
0.5% NaCl + Profenofos 100 mg/L	3.0	1.0	0.8

5.3.2.3 Capillary chemotaxis assay

The chemotactic response of PF1 and GFJ2 were conducted in profenofos at the different concentrations. Yeast extract was used as the positive control. The experiment was studied according to Vangnai et al. (2013). The results in Figure 5.17 was the chemotactic response of PF1 and GFJ2 to profenofos at the different concentrations at 90 s and Figure 5.18 was the response along with the time.

From the results, chemotactic responses of PF1 and GFJ2 were totally different. GFJ2 was Acinetobacter sp. known as a non-motile bacterium (Jung and Park, 2015). Then, there was not any ability to attract or repel substrate even with positive control (Figure 5.17). The normalized cell numbers in every experiments of GFJ2 were approximately 1 (1 presented no movement). This indicated that GFJ2 had no ability for getting attractant or repellent by profenofos. For PF1, chemoattractant was obviously seen. The concentrations of profenofos induced chemotactic ability of PF1. The ratio of normalized cell number at concentration of 100 mg/L was approximately 2-times higher than that of the assay with 20 mg/L and positive control. This implied that the concentration gradient could sense to chemoreceptor protein in the PF1 cell (Shamim, Rehman, & Qazi, 2014).

According to Kato et al. (2008), there were many complex chemosensory systems containing with chemotaxis genes that (controlling by Che proteins) and chemoreceptors genes (controlling by methyl-accepting chemotaxis proteins, MCP) in Pseudomonas aeruginosa strain (Kato, Kim, Takiguchi, Kuroda, & Ohtake, 2008). The previous work used Pseudomonas aeruginosa PAO1 as a model bacterium. MCPs, CheA, CheW, and CheY genes are common functional genes for chemotactic in prokaryotes (Wadhams & Armitage, 2004; Wuichet & Zhulin, 2010). MCPs are membrane bound proteins which responsible for detection of extracellular substance. It also connects with a scaffold protein (CheW) and transduces signal to CheA. The signaled CheA is transformed to phosphorylated CheA (CheA-P) and transferred to CheY. This reacts to a regulator of CheY and forms activated CheY-P that related with the flagella motor switch protein for governing the direction of motor rotation. Until now, there was less insight information in other genomes except PAO1. It also found that P. putida F1, P. putida G7, P. fluorescens KU-7, P. stutzeri KC were able to attract by 4-hydroxybenzoate (PcaY), naphthalene (NahY), 2-nitrobenzoate (NbaY)

and carbon tetrachloride, respectively (Adadevoh, Triolo, Ramsburg, & Ford, 2016; Iwaki et al., 2007; Luu et al., 2015; Parales et al., 2015; Witt et al., 1999). Since PF1 belonged to Pseudomonas genus; its chemoattractant phenomenon may govern by Che and MCP proteins. The continued work on chemoattractant mechanism related to protein functions should be performed for insight information. This information will be used as fundamental knowledge on gene engineering to improve microorganism performance.

For GFJ2, the culture was no chemotactic response but it had a positive activity on other motility assays. There were many types of movement relating with motility of microorganisms by type-IV pili (Nait Chabane et al., 2014; Wadhams & Armitage, 2004). The microbial cells can attach and retract to surface leading to move forward of cell. The motility mechanism of GFJ2 is required.

The chemotaxis assay confirmed movement ability of PF1 and GFJ2. PF1 could be move and get attracted by contaminant (profenofos). As stated previously, this ability is a key factor to achieve on bioremediation. Two profenofos-degrading cultures studied were appropriate to different environmental treatment situations. The motility and chemotaxis assays provided in-depth understanding which will be useful for future application.

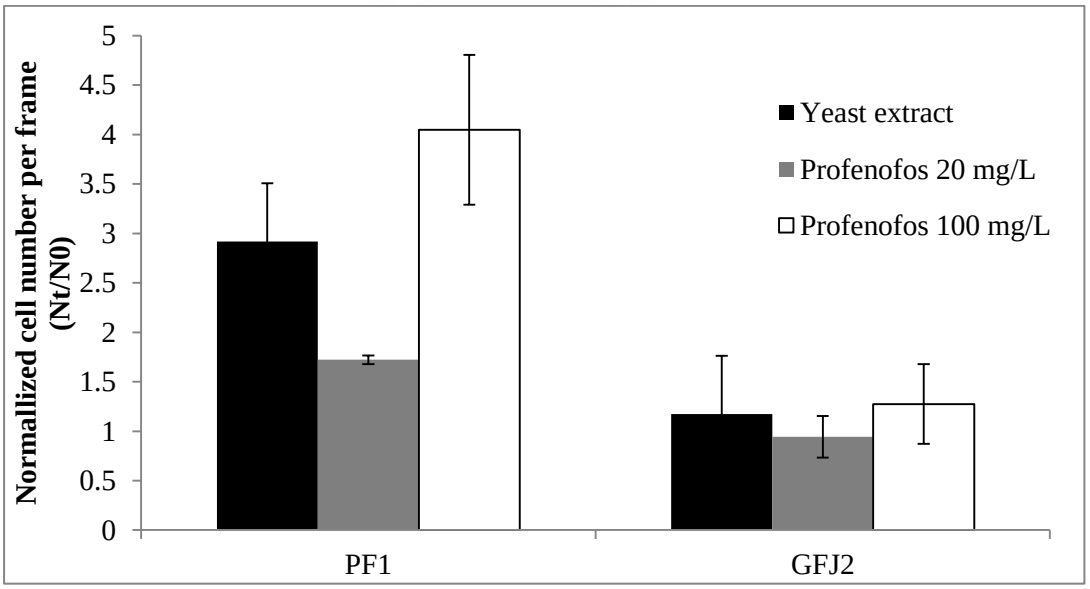


Figure 5.17 Chemotactic response of PF1 and GFJ2 to profenofos at the different concentrations comparing with yeast extract as positive control at 90-s time period

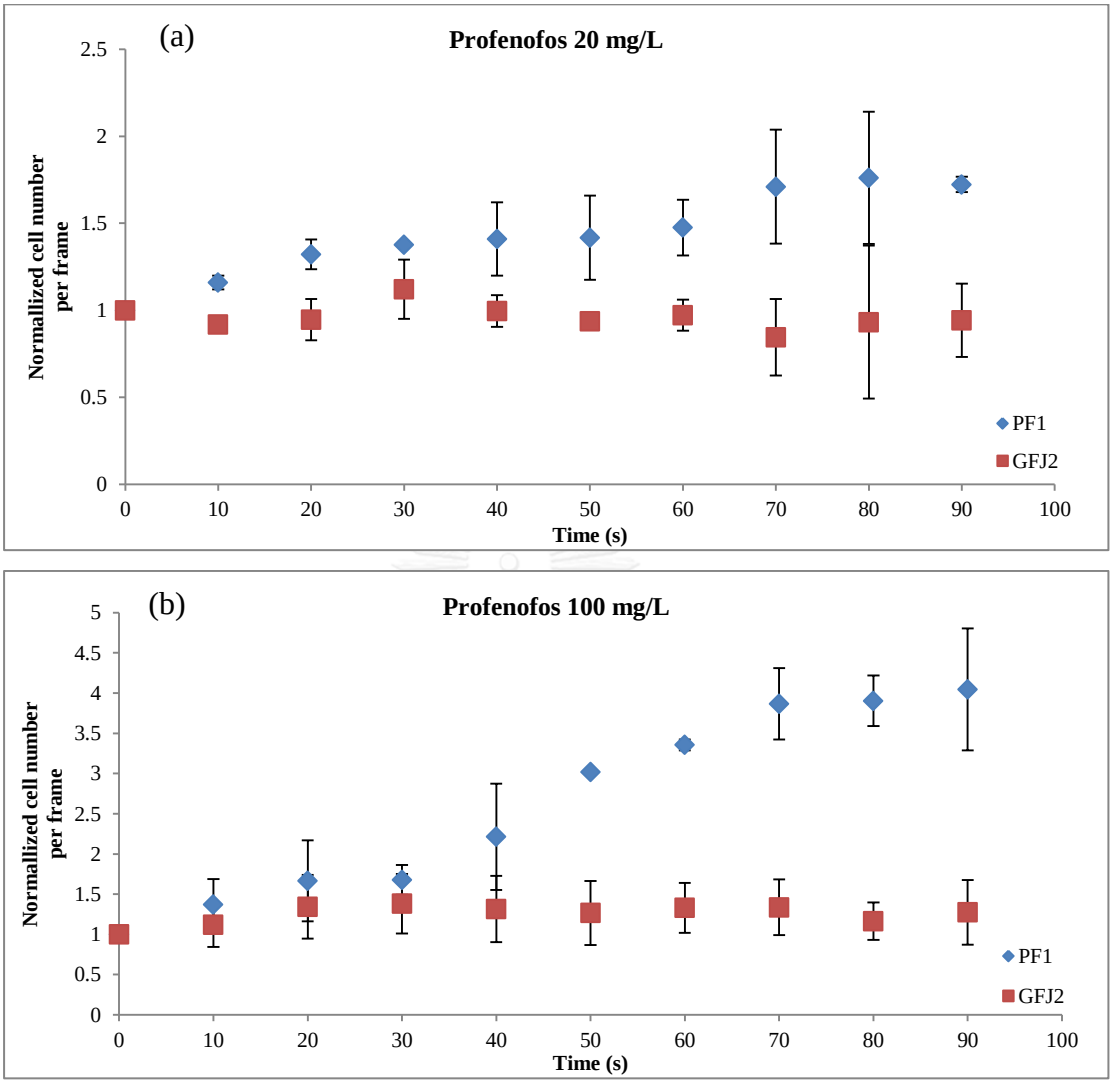


Figure 5.18 Chemotactic response of PF1 and GFJ2 to profenofos at the different concentrations along with the time for the tests at: a) 20 mg/L and b) 100 mg/L

5.4 Summary

The free and immobilized PF1 and GFJ2 well removed profenofos (50-88%). The degradation kinetics followed the first order kinetic reaction with the rates of 0.0017-0.0516 1/h. The biodegradation by the immobilized PF1 (50-90%) was better than one by the free cells (35-50%) but the profenofos removal efficiencies by the free and immobilized GFJ2 were similar. The bead sizes, initial profenofos concentrations, and salt co-contamination influenced profenofos removal performance. The 4-mm immobilized bead was the suitable size for profenofos biodegradation. Based on kinetic coefficients, the immobilization technique was more suitable for PF1 than GFJ2 and supported PF1 to tolerate to higher concentration of profenofos.

Even though the free GFJ2 could reduce profenofos more than PF1 in batch study, the use of immobilized cells sounds more applicable in bioremediation practice. There were not many profenofos-degrading isolates. These two cultures, PF1 and GFJ2, were chosen as models for motile and non-motile bacteria because the cultures well degraded profenofos. Also, movement ability of the strains was preliminarily tested. Substrate accessibility influenced on performance of contaminant removal. The cell movement assays supported the biodegradation phenomenon found in this study. The assays proved that the immobilized PF1 obviously moved out of the immobilization material since PF1 was attracted by profenofos while GFJ2 with no chemotactic response still stayed in the material. Therefore, for site remediation application, PF1 may be more potential. Influence of operational factors is needed for further study before the real application. Also, the continued work on chemoattractant mechanism of PF1 should be performed for improving microorganism performance later on.

CHAPTER 6
Profenofos Pesticide Removal by Immobilized Cells in Sand Column Experiment: Comparative Study of Motile and Non-motile Cells

6.1 Introduction

Profenofos is one of the most extensively used organophosphorus insecticides. It was applied to control pests for agricultural purpose. The extensive use resulted in contamination of profenofos into environment and agricultural products. It was found profenofos contamination of up to 1 mg/L in water and could distribute to other environmental media and pass along the food chain to affect human and animals in the long run (Harnpicharnchai et al., 2013).

