DEGRADATION OF CHLOROANILINES BY IMMOBILIZED *ACINETOBACTER BAUMANNII* STRAIN GFJ1

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การกำจัดสารกลุ่มคลอร์โรแอนาลีนโดย *Acinetobacter baumannii* GFJ1

นายฮาดัน ดุค

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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CONTENTS

Page

Page

Page

Page

Page

Page

TABLE CONTENT

FIGURE CONTENT

LIST OF ABBREVIATIONS

µl: microliter

23DCA: 2,3-dichloroaniline

246TCA: 2,4,6-trichloroaniline

24DCA: 2,4-dichloroaniline

25DCA: 2,5-dichloroaniline

2CA: 2-cloroaniline

34DCA: 3,4-dichloroaniline

35DCA: 3,5-dichloroaniline

3CA: 3-cloroaniline

4CA: 4-chloroaniline

50NO3: 50 mg/l NO_3^- as an electron acceptor

50SO4: 50 mg/l SO_4^{2-} as an electron acceptor

CAs: chloroanilines

DI water: *deionized water*

EPA: Environmental Protection Agency

HPLC = High performance liquid chromatography

Ki: inhibition coefficient

Kmax: maximum the degradation rate

Ks: half-saturation constant

LB = Luria bertani

mg: milligram

min: minute

ml: milliliter

mM: millimolar

MM: mineral medium

MYSA: MM supplemented with 0.01% YE, 0.1% succinate and 0.1% ammonium sulfate

NJ: neighbor joining

OD = Optical density

OD600: OD measurement number at 600 nm

PEG: polyethylene glycol

PEG1000: polyethylene glycol with *molecular weight* of 1000

PEG6000: polyethylene glycol with *molecular weight* of 6000

ppm: part per million

PVA: polyvinyl alcohol

rpm: revolution per minute

TLC: thin layer chromatography

UV: *ultraviolet*

v/v: volume/volume

w/v: weight/volume

YE: Yeast extract

CHAPTER 1 INTRODUCTION

1.1 Statement of problem

Chloroanilines (CAs) are chlorinated aromatic amines, which are the main materials for production of azo dyes, rubbers, photographic chemicals, varnishes, and herbicides. (Boon et al., 2001; Gheewala and Annachhatre, 1997; Gosetti et al., 2010b) CAs are also main intermediate during degradations of acetamide and urea herbicides (Lacorte et al., 1999). The widespread usage of these compounds in agriculture has undoubtedly caused contaminated agriculture soils, surface and subsurface water (shown in 2.1.4). Some of them such as 34DCA were even more frequently detected than the parent herbicides (Giacomazzi and Cochet, 2004). Besides CAs, wastewater, contaminated water and groundwater may contain other organic and inorganic compounds. The investigation of other components, which usually present in the environment, is important to determine their effects on biodegradation of CAs.

Because of toxicity and recalcitrance, CAs are considered as important environmental pollutants (Meyer, 1981). CAs are toxic chemicals, they cause cancer and other diseases. Moreover, they are in priority compounds listed in the 76/464/CEE Council Directive (Community, 1976), and Priority Pollutant List of the U.S. Environmental Protection Agency (Register, 1979). Because CAs are toxic and widely detected in the environments, the removal of these toxicants is necessary. One of the most efficient ways to eliminate CAs from the environment is biodegradation,

in which the removal process depends on the biodegradation ability of bacteria and environmental conditions. The isolation and application of microorganisms play an important role to clean up the toxicants in environments.

There are a number of reports on remediation of CAs contaminated environment. *Moraxella* sp. was the first to be reported that this bacterial strain could aerobically utilize CAs as sole sources of carbon, nitrogen and energy (Zeyer and Kearney, 1982). Some bacterial strains could degrade both mono- and di-chloroanilines such as *Pseudomonas diminuta* (Surovtseva et al., 1985), and *A. baylyii* GFJ2 (Hongsawat and Vangnai, 2011). Although a number of studies have investigated the degradation of CAs, there is only one report describing the utilization of mono-, di- and trichloroanilines as growth substrates by a bacterial mixture (Lu et al., 2009). The first pure culture, *Paracoccus* sp., had the capacity to degrade both mono- and dichloroanilines under aerobic and anaerobic conditions with the simultaneous reduction of nitrate to nitrite (Bollag and Russel, 1976). However, no study describing the utilization of CAs as sole carbon and energy sources in both aerobic and anaerobic conditions has been reported.