One of the effective processes for site remediation was microbial degradation. The technique was to use microbial cells to remove contaminants *via* metabolism or co-metabolism processes. Several bacteria including strains in *Pseudomonas*, *Bacillus*, *Sphingomonas*, *Flavobacterium*, and *Acinetobacter* genara were reported degradability of organophosphorus and organochlorine pesticides (Akbar, Sultan, & Kertesz, 2015; Chanika et al., 2011; Deng et al., 2015; Karpouzas & Singh, 2006; R. Li et al., 2009; Munir et al., 2016). For profenofos degradation, *Pseudomonas plecoglossicida* strain PF1 (PF1) and *Acinetobacter baylyi* strain GFJ2 (GFJ2) were potential strains. Previously, it was published profenofos degradation by PF1 (Siripattanakul-Ratpukdi et al., 2014) while effective profenofos degradation by GFJ2 was preliminarily tested. Although microbial degradation has been reported achievement of site remediation, limitations of the technique including toxicity of contaminant, environmental stresses, and cell leaching from the contaminated site were stated (Ahmad et al., 2012; Tallur et al., 2015; Zhang, Xu, Liu, & Zhao, 2014). Microbial cell immobilization was initiated to deal with these problems. The immobilization techniques were successfully applied for pesticide biodegradations (Pradeep & Subbaiah, 2015; Juliana M. Saez, Aparicio, Amoroso, & Benimeli, 2015; Yáñez-Ocampo, Sánchez-Salinas, & Ortiz-Hernández, 2011).

According to Parales and Harwood (2002), one important factor influencing environmental biodegradation was chemotactic ability. Chemotaxis is the ability of motile bacteria to response chemicals. The response could be attractant or repellant leading to improve or decrease the biodegradation, respectively (Pandey et al., 2012). For bioremediation, it was claimed that bacteria with the chemotactic ability were better remove the contaminants since it was able to move to substrate. Thus far, no study related to bioremediation by the immobilized cells was published. Therefore, it was no answer whether this chemotactic ability play role on contaminant removal.

To elucidate this gap of knowledge, the comparative study of profenofos biodegradation by motile (PF1) and non-motile (GFJ2) immobilized cells were investigated. Cell immobilization by calcium alginate which is the most common method was selected. Column experiments with different profenofos concentrations were conducted to investigate the long term profenofos degradation performance. The free cell and only immobilization material tests were completed along with the tests with the immobilized cells. The experiment including the biodegradation tests, cell leaching monitoring, and chemotaxis and cell viability assays after utilization of the immobilized cells was accomplished. The information from this study will be useful for the bioremediation application by the immobilized cells in the future.

6.2 Materials and methods

6.2.1 Chemicals

Commercial profenofos (50% EC w/v) from a local distributer (Syngenta Crop Protection Co., Thailand) was applied. The other chemicals for bacterial medium and chemical analysis were supplied from Himedia (India), Ajax (Australia), and RCI Labscan (Australia). The standard profenofos was obtained from Dr. Ehrenstorfer GmbH (LGC Standards, UK). Alginic acid sodium salt (Sigma-Aldrich, Singapore) was applied for cell immobilization.

6.2.2 Microorganisms and cultural condition

Pseudomonas plecoglossicida strain PF1 (GenBank accession number KJ620776) was previously isolated from profenofos-contaminated chili farm soil

(Siripattanakul-Ratpukdi et al., 2014). The strain was subcultured in minimal salt medium (MSM) in every 4 days for 2 weeks before used. The formulation of MSM (pH of 7.0) was $Na_2HPO_4 \cdot 2H_2O$ 6.82 g, KH_2PO_4 3 g, NaCl 0.5 g, NH_4Cl 2 g, and $MgCl_2 \cdot 7H_2O$ 0.51 g in 1000 mL of phosphate buffer at pH 6.80 ($NaH_2PO_4 \cdot 2H_2O$ 0.83 g and $Na_2HPO_4 \cdot 2H_2O$ 1.7 g) (Siripattanakul-Ratpukdi et al., 2014). *Acinetobacter baylyi* strain GFJ2 cells (GenBank accession number HQ612277) from Luria-Bertani (LB) agar plate was inoculated into LB broth with shaking condition at 200 rpm and 30 °C for overnight before enrichment. MMSAY medium following Hongsawat and Vangnai (2011) was modified and used as the enrichment medium for GJF2 with adding 0.47 g/L of succinic acid, 0.13 g/L of ammonium sulfate and 1 g/L of yeast extract.

The filtered sterile profenofos (the concentration of 20 mg/L in bacterial medium) was applied as the main carbon source on the enrichment process for both strains. After enrichment, the strains were harvested by centrifugation (5,000 rpm, 30 min, and 4 °C). The pellets were washed and resuspended with 0.85% NaCl. The cells of approximately 1.5×10^{12} CFU/mL were used for the profenofos biodegradation assay by the free and immobilized cells.

6.2.3 Cell immobilization procedure

A 90-mL sterile sodium alginate solution (3%, w/v) was homogeneously mixed with the suspension of PF1 and GFJ (18 g-wet cells each). The cell-alginate mixture was dropped into a $CaCl_2$ solution of 3.5% (w/v) by a peristaltic pump (Masterflex L/S Tubing Pumps, Cole-Palmer Instrument Company, IL, USA) at flow rate of 60 mL/min (bead size of 4 mm). The beads were hardened in the $CaCl_2$ solution for 4 h with gentle agitation. The hardened beads were washed before used. The washing solution containing Tris-HCl (1.2 g/L, pH 7.0) (designated as TSM) was applied to avoid de-entrapment of the immobilized cells.

6.2.4 Column experiment

6.2.4.1 Synthetic infiltrate

Synthetic groundwater infiltrate comprised different types of salts as following: Tris-HCl 1.22 g/L, NaCl 0.5 g/L, NH_4Cl 2 g/L, and $MgSO_4 \cdot 7H_2O$ 0.51 g/L at pH 7.0 (designated as TSM medium). The medium was autoclaved before applying into in columns. Commercial profenofos in absolute ethanol (RCI Labscan, Australia) was added and mixed with TSM. The profenofos concentrations of 20 or 100 mg/L were spiked before adding the infiltrate into column.

6.2.4.2 Sand and column preparation

The sand-bed column was applied to simulate the contaminated soil. The sterile and heat-cleaned silica quartz sand was selected to eliminate influence of indigenous cultures on profenofos biodegradation and profenofos adsorption by organic and clay portions in soil. Industrial silica-quartz sand was applied in this study. The sand was washed with tap water for 3 h and dried at 105 °C for 2 d. The washed sand was burned at 550 °C, 15 min for two times to remove organic carbon. The sand was sterile by autoclaving at 121 °C, 15 min for three times.

A plastic column with inside diameter of 4.2 cm and working depth of 20.0 cm was applied. The column was rinsed with 70% ethanol and washed with autoclaved de-ionized water. The 400 g of sterile sand was filled in the column and defined as a control test. After filling the sand, pore volume of column was 110 mL. For the other tests, free (11-mL inoculum) and immobilized (55-mL beads) PF1 and GFJ2 were mixed with sterile sand and packed into the column. The cell numbers inside the free and immobilized cell columns were approximately 10^{15} CFU/mL. For the sand column operation, the sterile sand was saturated with the TSM medium. The synthetic infiltrate supplemented with profenofos was gravitational flow from the top of column with the infiltration rate of 5 cm/d. It is noted that this infiltration rate used in this study was applied based on rainfall information during 2012-2016 in Thailand. Figure 6.1 presents the column setup.

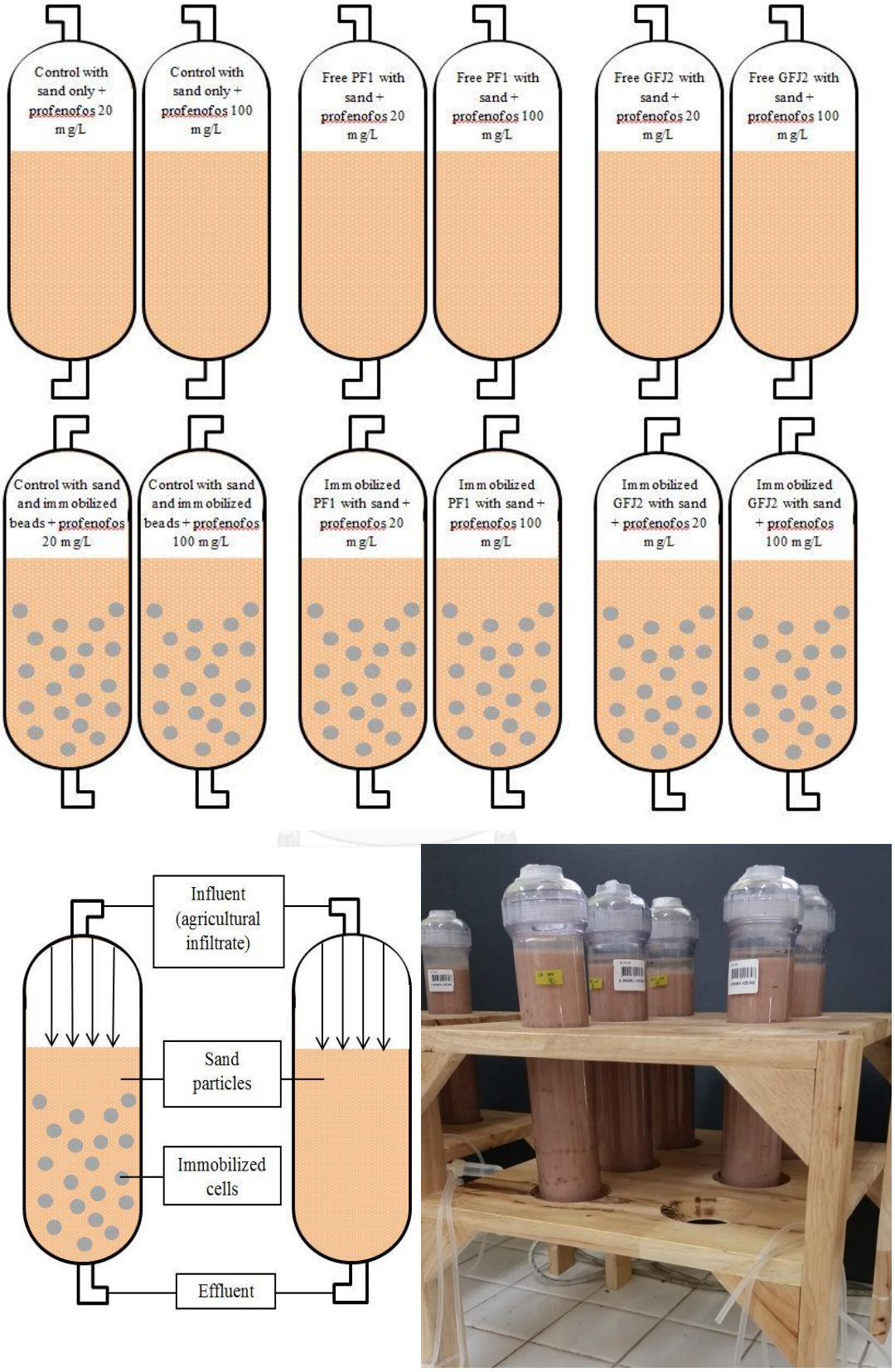


Figure 6.1 Profenofos biodegradation in column experiment in free and immobilized cells at different concentration of profenofos

6.2.4.3 Profenofos biodegradation and immobilized cell leaching in column experiment

The duplicate profenofos biodegradation experiments in sand columns were conducted. The two concentration of profenofos (20 and 100 mg/L) were tested. Profenofos removal efficiencies and leaching cell number were monitored for 15 pore volume (PV). The effluent was sampled and analyzed profenofos concentration for every 0.33PV while viable cell counting by spread plate count technique was done 1PV once.

6.2.5 Capillary chemotaxis assay

The capillary chemotaxis assay was modified from Vangnai et al. (2013). The 0-PV and 15-PV samples (before and after testing) from the middle of 100 mg/L in the column tests with free and immobilized PF1 and GFJ2 were selected. The free and immobilized cells were centrifuged at 10,000 rpm for 5 min. The cell pellet was washed with TSM medium for 2 times. The washed cells were re-suspended with 5 mL of TSM medium and kept for 4 h at 30 °C. For the immobilized cells, the bead was physically squeezed to obtain the de-entrapped cells without chemical used. The squeezed cells were directly explored for chemotaxis ability. The free and squeezed cells were transferred into 2.38 g/L of HEPES buffer (pH 7.0). The 20 µL of fresh cells was placed onto a coverslip. The amount of explored cells was also diluted with HEPES buffer into the same amount at the starting point of approximately 100 cells/frame. Profenofos concentrations of 100 mg/L (or yeast extract at 2% (w/v) as a positive control) at the mouth of the capillary tubes were solidified with 1% (w/v) agarose. The cell movement was recorded for 120 sec under an inverted microscope. The digital images were continuously captured. The bacterial cell number was counted for each image. The ratio of the cell number at 120-sec and starting time period was represented of the chemotactic response to the substrate.

6.2.6 Analytical methods

6.2.6.1 Profenofos analysis

Profenofos remaining was monitored by a gas chromatography (GC) with electron capture detector (Agilent 4890, Agilent, USA). The effluent from column was extracted using liquid/liquid extraction with hexane supplemented with 0.01% acetic acid (ratio 1:1). The mixture between effluent and extraction solution were vigorously mixed for 10 min and centrifuged at 10,000 rpm for 5 min. The organic phase was located in the upper phase and filtered by 0.22 µm filter nylon.

One µL of the filtered sample was injected into GC with a HP-5 column (30-m length, 0.25-mm i.d., and 0.25-µm film thickness). The GC condition was splitless, injection temperature of 240 °C, and helium gas flow of 1.5 mL min^{-1}. The GC temperature program was 10 min period. The temperature started at 180 °C and hold for 2.00 min, increased to 250 °C with the rate of 40 °C min^{-1} and hold for 6.25 min. Profenfos peak came out at 8.78 min.

6.2.6.2 Cell viability

The 0-PV and 15-PV samples (before and after testing) from the tests with free and immobilized PF1 and GFJ2 at 100 mg/L of the initial profenofos concentration were selected. The immobilized cells were extracted by squeezing and diluting in 0.85% NaCl. Both free and de-immobilized cells were washed with 0.85% NaCl for 2 times. The 0.5 mL-washed cells were aliquoted into 1.7 mL of microcentrifuge tubes. A staining kit (LIVE/DEAD®BacLight™ Bacterial Viability, Molecular Probes, Invitrogen) was applied for analysis of cell viability. The observation was conducted under confocal laser scanning microscopy (Fluo View FV10i, Olympus, Japan).

The differentiation between live and dead cells was determined by using the SYTO9 representing for all bacteria cells (green fluorescent dye) and propidium iodide (PI, red fluorescent dye) indicating only dead cells. The proportional between dyes and cells followed from manufacturer's protocol. The excitation wavelengths for SYTO9 and PI strains were 480 and 490 nm, respectively. The emission wavelengths for SYTO9 and PI strains were 500 and 653 nm, respectively. The least-five images

of live and dead cells were applied for area calculation by using ImageJ software (Giao et al., 2017).

6.2.6.3 Immobilized cell morphology

The 0-PV and 15-PV samples (before and after testing) from the tests with free and immobilized PF1 and GFJ2 at 100 mg/L of the initial profenofos concentration were selected. The 100 µL of the suspended cells in sand particles from column and a bead of the immobilized cells were dehydrated by oven at 100 °C for 24 h before observation on scanning electron microscope (SEM). The cells were attached to the stub and coated with gold. The cell surfaces were observed by SEM (S-3000N, Hitachi, Japan).

6.3 Results and discussion

6.3.1 Profenofos biodegradation and immobilized cell leaching in column experiment

6.3.1.1 Profenofos biodegradation in column experiment

Profenofos biodegradation in column experiments with two concentrations (20 and 100 mg/L) were conducted. The breakthrough curves were illustrated in Figure 6.2. For the control columns (Figure 6.1 (a)), the breakthrough of profenofos were shifted from about 5 PV for sand column only to 8 PV for sand with calcium alginate beads. This indicated that the column with the alginate beads adsorbed profenofos causing retardation of profenofos breakthrough (Gentry et al., 2010). It is notice that the role of profenofos adsorption by the alginate beads was not obvious in the tests with higher initial concentration (100 mg/L) (Figure 6.2 (b)).

According to Figures 6.1 (c)-(f), the breakthrough curves of profenofos biodegradation of the free and immobilized PF1 and GFJ2 were in the same trends at the different concentrations of profenofos. At the initial period, the breakthrough curves were delayed to reach plateau at 8-9 PV, this indicated that biodegradation was occurred. After that, the free and immobilized cells substantially degraded profenofos (60-90%) for 15 PV.

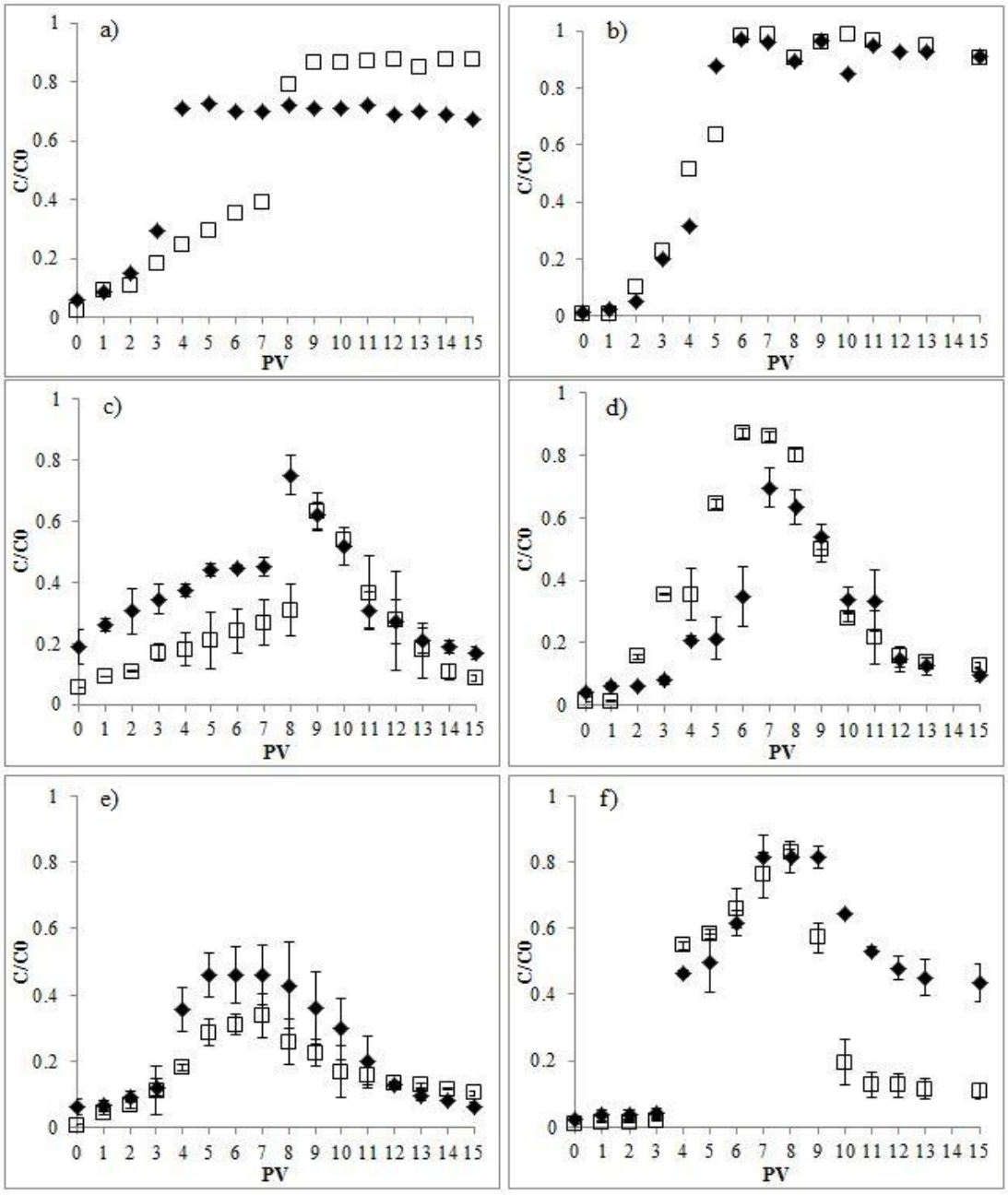


Figure 6.2 Breakthrough curves of profenofos biodegradation in column experiments at profenofos of 20 mg/L (a, c, and e) and profenofos of 100 mg/L (b, d, and f). Figures a and b were from the tests with only sand (◆) and calcium alginate beads (□). Figures c and d were from the tests with free (◆) and immobilized (□) PF1cells. Figures e and f were from the tests with free (◆) and immobilized (□) GFJ2 cells.

The free PF1, immobilized PF1, and immobilized GFJ2 successfully removed profenofos. This is because after a certain period, the microbial cultures well grew in the column resulting in maintain profenofos removal capacity. At 100 mg/L of profenofos, the profenofos biodegradation performance of the free GFJ2 was low comparing to the others. This result was confirmed by the previous result from batch experiment (5.3.1.2). High concentration of profenofos may be toxic and inhibition biodegradation activity by GFJ2.

According to Mertens et al. (2006), the immobilization technique was a slow-releasing inoculation process to support catabolic activity for bioaugmentation (Mertens, Boon, & Verstraete, 2006). The results from the immobilized cell tests were as expected while the tests with the free cells also well performed. This should be further explored.

6.3.1.2 Immobilized cell leaching in column experiment

The amounts of cell leaching from free and immobilized cells in PF1 and GFJ2 with the different concentrations of profenofos were conducted (Figure 6.3 (a)-(d)). Most of the tests showed similar trend (Figure 6.3 (a), (b), and (d)). At the first 3 PV, leaching cell numbers from the free cell columns were high while the leaching cell numbers was constant during later period. The leaching cell results were well correlated to the profenofos biodegradation performance. Since there was similar leaching cell number in later period, the biodegradation performances were high for either free or immobilized cell tests.

The leaching cells from the tests of GFJ2 at 20 mg/L attained the different result. The leaching cell numbers were higher than other tests. This indicated that the growth of GFJ2 occurred at low concentration of profenofos. This result well related to the result from batch experiment (5.3.1.2 and 5.3.2.2). From the previous parts, profenofos biodegradation of GFJ2 were reduced when increasing of the concentration to 100 mg/L. The motility behaviors of GFJ2 (swimming and swarming) were also inhibited even within rich medium (LB) containing profenofos at 100 mg/L. The immobilization technique was benefit for biodegradation in term of

toxic reduction leading to effective profenofos removal performance in all immobilized cell tests (Boon et al., 2002; Saez et al., 2012; Saez et al., 2015).