In bioremediation processes, immobilized cells offer several advantages over freely suspended cells, including more operational flexibility, high cell densities, protecting cells against toxicity, capability of recycling or reusing the microorganisms. Among the materials used for cell immobilization, polyvinyl alcohol (PVA) and alginate are popular (Chen and Lin, 1994; Ha et al., 2009). One of the drawbacks of immobilized cells is diffusion resistance. To enhance mass transfer, porogens were added into immobilized gels. Previous studies stated that using $CaCO_3$

(Bai et al., 2010) and polyethylene glycol (PEG) (Zhang and Ye, 2011) as pore-forming agents are economical ways to enhance the porosity of PVA-alginate beads.

Thus, a need exists for the determination of degradation toward CAs in different conditions and application of bacteria for remediation contaminated environments. In this study, the biodegradation toward CAs by freely suspended and immobilized cells and conditions affecting the degradation rates are investigated. The determination of 34DCA degradation by *A. baumannii* GFJ1 might provide potentials for bioremediation of 34DCA contaminated environments.

1.2 Research objectives

The overall objective in this study is to investigate the biodegradation ability toward chloroanilines by *A. baumannii* GFJ1. The objectives can be divided into parts:

1.2.1 To determine biodegradation characterization of *A. baumannii* GFJ1 toward CAs under aerobic conditions.

1.2.2 To determine the biodegradation toward CAs under anaerobic conditions.

1.2.3 To enhance biodegradation by entrapped cells of *A. baumannii* GFJ1 toward CAs by modifications of the carrier.

1.2.4 To determine the effects of cryoprotectants, freeze-drying process and storage conditions on CAs degradation rates of entrapped cells.

1.3 Hypothesis

1.3.1 *A. baumannii* GFJ1 can utilize CAs as sources of sole carbon, nitrogen and energy.

1.3.2 *A. baumannii* GFJ1 can degrade CAs under anaerobic conditions with the presences of electron acceptors.

1.3.3 The biodegradation toward CAs by entrapped cells in beads with modification and characteristics of modified beads:

1.3.3.1 The addition of porogens ($CaCO_3$ and PEG) enhances the porosity of beads and enhances the degradation rates of entrapped cells.

1.3.3.2 The physical and chemical characteristics of beads are stable with the addition of a specific amount of porogens.

1.3.4 The presence of cryoprotectants in beads increases the tolerance of entrapped cells during freeze-drying process and long-term storage.

1.4. Scope of study

1.4.1 Determination of biodegradation characterization of *A. baumannii* GFJ1 toward CAs under aerobic conditions:

The utilization of mono-, di- and trichloroanilines as sources of carbon, nitrogen and energy without supplementation of any co-substrate was investigated. In this experiment, the different concentrations of CAs were added in mineral medium to investigate the effects of chemical concentrations on degradation rates.

Effects of co-substrates, including nitrogen sources (YE, sodium nitrate, ammonium sulfate and ammonium chloride) and carbon sources (humate, succinate and citrate) on biodegradation and growth rates of *A. baumannii* GFJ1 were determined.

Intermediates in aerobic degradation and degradation pathways in *A. baumannii* GFJ1 for the biodegradation of CAs under aerobic degradation were investigated using TLC and LC-MS analysis.

1.4.2 Determination of biodegradation toward CAs under anaerobic conditions:

The utilization of 4CA and 34DCA (0.1 mM) as sources of carbon, nitrogen and energy, and effect of co-substrates (YE and succinate) on anaerobic degradation rates toward CAs of *A. baumannii* GFJ1 were determined.

The effects of electron acceptors (nitrate, sulfate) on CAs degradation, and the transformation of electron acceptors during anaerobic degradation were determined.

The determination of intermediates and degradation pathways for the degradation toward CAs under anaerobic degradation was carried out.

1.4.3 Determination of biodegradation toward 34DCA by entrapped cells in beads with modifications, and characteristics of modified beads:

The biodegradation of 34DCA (1.2 mM) by entrapped cells in PVA-alginate beads with and without the modification was determined. The modification of beads was carried out by the addition of porogens ($CaCO_3$ and PEG) at different concentrations (3%, 5%, 7% and 10%).