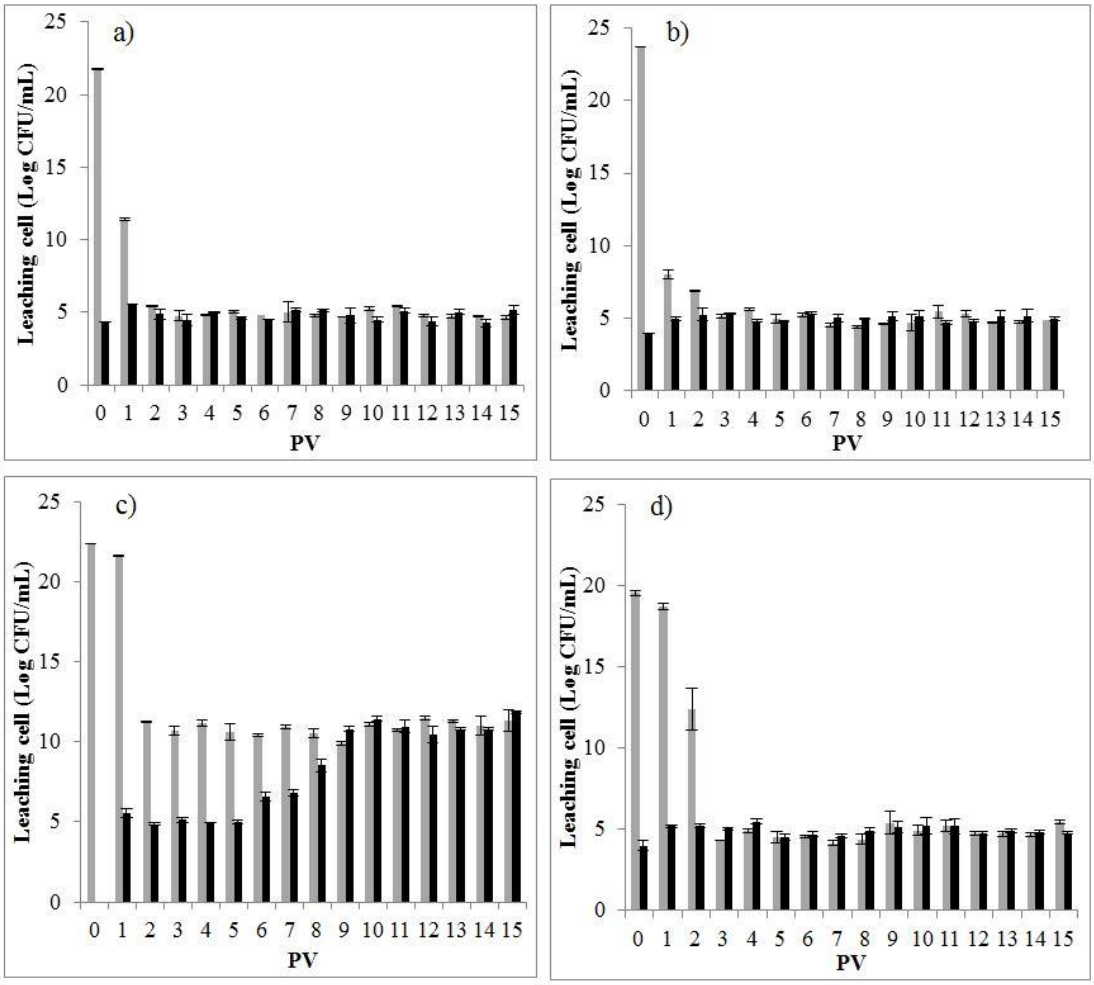


Figure 6.3 Leaching cells from free (light bar) and immobilized (dark bar) PF1 and GFJ2 in column: a) PF1 at 20 mg/L of profenofos, b) PF1 at 100 mg/L of profenofos, c) GFJ2 at 20 mg/L of profenofos, and d) GFJ2 at 100 mg/L

6.3.1.3 Immobilized cell morphology

The morphological observation of free and immobilized PF1 and GFJ2 were observed by SEM. At 0 PV of free cells, there was not any microbial cell colonization. This led to large numbers of the leaching cells in the medium at the first 3 PVs as presented previously. After that, the cell colonization on sand particles took place (Figure 6.4 (b)-(c)). This resulted in much lower leaching cells in all tests after 3

PV. Also, this should be the reason why the tests with the free cells well gave high profenofos biodegradation efficiencies.

The morphology of immobilized cells, after the long term usage (15PV), though the cells were well immobilized as can be seen from Figure 6.4 (d)-(e), the immobilized matrices of PF1 and GFJ2 were likely damage (Rathore, Desai, Liew, Chan, & Heng, 2013). This caused the leaching cell numbers similar to the free cell columns.

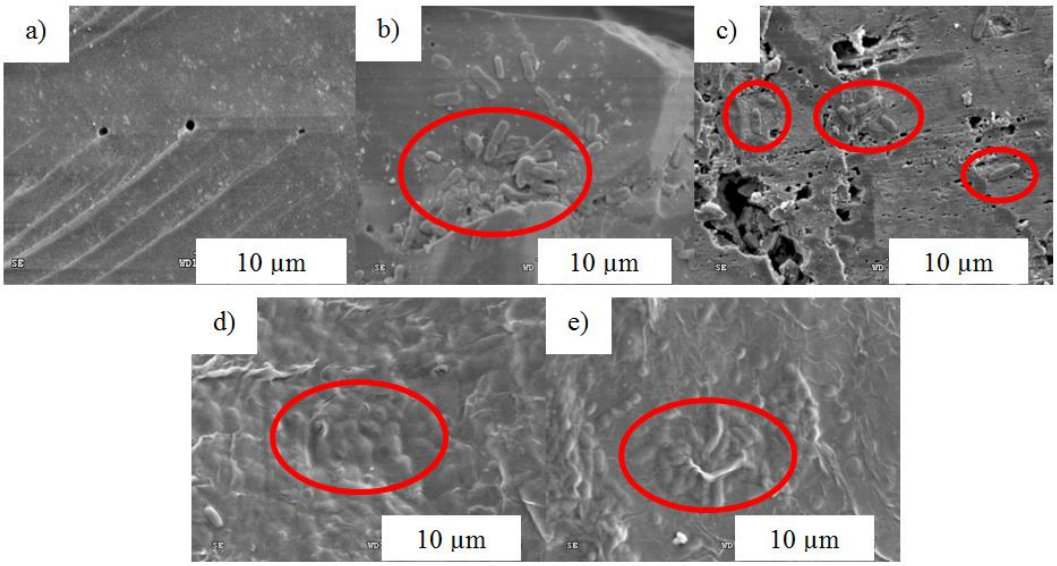


Figure 6.4 SEM observations from the surface of free and immobilized cells at 0 PV (magnification at 5,000×): a) sand particles at 0 PV, b) sand particles of PF1 at 15 PV, c) sand particles of GFJ2 at 15 PV, d) immobilized PF1 at 0 PV, e) immobilized PF1 at 0 PV

6.3.2 Capillary chemotaxis assay of free and de-immobilized cells

Capillary chemotaxis assay was conducted with the free and de-immobilized PF1 comparing to the de-immobilized GFJ2 after 15 PV. The suspended free and de-immobilized PF1 from the tests with the initial profenofos concentration of 100 mg/L were selected (Figure 6.5 (a)-(b)). According to the batch experiment, this concentration significantly impacted profenofos biodegradation performance. The change in motility ability could be clearly seen from the tests at this concentration.

GFJ2 is known as a non-motile bacterium. In this experiment, the de-immobilized GFJ2 (as a negative control) was not able to move after applying into the column for 15 PV as expected (normalized cell numbers were about 1 in all cases).

According to the test with the free PF1 (Figure 6.5 (a)), chemotactic response of PF1 obviously reduced after utilization in the tests with both yeast extract (rich medium) and profenofos of 100 mg/L for 15 PV. This indicated that the free PF1 was freely dispersed in the columns to reach substrate, therefore; chemotactic ability did not really play the main role under free dispersed condition. However, comparing to the non-motile bacterium, PF1 still showed slight chemotactic response. This result well correlated to the previous work by Witt et al. (1999). It was demonstrated that motile bacteria were more sensitive under saturated nutrient condition than non-motile ones. The motility characteristics related to higher cell migration throughout the porous medium in the column (Witt et al., 1999). This indicated that motile bacteria preferred to stay longer in the columns (Adadevoh et al., 2016; J. Liu, Ford, & Smith, 2011; Velasco-Casal, Wick, & Ortega-Calvo, 2008).

For the de-immobilized PF1, the cells were retained in the immobilization material. The cells did not directly contact to profenofos (Figure 6.3). Chemotactic responses of the de-immobilized PF1after testing for 15 PV were much higher than ones of the beginning period (0PV) (Figure 6.5). This indicated that chemotactic response did not well function after the preparation of immobilized cells (Hickey, Auty, Wilkinson, & Sheehan, 2015; Samuelsen, Badawi, Nybroe, Sørensen, & Aamand, 2016; R. Singh & Olson, 2008). However, after the cells were utilized in the confined and slow-releasing substrate condition, the chemotactic response obviously presented. This may be implied that the immobilized cells did not get sufficient substrate resulting in the chemotactic ability functioned. This also led to higher profenofos removal performance for the experiment with the immobilized cells as presented earlier.

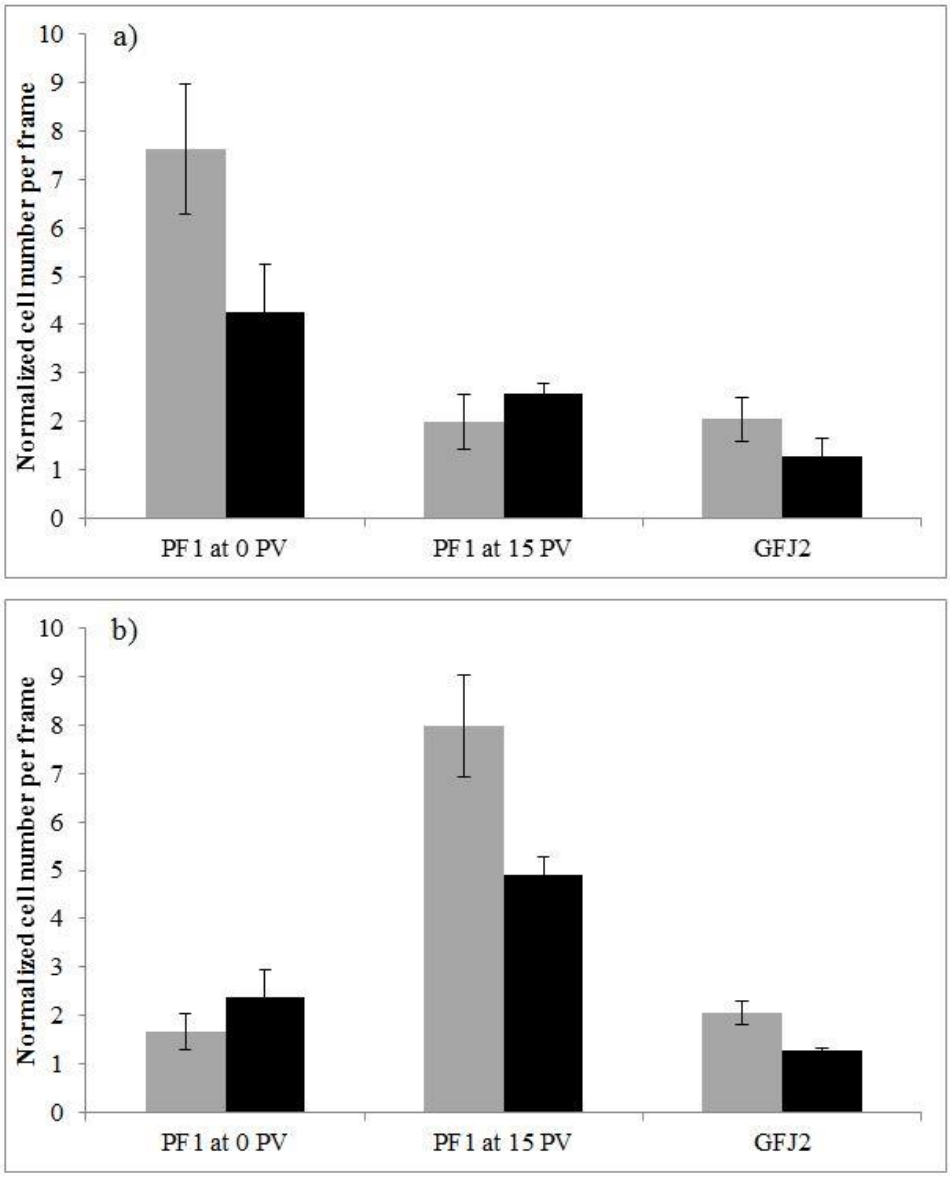


Figure 6.5 Chemotactic responses of PF1 and GFJ2 at the different substrates (yeast extract (light bar) and profenofos at 100 mg/L (dark bar)) : a) free cells and b) de-immobilized cells

6.3.3 Cell viability of free and de-immobilized cells

Cell viability of the free and de-immobilized PF1 and GFJ2 were performed. The percentage of the damaged cells based on membrane integrity at the beginning (0 PV) and after used for 15 PV from the tests at 100 mg/L of profenofos were presented in Figure 6.6. The amounts of the damaged free PF1 and GFJ2 after using for 15 PV were similar (50-60%). This indicated that profenofos could be injured to the cell membranes of the both strains. This viability result was not get along with profenofos removal presented earlier. The viability of two strains were similar while the profenofos removal by the free PF1 was higher than the free GFJ2. For the de-immobilized cells, the results of PF1 and GFJ2 were obviously different. The percentage of damaged de-immobilized PF1 was only 20% whereas the damaged GFJ2 was about 60%.

According to the results, it was only the proportion of live and dead cells. For better explanation, later live/dead study with continued cell number monitoring should be performed. This may not exactly described the reason of the different results. Further investigation on the function of profenofos removal and environmental influence by both strains should be performed.

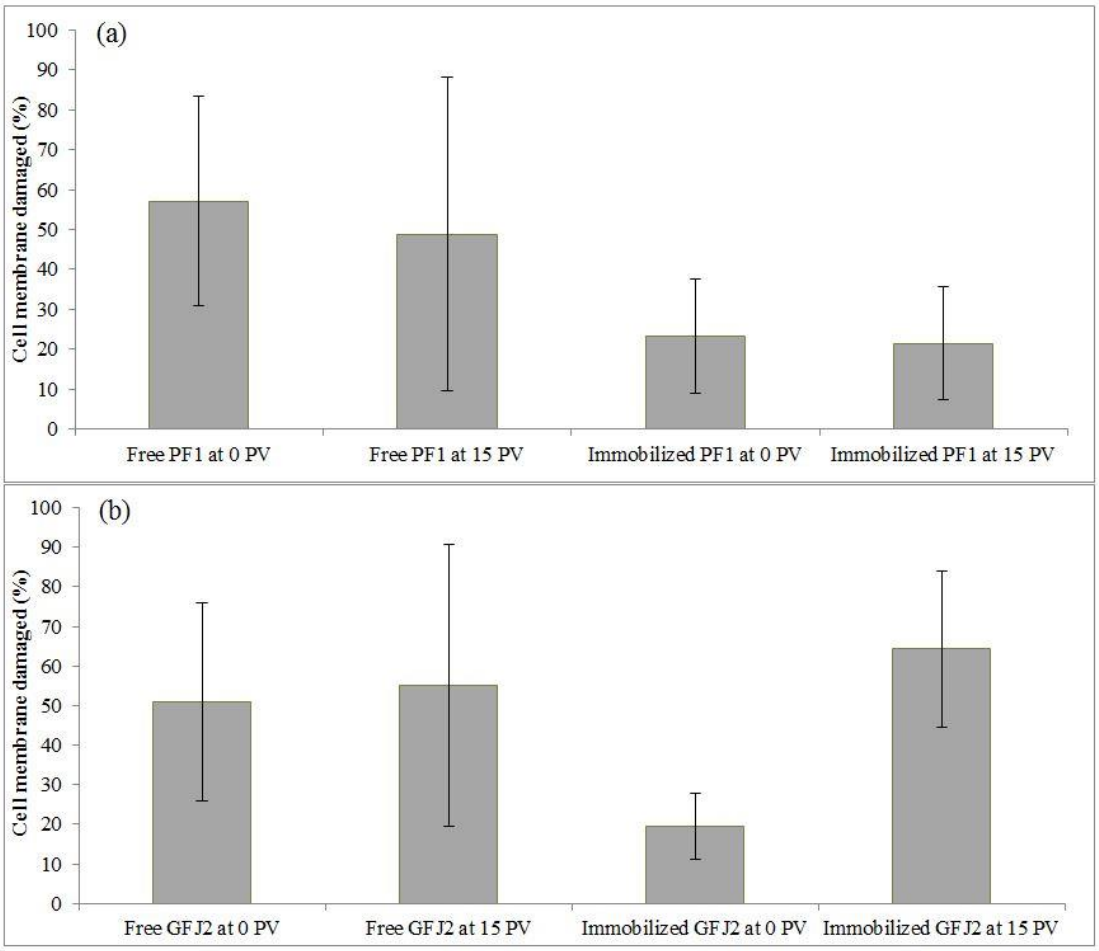


Figure 6.6 Percentage of damaged cells of free and de-immobilized PF1 (a) and GFJ2 (b) after testing for 15 PV

6.4 Summary

From the column experiment, the free and immobilized PF1 and GFJ2 were able to degrade profenofos at the high efficiencies (60-90%). At the low concentration of profenofos, both free and immobilized cell (both strains) well degraded profenofos while at higher concentration, the immobilized cells better treated profenofos. During the beginning of the test, large numbers of leaching free cells were found compared to the tests by the immobilized cells. After that, numbers of leaching free and immobilized cells were nearly the same because of natural cell colonization on sand occurring in the free cell tests. Based on the chemotactic result, it was found that the ability likely to play an important role for the profenofos biodegradation by the immobilized cells.

CHAPTER 7
Conclusions and Recommendations

7.1 Conclusions

Profenofos, a largely used pesticide, has been reported contamination in environment. This study investigated profenofos removal using the immobilized microbial cells. Cell movement ability related to biodegradation performance was emphasized. Two profenofos-degrading strains, *Pseudomonas plecoglossicida* strain PF1 (PF1) and *Acinetobacter baylyi* strain GFJ2 (GFJ2) which were motile and non-motile bacteria, respectively were characterized on the influence of environmental conditions (effects of profenofos concentration, pH, and temperature). In addition, the application of the immobilized cells in batch (effects of the immobilized cell sizes and inorganic salts) and column (effect of profenofos concentrations) tests were examined to compare the efficiency and behavior of the microorganisms with free cells. The results were concluded as followed.

1. PF1 and GFJ2 were the potential profenofos-degrading microorganism. The profenofos removal was 60-90%. The culture also well degraded BCP which was known as a toxic intermediate. The pH, temperature, and profenofos concentration parameters were significantly affected profenofos degradation kinetic rates. The optimum pH, temperature, and profenofos concentration were 5.30-7.87, 20-40 °C, and 20 mg/L, respectively. The ranges of the optimum parameters were mostly found in practice. However, there was no interrelationship between each parameter affecting the profenofos biodegradation kinetic rate.

2. Based on the batch experiment, the profenofos removal result showed that the immobilized PF1 (50-90%) degraded profenofos better than that of the free PF1 (35-50%) while the removal efficiencies by the free and immobilized GFJ2 were similar (60-69%). The bead size obviously affected the profenofos removal performance. The suitable size for profenofos biodegradation for both strains was 4 mm. The cell motility assay results well correlated the biodegradation experiment. PF1 had good motility in the plate and capillary (chemotactic ability) assays. PF1 got attraction by profenofos resulting in high removal efficiency. GFJ2 had a positive

activity on plate assays but it had no chemotactic response. The strains were able to work at the high inorganic salt conditions. There were not any apparent influences of inorganic salt types and concentrations on profenofos biodegradation by the free and immobilized PF1 and GFJ2.

3. Based on the column experiment, the free and immobilized PF1 and GFJ2 were able to degrade profenofos at the high efficiencies (60-90%). During the initial period, large numbers of free cells were leached out while in the later period, numbers of leaching free and immobilized cells were similar. The SEM observation confirmed natural cell colonization on sand in the free cell tests. Based on the chemotactic ability likely affect profenofos biodegradation, especially the treatment by the immobilized cells.

7.2 Recommendations

The key finding of this study will be benefit for the site remediation process by the immobilized cells. For short term remediation, a non-motile bacterium (GFJ2) also well degraded profenofos at the concentration of 5-50 mg/L while a profenofos-familiar motile bacterium (PF1) has chemoattractant ability supporting the profenofos biodegradation at higher concentration (100 mg/L). For the long term remediation, the immobilization technique is advantage for the both motile and non-motile bacteria.

Based on the biodegradation result, it is clear that during the tests with high profenofos concentrations, the immobilized cells worked better than the free cells because of higher cell retention. For comparison of motile and non-motile bacteria, profenofos removal ability was similar. Chemotactic ability did not obviously relate to the removal efficiency. In depth knowledge for profenofos biodegradation of the both strains are required including investigation of functional genes responsible for profenofos degradation, profenofos biodegradation and mineralization pathway, and mechanism of motile bacterium for adaptation with the immobilization technique.

REFERENCES

Adadevoh, J. S. T., Triolo, S., Ramsburg, C. A., & Ford, R. M. (2016). Chemotaxis Increases the Residence Time of Bacteria in Granular Media Containing Distributed Contaminant Sources. *Environmental Science and Technology, 50*(1), 181-187. doi:10.1021/acs.est.5b03956

Agudelo, R. M., Peñuela, G., Aguirre, N. J., Morató, J., & Jaramillo, M. L. (2010). Simultaneous removal of chlorpyrifos and dissolved organic carbon using horizontal sub-surface flow pilot wetlands. *Ecological Engineering, 36*(10), 1401-1408. doi:10.1016/j.ecoleng.2010.06.019

Ahmad, S. A., Shamaan, N. A., Arif, N. M., Koon, G. B., Shukor, M. Y. A., & Syed, M. A. (2012). Enhanced phenol degradation by immobilized Acinetobacter sp. strain AQ5NOL 1. *World Journal of Microbiology and Biotechnology, 28*(1), 347-352. doi:10.1007/s11274-011-0826-z

Akbar, S., Sultan, S., & Kertesz, M. (2015). Determination of Cypermethrin Degradation Potential of Soil Bacteria Along with Plant Growth-Promoting Characteristics. *Current Microbiology, 70*(1), 75-84. doi:10.1007/s00284-014-0684-7

Alonso, S., Rendueles, M., & Díaz, M. (2015). A novel approach to monitor stress-induced physiological responses in immobilized microorganisms. *Applied Microbiology and Biotechnology, 99*(8), 3573-3583. doi:10.1007/s00253-015-6517-1

Anwar, S., Liaquat, F., Khan, Q. M., Khalid, Z. M., & Iqbal, S. (2009). Biodegradation of chlorpyrifos and its hydrolysis product 3,5,6-trichloro-2-pyridinol by Bacillus pumilus strain C2A1. *Journal of Hazardous Materials, 168*(1), 400-405. doi:10.1016/j.jhazmat.2009.02.059

Bajet, C. M., Kumar, A., Calingacion, M. N., & Narvacan, T. C. (2012). Toxicological assessment of pesticides used in the Pagsanjan-Lumban catchment to selected non-target aquatic organisms in Laguna Lake, Philippines. *Agricultural Water Management, 106*, 42-49. doi:https://doi.org/10.1016/j.agwat.2012.01.009

Barrionuevo, M. R., & Vullo, D. L. (2012). Bacterial swimming, swarming and chemotactic response to heavy metal presence: Which could be the influence on wastewater biotreatment efficiency? *World Journal of Microbiology and Biotechnology, 28*(9), 2813-2825. doi:10.1007/s11274-012-1091-5

Basak, B., Bhunia, B., & Dey, A. (2014). Studies on the potential use of sugarcane bagasse as carrier matrix for immobilization of Candida tropicalis PHB5 for phenol biodegradation. *International Biodeterioration & Biodegradation, 93*, 107-117. doi:http://dx.doi.org/10.1016/j.ibiod.2014.05.012