The characterizations (physical and chemical stability, porosity, diffusion and swelling ratios) of beads with the addition of porogens were also determined.

1.4.4 Effects of cryoprotectants, freeze-drying process and storage conditions on CAs degradation rates of entrapped cells:

The effects of cryoprotectant types (glycerol, sucrose, sorbitol and mannitol) and concentrations of cryoprotectants (10% and 20%, w/v) on CAs degradation rates of entrapped cells before and after the freeze-drying process were determined.

Roles of these cryoprotectants in maintenance of CAs degradation rates of entrapped cells after one month storage at different temperatures: room temperature, 4 ^{o}C and minus 20 ^{o}C were carried out.

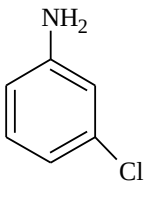
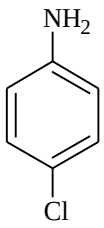
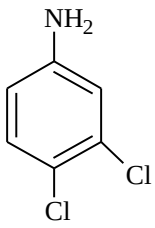
CHAPTER 2 THEORETICAL BACKGROUND AND LITERATURE REVIEW

2.1 Properties, production, toxicity and accumulation of CAs in environments

2.1.1 Properties of CAs

CAs are aromatic amines. The component of these chemicals includes a benzene ring, NH_2 group and one, two or more chlorine atoms. The physical and chemical properties among them are not the same. The properties of several CAs are shown in Table 2-1.

Table 2-1. Chemical and physical properties of several CAs (3CA, 4CA and 34DCA).

Properties	Chemicals		
	3CA (HSDB, 2011b)	4CA (Boehncke et al., 2003)	34DCA (Bureau, 2006)
Chemical formula	C_6H_6ClN	C_6H_6ClN	$C_6H_5Cl_2N$
Chemical structure			
Molecular weight	127.57 (g/mole)	127.57 (g/mole)	162.02 (g/mole)
Physical state	Liquid at room	Crystalline solid	Solid at room

	temperature		temperature
Color	Colorless to light amber, darken during storage	Colorless to slightly amber color	Brown
Melting point	-10.4 ^{o}C	72.5 ^{o}C	70 - 72.5 ^{o}C
Boiling point	230.5 ^{o}C	232 ^{o}C	270 ^{o}C
Density	1.215 g/cm^3	1.43g/cm^3	1.57 g/cm^3
Water solubility	5.4 g/l at 20 ^{o}C	2.6 g/l at 20 ^{o}C	580 mg/l at 20 ^{o}C
Vapor pressure	0.066 mm Hg at 25 ^{o}C	0.5 Pa at 10 ^{o}C	0.184 Pa at 20 ^{o}C
Henry's law constant	10^{-6} atm. m^3/mol at 25 ^{o}C	0.1 Pa.m^3/mol at 20 ^{o}C	0.05 Pa.m^3/mol at 20 ^{o}C
logK_{ow}	1.88	1.83	2.69

2.1.2 The production and release of CAs

CAs are ingredients for industrial production of polyurethanes, rubbers, dyes, pharmaceutics, photographic chemicals, vanishes and herbicides (Zhang et al., 2010a). Thousands of tons have been produced and released into the environment in Europe, USA, India, China and other countries (Table 2-2 and 2-3). CAs may release into soil, water, and air, which cause contaminated environments.

Table 2-2. The production of several CAs

Year	CA	Sources	Amount	References
1985	2CA	Production in European Chemicals Bureau communities	Up to 2000 tons/year	(Communities, 1985)
1985	3CA	Production in European Chemicals Bureau communities	Up to 4000 tons/year	(Communities, 1985)
1988	4CA	The global annual production of 4CA	3,500 tons	(Boehncke et al., 2003)
1990	4CA	Production in the former Federal of Germany in 1990	1,350 tons	(Boehncke et al., 2003)
		Pocessing in the German manufacturers	1,000 tons	(Boehncke et al., 2003)
1991	34DCA	Production in Western Europe	12,000 tons	(Bureau, 2006)
1996	34DCA	Production in Western Europe	13,500 – 15,500 tons/year	(Bureau, 2006)