Bastos, A. E. R., Cassidy, M. B., Trevors, J. T., Lee, H., & Rossi, A. (2001). Introduction of green fluorescent protein gene into phenol-degrading Alcaligenes faecalis cells and their monitoring in phenol-contaminated soil. *Applied Microbiology and Biotechnology, 56*(1-2), 255-260. doi:10.1007/s002530100614

Bergero, M. F., & Lucchesi, G. I. (2013). Degradation of cationic surfactants using Pseudomonas putida A ATCC 12633 immobilized in calcium alginate beads. *Biodegradation, 24*(3), 353-364. doi:10.1007/s10532-012-9592-3

Blanchoud, H., Moreau-Guigon, E., Farrugia, F., Chevreuil, M., & Mouchel, J. M. (2007). Contribution by urban and agricultural pesticide uses to water contamination at the scale of the Marne watershed. *Science of the Total Environment, 375*(1-3), 168-179. doi:10.1016/j.scitotenv.2006.12.009

Cassidy, M. B., Lee, H., & Trevors, J. T. (1996). Environmental applications of immobilized microbial cells: A review. *Journal of Industrial Microbiology, 16*(2), 79-101. doi:10.1007/BF01570068

Chandrasekar, N., Selvakumar, S., Srinivas, Y., John Wilson, J. S., Simon Peter, T., & Magesh, N. S. (2014). Hydrogeochemical assessment of groundwater quality along the coastal aquifers of southern Tamil Nadu, India. *Environmental Earth Sciences, 71*(11), 4739-4750. doi:10.1007/s12665-013-2864-3

Chanika, E., Georgiadou, D., Soueref, E., Karas, P., Karanasios, E., Tsiropoulos, N. G., . . . Karpouzas, D. G. (2011). Isolation of soil bacteria able to hydrolyze both organophosphate and carbamate pesticides. *Bioresource Technology, 102*(3), 3184-3192. doi:https://doi.org/10.1016/j.biortech.2010.10.145

Chen, R. R. (2007). Permeability issues in whole-cell bioprocesses and cellular membrane engineering. *Applied Microbiology and Biotechnology, 74*(4), 730-738. doi:10.1007/s00253-006-0811-x

Chin-Pampillo, J. S., Ruiz-Hidalgo, K., Masís-Mora, M., Carazo-Rojas, E., & Rodríguez-Rodríguez, C. E. (2015). Design of an optimized biomixture for the degradation of carbofuran based on pesticide removal and toxicity reduction of the matrix. *Environmental Science and Pollution Research, 22*(23), 19184-19193. doi:10.1007/s11356-015-5093-3

Chishti, Z., & Arshad, M. (2013). Growth linked biodegradation of chlorpyrifos by Agrobacterium and Enterobacter spp. *International Journal of Agriculture and Biology, 15*(1), 19-26.

Chung, S. Y., Venkatramanan, S., Kim, T. H., Kim, D. S., & Ramkumar, T. (2015). Influence of hydrogeochemical processes and assessment of suitability for groundwater uses in Busan City, Korea. *Environment, Development and Sustainability, 17*(3), 423-441. doi:10.1007/s10668-014-9552-7

Clarke, C. R., Hayes, B. W., Runde, B. J., Markel, E., Swingle, B. M., & Vinatzer, B. A. (2016). Comparative genomics of Pseudomonas syringae pathovar tomato reveals novel chemotaxis pathways associated with motility and plant pathogenicity. *PeerJ, 2016*(10). doi:10.7717/peerj.2570

Comoretto, L., Arfib, B., Talva, R., Chauvelon, P., Pichaud, M., Chiron, S., & Höhener, P. (2008). Runoff of pesticides from rice fields in the Ile de Camargue (Rhône river delta, France): Field study and modeling. *Environmental Pollution, 151*(3), 486-493. doi:10.1016/j.envpol.2007.04.021

Deng, S., Chen, Y., Wang, D., Shi, T., Wu, X., Ma, X., . . . Li, Q. X. (2015). Rapid biodegradation of organophosphorus pesticides by Stenotrophomonas sp. G1. *Journal of Hazardous Materials, 297*, 17-24. doi:http://dx.doi.org/10.1016/j.jhazmat.2015.04.052

Fabro, L., & Varca, L. M. (2012). Pesticide usage by farmers in Pagsanjan-Lumban catchment of Laguna de Bay, Philippines. *Agricultural Water Management, 106*, 27-34. doi:10.1016/j.agwat.2011.08.011

Fang, H., Wenrong, H., & Yuezhong, L. (2004). Investigation of isolation and immobilization of a microbial consortium for decoloring of azo dye 4BS. *Water Research, 38*(16), 3596-3604. doi:10.1016/j.watres.2004.05.014

FAOSTAT. (2013). Organophosphates Use. Retrieved from http://faostat3.fao.org/search/*/E

Filloux, A., & Ramos, J. L. (2014). Preface. Pseudomonas methods and protocols. *Methods in molecular biology (Clifton, N.J.), 1149*, v.

Furukawa, K., Ike, A., Ryu, S. L., & Fujita, M. (1993). Nitrification of NH4-N polluted sea water by immobilized acclimated marine nitrifying sludge (AMNS). *Journal of Fermentation and Bioengineering, 76*(6), 515-520. doi:10.1016/0922-338X(93)90251-3

Grimsley, J. K., Calamini, B., Wild, J. R., & Mesecar, A. D. (2005). Structural and mutational studies of organophosphorus hydrolase reveal a cryptic and functional allosteric-binding site. *Archives of Biochemistry and Biophysics, 442*(2), 169-179. doi:https://doi.org/10.1016/j.abb.2005.08.012

Hamilton, D. J., Ambrus, Á., Dieterle, R. M., Felsot, A. S., Harris, C. A., Holland, P. T., . . . Zeeh, B. W. (2003). Regulatory limits for pesticide residues in water (IUPAC technical report). *Pure and Applied Chemistry, 75*(8), 1123-1155.

Harding, C. M., Tracy, E. N., Carruthers, M. D., Rather, P. N., Actis, L. A., & Munson Jr, R. S. (2013). Acinetobacter baumannii strain M2 produces type IV Pili which play a role in natural transformation and twitching motility but not surface-associated motility. *mBio, 4*(4). doi:10.1128/mBio.00360-13

Harnpicharnchai, K., Chaiear, N., & Charerntanyarak, L. (2013). Residues of organophosphate pesticides used in vegetable cultivation in ambient air, surface water and soil in Bueng Niam subdistrict, Khon Kaen, Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health, 44*(6), 1088-1097.

He, J., Fan, M., & Liu, X. (2010). Environmental behavior of profenofos under paddy field conditions. *Bulletin of Environmental Contamination and Toxicology, 84*(6), 771-774. doi:10.1007/s00128-010-0023-z

Hickey, C. D., Auty, M. A. E., Wilkinson, M. G., & Sheehan, J. J. (2015). The influence of cheese manufacture parameters on cheese microstructure, microbial localisation and their interactions during ripening: A review. *Trends in Food Science & Technology, 41*(2), 135-148. doi:http://dx.doi.org/10.1016/j.tifs.2014.10.006

Hill, C. B., & Khan, E. (2008). A comparative study of immobilized nitrifying and co-immobilized nitrifying and denitrifying bacteria for ammonia removal from sludge digester supernatant. *Water, Air, and Soil Pollution, 195*(1-4), 23-33. doi:10.1007/s11270-008-9724-x

Hongsawat, P., & Vangnai, A. S. (2011). Biodegradation pathways of chloroanilines by Acinetobacter baylyi strain GFJ2. *Journal of Hazardous Materials, 186*(2–3), 1300-1307. doi:http://dx.doi.org/10.1016/j.jhazmat.2010.12.002

Horne, I., Harcourt, R. L., Sutherland, T. D., Russell, R. J., & Oakeshott, J. G. (2002). Isolation of a Pseudomonas monteilli strain with a novel phosphotriesterase.

FEMS Microbiology Letters, 206(1), 51-55. doi:10.1016/S0378-1097(01)00518-3

Hu, Z. C., Korus, R. A., Levinson, W. E., & Crawford, R. L. (1994). Adsorption and biodegradation of pentachlorophenol by polyurethane- immobilized Flavobacterium. *Environmental Science and Technology, 28*(3), 491-496.

Ibrahim, S., Shukor, M. Y., Syed, M. A., Johari, W. L. W., Shamaan, N. A., Sabullah, M. K., & Ahmad, S. A. (2016). Enhanced caffeine degradation by immobilised cells of Leifsonia sp. strain SIU. *Journal of General and Applied Microbiology, 62*(1), 18-24. doi:10.2323/jgam.62.18

Irie, M. (2008). Profenofos. Retrieved from http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JM PR/Evaluation08/Profenofos.pdf

Iwaki, H., Muraki, T., Ishihara, S., Hasegawa, Y., Rankin, K. N., Sulea, T., . . . Lau, P. C. K. (2007). Characterization of a pseudomonad 2-nitrobenzoate nitroreductase and its catabolic pathway-associated 2-hydroxylaminobenzoate mutase and a chemoreceptor involved in 2-nitrobenzoate chemotaxis. *Journal of Bacteriology, 189*(9), 3502-3514. doi:10.1128/JB.01098-06

Jabeen, H., Iqbal, S., Anwar, S., & Parales, R. E. (2015). Optimization of profenofos degradation by a novel bacterial consortium PBAC using response surface methodology. *International Biodeterioration and Biodegradation, 100*, 89-97. doi:10.1016/j.ibiod.2015.02.022

Jaipieam, S., Visuthismajarn, P., Sutheravut, P., Siriwong, W., Thoumsang, S., Borjan, M., & Robson, M. (2009). Organophosphate pesticide residues in drinking water from artesian wells and health risk assessment of agricultural communities, Thailand. *Human and Ecological Risk Assessment, 15*(6), 1304-1316. doi:10.1080/10807030903306984

Jesitha, K., Nimisha, K. M., Manjusha, C. M., & Harikumar, P. S. (2015). Biodegradation of Endosulfan by Pseudomonas fluorescens. *Environmental Processes, 2*(1), 225-240. doi:10.1007/s40710-015-0059-5

John, E. M., & Shaike, J. M. (2015). Chlorpyrifos: pollution and remediation. *Environmental Chemistry Letters, 13*(3), 269-291. doi:10.1007/s10311-015-0513-7

Jung, J., & Park, W. (2015). Acinetobacter species as model microorganisms in environmental microbiology: current state and perspectives. *Applied Microbiology and Biotechnology, 99*(6), 2533-2548. doi:10.1007/s00253-015-6439-y

Kang, Y.-S., & Park, W. (2010). Protection against diesel oil toxicity by sodium chloride-induced exopolysaccharides in Acinetobacter sp. strain DR1. *Journal of Bioscience and Bioengineering, 109*(2), 118-123. doi:http://dx.doi.org/10.1016/j.jbiosc.2009.08.001

Karpouzas, D. G., & Singh, B. K. (2006) Microbial Degradation of Organophosphorus Xenobiotics: Metabolic Pathways and Molecular Basis. *Vol. 51. Advances in Microbial Physiology* (pp. 119-185,225).

Karpouzas, D. G., & Walker, A. (2000). Factors influencing the ability of Pseudomonas putida strains epI and II to degrade the organophosphate ethoprophos. *Journal of Applied Microbiology, 89*(1), 40-48. doi:10.1046/j.1365-2672.2000.01080.x

Kathiravan, M. N., Praveen, S. A., Gim, G. H., Han, G. H., & Kim, S. W. (2014). Biodegradation of Methyl Orange by alginate-immobilized Aeromonas sp. in a packed bed reactor: External mass transfer modeling. *Bioprocess and Biosystems Engineering, 37*(11), 2149-2162. doi:10.1007/s00449-014-1192-7

Kato, J., Kim, H. E., Takiguchi, N., Kuroda, A., & Ohtake, H. (2008). Pseudomonas aeruginosa as a model microorganism for investigation of chemotactic behaviors in ecosystem. *Journal of Bioscience and Bioengineering, 106*(1), 1-7. doi:10.1263/jbb.106.1

Lacal, J., Muñoz-Martínez, F., Reyes-Darías, J. A., Duque, E., Matilla, M., Segura, A., . . . Ramos, J. L. (2011). Bacterial chemotaxis towards aromatic hydrocarbons in Pseudomonas. *Environmental Microbiology, 13*(7), 1733-1744. doi:10.1111/j.1462-2920.2011.02493.x

Lee, J.-Y. (2006). Characteristics of ground and groundwater temperatures in a metropolitan city, Korea: considerations for geothermal heat pumps. *Geosciences Journal, 10*(2), 165-175. doi:10.1007/BF02910361

Lewis, K. A., Tzilivakis, J., Warner, D. J., & Green, A. (2016). An international database for pesticide risk assessments and management. *Human and Ecological Risk Assessment, 22*(4), 1050-1064. doi:10.1080/10807039.2015.1133242

Li, R., Guo, X., Chen, K., Zhu, J., Li, S., & Jiang, J. (2009). Isolation of an isocarbophos-degrading strain of Arthrobacter sp. scl-2 and identification of the degradation pathway. *Journal of Microbiology and Biotechnology, 19*(11), 1439-1446. doi:10.4014/jmb.0811.0626

Li, X., He, J., & Li, S. (2007). Isolation of a chlorpyrifos-degrading bacterium, Sphingomonas sp. strain Dsp-2, and cloning of the mpd gene. *Research in Microbiology, 158*(2), 143-149. doi:10.1016/j.resmic.2006.11.007

Liu, H., Guo, L., Liao, S., & Wang, G. (2012). Reutilization of immobilized fungus Rhizopus sp. LG04 to reduce toxic chromate. *Journal of Applied Microbiology, 112*(4), 651-659. doi:10.1111/j.1365-2672.2012.05257.x

Liu, J., Ford, R. M., & Smith, J. A. (2011). Idling time of motile bacteria contributes to retardation and dispersion in sand porous medium. *Environmental Science and Technology, 45*(9), 3945-3951. doi:10.1021/es104041t

Lozinsky, V. I., & Plieva, F. M. (1998). Poly(vinyl alcohol) cryogels employed as matrices for cell immobilization. 3. Overview of recent research and developments. *Enzyme and Microbial Technology, 23*(3-4), 227-242. doi:10.1016/S0141-0229(98)00036-2

Luu, R. A., Kootstra, J. D., Nesteryuk, V., Brunton, C. N., Parales, J. V., Ditty, J. L., & Parales, R. E. (2015). Integration of chemotaxis, transport and catabolism in Pseudomonas putida and identification of the aromatic acid chemoreceptor PcaY. *Molecular Microbiology, 96*(1), 134-147. doi:10.1111/mmi.12929

Ma, C., Qin, D., Sun, Q., Zhang, F., Liu, H., & Yu, C. P. (2016). Removal of environmental estrogens by bacterial cell immobilization technique. *Chemosphere, 144*, 607-614. doi:10.1016/j.chemosphere.2015.09.014

Malghani, S., Chatterjee, N., Hu, X., & Zejiao, L. (2009). Isolation and characterization of a profenofos degrading bacterium. *Journal of Environmental Sciences, 21*(11), 1591-1597. doi:10.1016/S1001-0742(08)62460-2

Mansouriieh, N., Sohrabi, M. R., & Khosravi, M. (2016). Adsorption kinetics and thermodynamics of organophosphorus profenofos pesticide onto Fe/Ni bimetallic nanoparticles. *International Journal of Environmental Science and Technology, 13*(5), 1393-1404. doi:10.1007/s13762-016-0960-0

Technol, 63, pp. 741-746. , COI: 1:CAS:528:DC%2BC3MXks1Gjs7s%3D

Maya, K., Singh, R. S., Upadhyay, S. N., & Dubey, S. K. (2011). Kinetic analysis reveals bacterial efficacy for biodegradation of chlorpyrifos and its hydrolyzing metabolite TCP. *Process Biochemistry, 46*(11), 2130-2136. doi:10.1016/j.procbio.2011.08.012

Mekuto, L., Ntwampe, S. K. O., & Jackson, V. A. (2015). Biodegradation of free cyanide and subsequent utilisation of biodegradation by-products by Bacillus consortia: Optimisation using response surface methodology. *Environmental Science and Pollution Research, 22*(14), 10434-10443. doi:10.1007/s11356-015-4221-4

Mertens, B., Boon, N., & Verstraete, W. (2006). Slow-Release Inoculation Allows Sustained Biodegradation of γ-Hexachlorocyclohexane. *Applied and Environmental Microbiology, 72*(1), 622-627. doi:10.1128/aem.72.1.622-627.2006

Mulbry, W. W., Kearney, P. C., Nelson, J. O., & Karns, J. S. (1987). Physical comparison of parathion hydrolase plasmids from Pseudomonas diminuta and Flavobacterium sp. *Plasmid, 18*(2), 173-177. doi:10.1016/0147-619X(87)90046-1

Munir, S., Khalid, M., Bilal, M., Irshad, U., Shah, M. M., Hussain, J., & Naqvi, T. A. (2016). Biodegradation of organophosphorus insecticides by indigenous soil bacterium isolated from agricultural contaminated soil. *Minerva Biotecnologica, 28*(3), 146-152.

Na, I. Y., Chung, E. S., Jung, C. Y., Kim, D. H., Shin, J., Kang, K. J., . . . Ko, K. S. (2016). Comparison of the virulence-associated phenotypes of five species of acinetobacter baumannii complex. *Journal of Microbiology and Biotechnology, 26*(1), 171-179. doi:10.4014/jmb.1507.07076

Nair, P. R. A., & Sujatha, C. H. (2012). Organic pollutants as endocrine disruptors: Organometallics, PAHs, organochlorine, organophosphate and carbamate insecticides, phthalates, dioxins, phytoestrogens, alkyl phenols and bisphenol A *Environmental Chemistry for a Sustainable World* (Vol. 1, pp. 259-309): Springer Netherlands.

Nait Chabane, Y., Mlouka, M. B., Alexandre, S., Nicol, M., Marti, S., Pestel-Caron, M., . . . Dé, E. (2014). Virstatin inhibits biofilm formation and motility of Acinetobacter baumannii. *BMC Microbiology, 14*(1). doi:10.1186/1471-2180-14-62

Nasrabadi, T., Bidhendi, G. N., Karbassi, A., Grathwohl, P., & Mehrdadi, N. (2011). Impact of major organophosphate pesticides used in agriculture to surface water and sediment quality (Southern Caspian Sea basin, Haraz River). *Environmental Earth Sciences, 63*(4), 873-883. doi:10.1007/s12665-010-0757-2

Nikata, T., Sumida, K., Kato, J., & Ohtake, H. (1992). Rapid method for analyzing bacterial behavioral responses to chemical stimuli. *Applied and Environmental Microbiology, 58*(7), 2250-2254.

NLM. (2002). Hazardous Substances Data Bank. Retrieved from http://toxnet.nlm.nih.gov/cgi-bin/sis/search/a?dbs+hsdb:@term+@DOCNO+6992

Ondo Zue Abaga, N., Alibert, P., Dousset, S., Savadogo, P. W., Savadogo, M., & Sedogo, M. (2011). Insecticide residues in cotton soils of Burkina Faso and effects of insecticides on fluctuating asymmetry in honey bees (Apis mellifera Linnaeus). *Chemosphere, 83*(4), 585-592. doi:10.1016/j.chemosphere.2010.12.021

Pandey, J., Sharma, N. K., Khan, F., Ghosh, A., Oakeshott, J. G., Jain, R. K., & Pandey, G. (2012). Chemotaxis of Burkholderia sp. Strain SJ98 towards chloronitroaromatic compounds that it can metabolise. *BMC Microbiology, 12*. doi:10.1186/1471-2180-12-19

Parales, R. E., Luu, R. A., Hughes, J. G., & Ditty, J. L. (2015). Bacterial chemotaxis to xenobiotic chemicals and naturally-occurring analogs. *Current Opinion in Biotechnology, 33*, 318-326. doi:10.1016/j.copbio.2015.03.017

Patel, B. P., & Kumar, A. (2016). Optimization study for maximizing 2,4-dichlorophenol degradation by Kocuria rhizophila strain using response surface methodology and kinetic study. *Desalination and Water Treatment, 57*(39), 18314-18325. doi:10.1080/19443994.2015.1091988

Pino, N. J., Muñera, L. M., & Peñuela, G. A. (2016). Bioaugmentation with Immobilized Microorganisms to Enhance Phytoremediation of PCB-Contaminated Soil. *Soil and Sediment Contamination, 25*(4), 419-430. doi:10.1080/15320383.2016.1148010

Pradeep, V., & Subbaiah, U. M. (2015). Repeated batch and continuous degradation of chlorpyrifos by Pseudomonas putida. *Journal of Environmental Science and Health - Part B Pesticides, Food Contaminants, and Agricultural Wastes, 50*(5), 346-360. doi:10.1080/03601234.2015.1000180

Pramanik, S., & Khan, E. (2008). Effects of cell entrapment on growth rate and metabolic activity of mixed cultures in biological wastewater treatment. *Enzyme and Microbial Technology, 43*(3), 245-251. doi:http://dx.doi.org/10.1016/j.enzmictec.2008.04.004

Rathore, S., Desai, P. M., Liew, C. V., Chan, L. W., & Heng, P. W. S. (2013). Microencapsulation of microbial cells. *Journal of Food Engineering, 116*(2), 369-381. doi:http://dx.doi.org/10.1016/j.jfoodeng.2012.12.022

Romeh, A. A., Mekky, T. M., Ramadan, R. A., & Hendawi, M. Y. (2009). Dissipation of Profenofos, imidacloprid and penconazole in tomato fruits and products. *Bulletin of Environmental Contamination and Toxicology, 83*(6), 812-817. doi:10.1007/s00128-009-9852-z

Saez, J. M., Álvarez, A., Benimeli, C. S., & Amoroso, M. J. (2014). Enhanced lindane removal from soil slurry by immobilized Streptomyces consortium. *International Biodeterioration and Biodegradation, 93*, 63-69. doi:10.1016/j.ibiod.2014.05.013

Saez, J. M., Aparicio, J. D., Amoroso, M. J., & Benimeli, C. S. (2015). Effect of the acclimation of a Streptomyces consortium on lindane biodegradation by free and immobilized cells. *Process Biochemistry, 50*(11), 1923-1933. doi:https://doi.org/10.1016/j.procbio.2015.08.014

Saez, J. M., Benimeli, C. S., & Amoroso, M. J. (2012). Lindane removal by pure and mixed cultures of immobilized actinobacteria. *Chemosphere, 89*(8), 982-987. doi:10.1016/j.chemosphere.2012.06.057

Sampedro, I., Parales, R. E., Krell, T., & Hill, J. E. (2015). Pseudomonas chemotaxis. *FEMS Microbiology Reviews, 39*(1), 17-46. doi:10.1111/1574-6976.12081

Samuelsen, E. D., Badawi, N., Nybroe, O., Sørensen, S. R., & Aamand, J. (2016). Adhesion to sand and ability to mineralise low pesticide concentrations are required for efficient bioaugmentation of flow-through sand filters. *Applied Microbiology and Biotechnology*, 1-11. doi:10.1007/s00253-016-7909-6

Santos da Silva, S., Chiavone-Filho, O., de Barros Neto, E. L., Foletto, E. L., & Mota, A. L. N. (2013). Effect of Inorganic Salt Mixtures on Phenol Mineralization by Photo-Fenton-Analysis via an Experimental Design. *Water, Air, & Soil Pollution, 225*(1), 1-10. doi:10.1007/s11270-013-1784-x