Table 2-3. The amount of several CAs releasing into environments

CAs	Sources	Amount	References
4CA	The releases from the processing of 4CA in Germany in 1990:		(Boehncke et al., 2003)
	In air	25 g/ton	
	In water	240 g/ton	
	In wastes	Maximum of 695 g/ton	
4CA	Released in USA in 1995	500 kg	(Boehncke et al., 2003)
4CA	Released in USA in 1998	2,814 kg	(Boehncke et al., 2003)
4CA	Released in USA in 1999	212 kg	(Boehncke et al., 2003)
34DCA	Emissions into the hydrosphere during production in Europe	1,700 kg/year	(Bureau, 2006)
34DCA	Emissions into the atmosphere during production in Europe	37 kg/year	(Bureau, 2006)
34DCA	Emissions into the atmosphere during use of trichlorocarbanilide in Europe	1,840 kg/year	(Bureau, 2006)
34DCA	Emissions into surface waters during processing of 34DCA to propanil in Europe	4.9 kg/year	(Bureau, 2006)

34DCA	Releases during use of plant protecting agents and biocides in Germany	310 kg/year	(Bureau, 2006)

CAs distribute among the environmental compartments after releasing into the aquatic environment. The main part distributes between the water and sediment compartments, other parts may volatilize into the air and may be degraded by photo-degradation (Communities, 1985). CAs released into environments result from industrial productions and agricultural practices as ingredients and intermediate degradation products.

Herbicides play an important role to increase the agricultural production because they contribute effectively and profitably to weed control, increase crop production and reduce the cost of farming. But the wide usage of herbicides causes contaminated environments and badly affects human and animal health. The amount of herbicides consumed is 47.5% of the 2 million tons of pesticide worldwide consumed each year (Sopeña et al., 2009). Phenylurea herbicides such as diuron and propanil have been widely applied for weed control. For examples, the amount of diuron used as the plant protection agent was 112 tons in the Netherlands in 1995, linuron used in the UK was 92.2 tons/year from 1994 to 1997 (Bureau, 2006).

Other chemicals are involved in CAs such as azo dyes. Every year, worldwide production of azo dyes is about 450,000 tons/year, and almost 50,000 tons/year are lost in effluent during application and manufacture (Sawhney and Kumar, 2011).

2.1.3 The toxicity of CAs

CAs are toxic to human and non-target organisms. CAs can be absorbed into the body *via* dermal, gastrointestinal or respiratory ways (Bureau, 2006; HSDB, 2011b). For example, 4CA causes acute and chronic effects after absorption into the body. For acute symptoms, the chemical causes skin blisters, cyanosis, headache, dizziness, irritate and burn eyes (HSDB, 2011b, c). CAs bind covalently to hemoglobin and protein which the main target tissues being liver, kidney and urinary bladder (Giacomazzi and Cochet, 2004). For a long time, the chemicals may cause cancer, reproductive damage, and bad effects on the nervous system (Bureau, 2006).

The investigation of CAs toxicity on animals showed that LD50 of Mouse and Rat oral toward 3CA was 334 and 256 mg/kg, respectively (HSDB, 2011b). Rats were cyanosis and lethargy for up to 24 hours after absorption of 4CA. LC_{50} (lethal concentration) of rat was 2,340 mg/m^3 *via* inhalation (Boehncke et al., 2003), and 2/6 rats died after exposed 0.25 hours at 65 mg/m^3 of 34DCA (Bureau, 2006). Oral LD50 values of 228 - 500 mg/kg, and 350 mg/kg body weight for mice and guinea pigs were reported, respectively (Boehncke et al., 2003).

The toxicity of CAs on aquatic species has been studied. For 3CA, EC50 of *Daphnia magna* and *Tetrahymena pyrifonnis* was 0.4 mg/1 and 64 mg/l, respectively (Communities, 1985). 34DCA causes harmful effects in the aquatic compartment including vertebrates, invertebrates and microorganisms. Zebrafish (*Brachydanio rerio)* has 96-hour LC50 at 8.5 mg/l, water flea *Daphnia magna* has 48-hour LC50 at 0.23 mg/l and 96-hour LC50 at 0.16 mg/l (Bureau, 2006).

2.1.4 The detection and accumulation of CAs in environments

CAs are widely detected in environments, including in air, soil, sediment and especially in water. In water, CAs were detected in surface water, tap water, wastewater and groundwater. In freshwater and marine water, the maximal permissible concentrations were proposed of 1 to 5 µg/l for all CAs (Communities, 1985).

In surface water, Wegman and Corte (1981) showed that 3CA, 4CA and 34DCA were detected in 83%, 100% and 100% with concentration up to 1.8, 0.74, 1.2 µg/l (0.014, 0.006 and 0.0074 µM) of all samples collected from the tributaries of the Rhine River in 1979, respectively (Wegman and Corte, 1981). The 34DCA concentrations in water from irrigated rice farming in Brazil were from 1.0 to 567.5 µg/l (0.006 – 3.5 µM) after 3 days application with herbicide propanil (Ednei et al., 2007).

In groundwater, concentration of aniline, 2CA, 3CA, 4CA and 34DCA in contaminated groundwater collected underneath a former industrial complex were up to 17.0, 44.4, 5.48, 3.84 and 10.9 mg/l (0.13, 0.34, 0.04, 0.03 and 0.067 mM), respectively (Boyd et al., 1997). 4CA concentration in groundwater was detected from smaller than 10 µg/l (0.078 µM) to 50 µg/l (0.392 µM) at 5.5 and 8.5 m in depth into the ground surface below a Danish landfill site containing domestic wastes and wastes from pharmaceutical production, respectively (Holm et al., 1995).

In wastewater, aniline was detected at 2,480 mg/l (26.6 mM) in the effluent of a pharmaceutical industry (Li et al., 2010). In wastewater from the Rhone Poulenc Chemical plant (United Kingdom), the concentrations of aniline, 4CA, 23DCA, 25DCA and 34DCA were 11.6, 21.4, 98.6, 3.1 and 260 mg/l (0.12, 0.17, 0.61, 0.02

and 1.6 mM) respectively (Livingston and Willacy, 1991a). Aniline, 4CA, 23DCA and 34DCA were also detected at this site at 36.0, 24.0, 62.0 and 194.0 mg/l (0.39, 0.19, 0.38 and 1.2 mM) in another time, respectively (Brookes and Livingston, 1993). 2CA was detected at 2.56 mg/l (0.02 mM) in the **effluent** of pharmaceutical industry (HSDB, 2011a). 3CA was detected at 0.6 mg/l (0.005 mM) in the **effluent** of pharmaceutical industry (HSDB, 2011a).

In addition, CAs have been detected in tap and drinking water. 4CA was detected in German drinking-water samples (BUA, 1995), 3CA and 34DCA was detected in German and Dutch tap water (HSDB, 2011b, c).

In soil, CAs may be contaminated due to release directly from chemical processes or intermediate degradation of herbicides, azo dye and other compounds. 4CA in soil was 0.001 – 0.27 mg/kg detected in Japan and up to 0.968 mg/kg in Germany (Boehncke et al., 2003). In sediment, 4CA was detected at 3.30 mg/kg in USA (EPA, 2010), or 0.001 – 0.27 mg/kg in Japan (Boehncke et al., 2003). 34DCA was detected at 0.15 mg/kg sediment in the Netherlands (Bureau, 2006).

2.2 Legislation

Because of their toxicity and recalcitrant properties, CAs are considered as important environmental pollutants and are subject to legislative control by the 76/464/EEC Directive and environmental protection agency of the United States (Boon et al., 2001). Besides, they are in the list of priority pollutants on the black list of EEC (Wolf et al., 1994).

Other chemicals relating to CAs are subject to legislative control such as diuron and azo dye. Diuron is in the list of priority substances for European freshwater resources of the European Commission and on the U.S. Environmental Protection Agency's Second Drinking Water Contaminant Candidate List (Sorensen et al., 2008). The products containing 4CA-based azo dyes were recently banned to market and use by the European Union (EC, 2000).