Sapbamrer, R., & Hongsibsong, S. (2014). Organophosphorus pesticide residues in vegetables from farms, markets, and a supermarket around Kwan Phayao Lake of Northern Thailand. *Archives of Environmental Contamination and Toxicology, 67*(1), 60-67. doi:10.1007/s00244-014-0014-x

Sedighi, M., Zamir, S. M., & Vahabzadeh, F. (2016). Cometabolic degradation of ethyl mercaptan by phenol-utilizing Ralstonia eutropha in suspended growth and gas-recycling trickle-bed reactor. *Journal of Environmental Management, 165*, 53-61. doi:10.1016/j.jenvman.2015.09.006

Shamim, S., Rehman, A., & Qazi, M. H. (2014). Swimming, swarming, twitching, and chemotactic responses of Cupriavidus metallidurans CH34 and Pseudomonas putida mt2 in the presence of cadmium. *Archives of Environmental Contamination and Toxicology, 66*(3), 407-414. doi:10.1007/s00244-013-9966-5

Singaraja, C., Chidambaram, S., Anandhan, P., Prasanna, M. V., Thivya, C., & Thilagavathi, R. (2015). A study on the status of saltwater intrusion in the coastal hard rock aquifer of South India. *Environment, Development and Sustainability, 17*(3), 443-475. doi:10.1007/s10668-014-9554-5

Singaraja, C., Chidambaram, S., Anandhan, P., Prasanna, M. V., Thivya, C., Thilagavathi, R., & Sarathidasan, J. (2014). Determination of the utility of groundwater with respect to the geochemical parameters: A case study from Tuticorin District of Tamil Nadu (India). *Environment, Development and Sustainability, 16*(3), 689-721. doi:10.1007/s10668-013-9502-9

Singh, B. K. (2009). Organophosphorus-degrading bacteria: Ecology and industrial applications. *Nature Reviews Microbiology, 7*(2), 156-164. doi:10.1038/nrmicro2050

Singh, B. K., & Walker, A. (2006). Microbial degradation of organophosphorus compounds. *FEMS Microbiology Reviews, 30*(3), 428-471. doi:10.1111/j.1574-6976.2006.00018.x

Singh, B. K., Walker, A., & Wright, D. J. (2006). Bioremedial potential of fenamiphos and chlorpyrifos degrading isolates: Influence of different environmental conditions. *Soil Biology and Biochemistry, 38*(9), 2682-2693. doi:10.1016/j.soilbio.2006.04.019

Singh, R., & Olson, M. S. (2008). Application of bacterial swimming and chemotaxis for enhanced bioremediation *Emerging Environmental Technologies* (pp. 149-172): Springer Netherlands.

Siripattanakul-Ratpukdi, S., Vangnai, A. S., Sangthean, P., & Singkibut, S. (2014). Profenofos insecticide degradation by novel microbial consortium and isolates enriched from contaminated chili farm soil. *Environmental Science and Pollution Research, 22*(1), 320-328. doi:10.1007/s11356-014-3354-1

Siripattanakul, S., & Khan, E. (2010). Fundamentals and applications of entrapped cell bioaugmentation for contaminant removal *Emerging Environmental Technologies* (Vol. 2, pp. 147-169): Springer Netherlands.

Siripattanakul, S., Wirojanagud, W., McEvoy, J. M., Casey, F. X. M., & Khan, E. (2008) Atrazine remediation in agricultural infiltrate by bioaugmented polyvinyl alcohol immobilized and free Agrobacterium radiobacter J14a. *Vol. 58. Water Science and Technology* (pp. 2155-2163): IWA Publishing.

Suwannaruang, T., & Wantala, K. (2016). Single-step uncalcined N-TiO2 synthesis, characterizations and its applications on alachlor photocatalytic degradations. *Applied Surface Science, 380*, 257-267. doi:10.1016/j.apsusc.2016.01.117

Takeno, K., Yamaoka, Y., & Sasaki, K. (2005). Treatment of oil-containing sewage wastewater using immobilized photosynthetic bacteria. *World Journal of Microbiology and Biotechnology, 21*(8-9), 1385-1391. doi:10.1007/s11274-005-5739-2

Tallur, P. N., Mulla, S. I., Megadi, V. B., Talwar, M. P., & Ninnekar, H. Z. (2015). Biodegradation of cypermethrin by immobilized cells of micrococcus sp. Strain CPN 1. *Brazilian Journal of Microbiology, 46*(3), 667-672. doi:10.1590/S1517-838246320130557

Talwar, M. P., & Ninnekar, H. Z. (2015). Biodegradation of pesticide profenofos by the free and immobilized cells of Pseudoxanthomonas suwonensis strain HNM. *Journal of Basic Microbiology, 55*(9), 1094-1103. doi:10.1002/jobm.201400978

Tampion, J. T., MD. (1987). *Immobilized Cells: Principles and Applications* (C. U. Press Ed.). UK, Cambridge: Cambridge Studies in Biotechnology 5.

Teklu, B. M., Adriaanse, P. I., & Van den Brink, P. J. (2016). Monitoring and risk assessment of pesticides in irrigation systems in Debra Zeit, Ethiopia. *Chemosphere, 161*, 280-291. doi:10.1016/j.chemosphere.2016.07.031

Teklu, B. M., Hailu, A., Wiegant, D. A., Scholten, B. S., & Van den Brink, P. J. (2016). Impacts of nutrients and pesticides from small- and large-scale agriculture on the water quality of Lake Ziway, Ethiopia. *Environmental Science and Pollution Research*, 1-10. doi:10.1007/s11356-016-6714-1

Toan, P. V., Sebesvari, Z., Bläsing, M., Rosendahl, I., & Renaud, F. G. (2013). Pesticide management and their residues in sediments and surface and drinking water in the Mekong Delta, Vietnam. *Science of the Total Environment, 452-453*, 28-39. doi:10.1016/j.scitotenv.2013.02.026

USEPA. (2006). Reregistration eligibility decision for profenofos. Retrieved from http://www.epa.gov/oppsrrd1/REDs/profenofos_red.pdf

Van Dyk, J. S., & Pletschke, B. (2011). Review on the use of enzymes for the detection of organochlorine, organophosphate and carbamate pesticides in the

environment. *Chemosphere, 82*(3), 291-307. doi:10.1016/j.chemosphere.2010.10.033

Vangnai, A. S., Takeuchi, K., Oku, S., Kataoka, N., Nitisakulkan, T., Tajima, T., & Kato, J. (2013). Identification of CtpL as a Chromosomally Encoded Chemoreceptor for 4-Chloroaniline and Catechol in Pseudomonas aeruginosa PAO1. *Applied and Environmental Microbiology, 79*(23), 7241-7248. doi:10.1128/aem.02428-13

Velasco-Casal, P., Wick, L. Y., & Ortega-Calvo, J. J. (2008). Chemoeffectors decrease the deposition of chemotactic bacteria during transport in porous media. *Environmental Science and Technology, 42*(4), 1131-1137. doi:10.1021/es071707p

Verma, J., Jaiswal, D., & Sagar, R. (2014). Pesticide relevance and their microbial degradation: a-state-of-art. *Reviews in Environmental Science and Bio/Technology, 13*(4), 429-466. doi:10.1007/s11157-014-9341-7

Wadhams, G. H., & Armitage, J. P. (2004). Making sense of it all: Bacterial chemotaxis. *Nature Reviews Molecular Cell Biology, 5*(12), 1024-1037. doi:10.1038/nrm1524

Weir, S. C., Dupuis, S. P., Providenti, M. A., Lee, H., & Trevors, J. T. (1995). Nutrient-enhanced survival of and phenanthrene mineralization by alginate-encapsulated and free Pseudomonas sp. UG14Lr cells in creosote-contaminated soil slurries. *Applied Microbiology and Biotechnology, 43*(5), 946-951. doi:10.1007/BF02431932

Witt, M. E., Dybas, M. J., Worden, R. M., & Criddle, C. S. (1999). Motility-enhanced bioremediation of carbon tetrachloride-contaminated aquifer sediments. *Environmental Science and Technology, 33*(17), 2958-2964. doi:10.1021/es981280+

Wuichet, K., & Zhulin, I. B. (2010). Origins and diversification of a complex signal transduction system in prokaryotes. *Science Signaling, 3*(128). doi:10.1126/scisignal.2000724

Xu, G., Zheng, W., Li, Y., Wang, S., Zhang, J., & Yan, Y. (2008). Biodegradation of chlorpyrifos and 3,5,6-trichloro-2-pyridinol by a newly isolated Paracoccus sp. strain TRP. *International Biodeterioration and Biodegradation, 62*(1), 51-56. doi:10.1016/j.ibiod.2007.12.001

Yan, J. H., Y.Y. (2009). Partial nitrification to nitrite for treating ammonium-rich organic wastewater by immobilized biomass system. *Bioresource Technology, 100*(8), 2341-2347.

Yáñez-Ocampo, G., Sánchez-Salinas, E., & Ortiz-Hernández, M. L. (2011). Removal of methyl parathion and tetrachlorvinphos by a bacterial consortium immobilized on tezontle-packed up-flow reactor. *Biodegradation, 22*(6), 1203-1213. doi:10.1007/s10532-011-9475-z

Yasukawa, K., Uchida, Y., Tenma, N., Taguchi, Y., Muraoka, H., Ishii, T., . . . Nguyen, T. H. (2009). Groundwater Temperature Survey for Geothermal Heat Pump Application in Tropical Asia. *BULLETIN OF THE GEOLOGICAL SURVEY OF JAPAN, 60*(9-10), 459-467. doi:10.9795/bullgsj.60.459

Zhang, Y. H., Xu, D., Liu, J. Q., & Zhao, X. H. (2014). Enhanced degradation of five organophosphorus pesticides in skimmed milk by lactic acid bacteria and its

potential relationship with phosphatase production. *Food Chemistry, 164*, 173-178. doi:10.1016/j.foodchem.2014.05.059

Zheng, C., Zhou, J., Wang, J., Qu, B., Wang, J., Lu, H., & Zhao, H. (2009). Aerobic degradation of nitrobenzene by immobilization of Rhodotorula mucilaginosa in polyurethane foam. *Journal of Hazardous Materials, 168*(1), 298-303. doi:10.1016/j.jhazmat.2009.02.029

VITA

Miss Chutima Ploychankul graduated Ph.D. and Maste's Degree from the International Postgraduate Programs in Environmental Management, Chulalongkorn University. She finished her bachelor's degree from the Department of Biochemistry, Faculty of Science, Chulalongkorn University, She had chance to pass the examination from the International Association for the Exchange of Students for Technical Experience (IAESTE). This opportunity offered her to make an internship at Max-Planck Institute of Molecular Plant Physiology, Potsdam, Germany for six months in 2006.

During the time she was in Ph.D., she has already submitted on two journal articles on the topic of Characterization of Profenofos Degradation by Pseudomonas plecoglossicida Strain PF1 Using Surface Response Methodology (Desalination and Water Treatment) and Profenofos Removal by Acinetobacter baylyi Strain GFJ2: Biodegradation Kinetics and Influence of Environmental Conditions (Environmental Earth Sciences). In addition, she also have two presentation on international conferences. The topic of Profenofos biodegradation by Pseudomonas plecoglossicida PF1 was presented on the 3rd Joint Symposium CU-NUT, Jan 5th, 2015, Chulalongkorn University, Bangkok, Thailand. The other was presented on the 3rd Farm Engineering and Automation Technology Research Group (FEAT), Nov 25th , 2016, Khon Kaen University, Khon Kaen, Thailand. The topic for this conference was the Effect of Inorganic Salts on Profenofos Pesticide Degradation of Immobilized Pseudomonas plecoglossicida PF1 and Acinetobacter baylyi GFJ2. In this confence, she got reward for the best presentation in the session.