2.3 Treatment of CAs

2.3.1 CAs degradation of CAs by physical and chemical treatments

The decomposition of 4CA in aqueous solution was studied by ozonolysis as well as by γ-rays in the presence of ozone under the combination condition (Sánchez et al., 2002). The result showed that the combination of radiolysis and ozonolysis enhanced the degradation rate in comparison to only ozonolysis treatment. The combination of radiolysis (74 Gy/min) and ozonolysis (0.8 mg O_3/l) was the most effective (Sánchez et al., 2002).

Gosetti et al. (2010) presented that degradation of 4CA by sunlight naturally underwent in water without any addition of organic solvents. 4CA degradation by photoradiation eliminated the chlorine atom, but ring-cleavage did not occur and photoproducts of 4CA had a toxicity level significantly greater than the precursor (Gosetti et al., 2010a).

2.3.2 Natural attenuation of CAs

The dissipation of toxicants by natural processes occurs by biodegradation, volatilization, dispersion, dilution and sorption (EPA., 1999). Tongarun showed that 4CA degraded in loam soil in Nakornnayok province was poor (<10%), but it was fairly effective (40%) in loam soil in Chiangmai province (Thailand) (Tongarun et al., 2008). This method is easy and cheap; however, it requires a long time to completely degrade the chemicals because of low population of indigenous degrading microorganisms.

2.3.3 Biostimulation method

Biostimulation enhances the natural attenuation processes. Nutrients, electron donors and electron acceptors such as carbon, nitrogen, phosphorus and oxygen are added to contaminated sites to stimulate indigenous microorganisms for degradation (Mrozik and Piotrowska-Seget, 2010).

2.3.4 Bioaugmentation method

Bioaugmentation is considered one of the most effective methods to treat contaminants in environment. The method includes the application of a single strain or consortia of microorganisms which are capable to degrade toxic compounds to augment biodegradation at contaminated sites.

2.4 Application of *A. baumannii* for biodegradation

A. baumannii was reported on biodegradation processes of toxic chemicals. For instances, *A. baumannii* strain PL-2 and PL-3 isolated from municipal solid waste

landfill could degrade polyethylene (Pramila et al., 2012); *A. baumannii* isolated from sludge of a pulp wastewater treatment plant was able to degrade phenanthrene (Kim et al., 2009); or *A. baumannii* CA2 isolated from soil degraded 4CA *via ortho*-cleavage (Vangnai and Petchkroh, 2007).

In this study, *A. baumannii* GFJ1 will be investigated for degradation of CAs and its application to clean up the chemicals contaminated environments.

2.5 CAs degradation under aerobic conditions

2.5.1 Reports on biodegradation toward CAs by bacteria

There are a number of reports on biodegradation of CAs by pure cultures (Table 2-4). Most bacteria were isolated from soil and used as freely suspended cells for degradation of CAs. However, there are some reports on using immobilized cells for CAs degradation; for examples, *P. acidovorans* CA28 immobilized in alginate and cultivated in an air-lift fermentor degraded aniline, 3CA and 4CA (Ferschl et al., 1991), cells immobilized on celite diatomaceous earth (Livingston and Willacy, 1991a) and membrane bioreactor (Brookes and Livingston, 1993) were applied for degradation of 34DCA. The degradation of 4CA and 34DCA by aerobic granules was reported (Zhu et al., 2012; Zhu et al., 2011a).

Table 2-4. Some CAs-degrading bacteria have been reported

Bacteria strain	Degradation forms	CAs degradation	Intermediate products	Characteristic degradation	Reference
P. muttivorans An 1	Free cell	Aniline > 2CA > 3CA > 4CA	3-Chlorocatechol, 4-chlorocatechol	Bacteria utilized aniline as a sole carbon source. Bacteria could transform 3CA, 4CA with the presence of aniline	(Reber et al., 1979)
Pseudomonas putida	Free cell	34DCA	3,4-dichloromuconate, 3-chlorobutenolide, and 3-chlorolevulinic	Bacteria could not utilize 34DCA as sole carbon, nitrogen and energy source, substrate analogues such aniline or propionanilide	(You and Bartha, 1982a)
Pseudomonas sp.,	Resting cell	Aniline > 34DCA	4-chlorocatechol and catechol	Specific degradation rate of 4CA at 3 mM (resting cell) = 12.7 μmol[min.g protein]$^{-1}$	(Zeyer and Kearney, 1982)
Moraxella sp. G	Free cell	Aniline > 4CA > 3CA > 2CA	Chlorocatechol and other products	Bacteria utilized 2CA, 3CA and4CA as sole carbon, nitrogen and energy sources, but not 34DCA	(Zeyer et al., 1985a)
Rhodococcus AM 144	Resting cell	Aniline > 3CA > 2CA > 34DCA	-	The degradation enhanced with the addition of glucose	(Janke and Ihn, 1989)
P. acidovorans	Free cell	Aniline > 3CA > 4CA	Chlorocatechol	Bacteria utilized aniline, 3CA and 4CA as sole carbon, nitrogen and energy sources	(Loidl et al., 1990)
Pseudomonas	Resting	Aniline >	Chlorocatechol	Bacteria could not utilize CAs	(Kodama

AW-2	cell	2CA > 3CA > 4CA	and catechol	as a sole carbon and nitrogen source, but could degrade CAs at higher concentration	et al., 1997)
Pseudomonas acidovorans CA28	Entrapment in alginate	Aniline > 3CA > 4CA > 2CA	-	The degradation was investigated in an air-lift fermentor	(Ferschl et al., 1991)
-	Free cell and biofilm	34DCA	-	The packed bed reactor was found to be capable of degrading over 98% of the 34DCA (250 mg/l) with residence times of less than 4 h.	(Livingston and Willacy, 1991b)
-	Biofilm reactors	4CA	4-chlorocatechol	Degradation of 4CA in the presence of aniline	(Radianingtyas et al., 2003)
Paracoccus denitrificans 3XA	Free cell	34DCA	-	Using activated carbon as an adsorbent resulted in accelerated biodegradation of 34DCA	(Vasilyeva et al., 2003)
P. fluorescens 26-K	Free cell	34DCA	*Ortho*-cleavage pathway.	In the presence of glucose, the bacterial strain degraded 170 mg/L of 34DCA during 2–3 days. At 250 mg/L led to degradation of 34DCA during 4 days.	(Travkin et al., 2003a)
A. baumannii CA2, *P. putida* CA16	Free cell	4CA	4-chlorocatechol and catechol	Bacteria used 4CA as a sole carbon, nitrogen and energy source	(Vangnai and Petchkroh,

and *Klebsiella* sp. CA17.					2007)
Delftia tsuruhatensis H1	Free cell	2CA, 3CA and 4CA	*Ortho*-cleavage pathway	*Delftia tsuruhatensis* H1 was able to degrade several CAs as individual compounds or a mixture. The additions of yeast extract, citrate or succinate appeared to accelerate CA degradation.	(Zhang et al., 2010a)
Bacillus megaterium IMT21	Free cell	34DCA and othe dichloroanilines	Degrade 34DCA *via* dichloroacetanilide	The strain utilized 34DCA as a sole carbon, nitrogen source.	(Yao et al., 2011a)
A. baylyi GFJ2	Free cell, resting cell	Mono- and dichloroanilines	*Ortho*-cleavage pathway	Degradation CAs with the presence of co-substrates. The degradation of 4CA followed saturation kinetics of a Michaelis–Menten-like relationship, and 34DCA followed Edwards model	(Hongsawat and Vangnai, 2011)
B. licheniformis ycsd02	Free cell	4CA	-	Bacteria could degrade 4CA under aerobic and anaerobic condition with higher rate in aerobic media	(Ding et al., 2011)
Myroides odoratimimus LWD09	Free cell	34DCA	-	Degradation of 34DCA in saline condition. The kinetics of DCA degradation was well described using the Andrews	(Li et al., 2012b)

				equation	
	Aerobic granules	4CA	*Meta*-cleavage pathway	Mixed-culture microbial granules under aerobic conditions in a sequencing airlift bioreactor, following Haldane model	(Zhu et al., 2011b)
Mixed-culture	Aerobic granules	Aniline > 4ClA > 2ClA > 3ClA > 34DClA	-	Mixed-culture microbial granules under aerobic conditions in a sequencing airlift bioreactor	(Zhu et al., 2012)

2.5.2 CAs degradation pathways under aerobic conditions

The degradation of CAs *via meta*-cleavage pathway has been reported. *Diaphorobacter* sp. PCA039 was able to completely metabolize 4CA to TCA-cycle intermediates *via* the *meta*-cleavage pathway (Zhang et al., 2010b) (Figure 2-1). *Comamonas testosteroni* I2 isolated from activated sludge was able to transform 3CA completely as a carbon and nitrogen source, and further mineralization was *via meta*-pathway (Boon et al., 2000). The *meta* pathway of degradation toward chromatic compounds usually appears greenish yellow-color compounds such as 2-hydroxymuconic semialdehyde (Schmidt et al., 1983; Urata et al., 2004; Zeyer et al., 1985b). The *meta* degradation pathway may lead to incomplete metabolisms due to the production of dead-end or suicide-metabolites (Schmidt et al., 1983).

Ortho-cleavage is the dominant cleavage mechanism in the degradation of chlorinated compounds (Zhang et al., 2010b). *A. baumannii* CA2, *Pseudomonas putida* CA16 and *Klebsiella* sp. CA17 degraded 4CA *via* an *ortho*-cleavage pathway

by chlorocatechol 1, 2-dioxygenase (Hongsawat and Vangnai, 2011). 34DCA was transformed to 4CA as the initial intermediate product by *A. baylyi* strain GFJ2, then 4CA was degraded *via* an *ortho* and modified *ortho*-pathway forming aniline, 4-chlorocatechol (Hongsawat and Vangnai, 2011) (Figure 2-2). *Moraxella* sp. strain G was able to degrade 4CA to 4-chlorocatechol by aniline oxygenase (Figure 2-3), then it was mineralized *via* a modified *ortho* ring cleavage (Zeyer et al., 1985b). The degradation of 3CA by *P. acidovorans* CA28 occurred *via* an *ortho* pathway (Hinteregger et al., 1992).

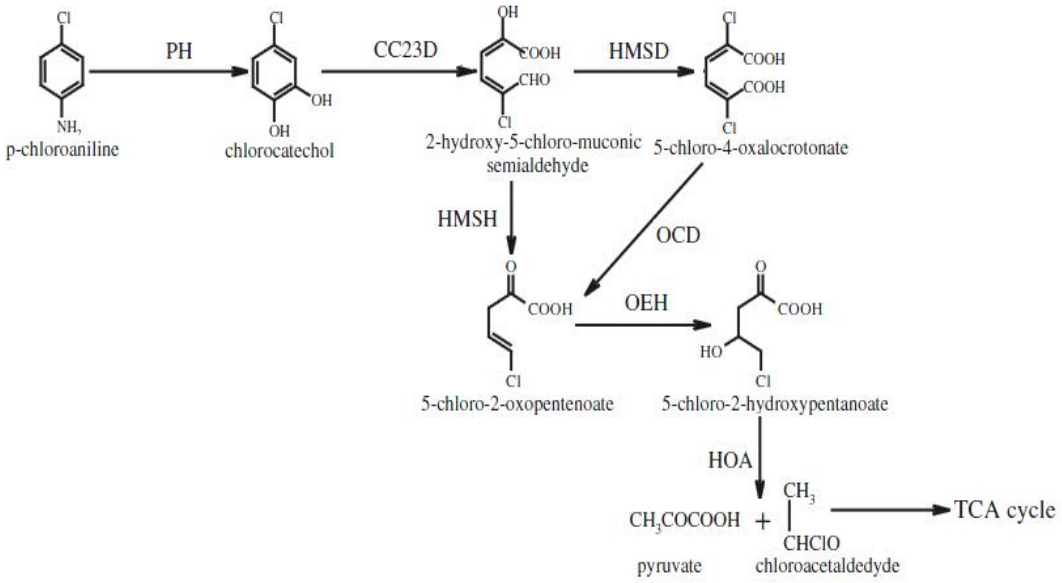


Figure 2-1. 4CA degradation pathway in *Diaphorobacter* sp. PCA039 (Zhang et al., 2010b). Enzyme activity: PH (Phenol hydroxylase); HMSD (2-hydroxymuconic semialdehyde dehydrogenase); HMSH (2-hydroxymuconic semialdehyde hydrolase); OCD (4-oxalocrotonate decarboxylase), OEH (2-oxopent-4-dienoate hydratase), HOA (4-hydroxy-2-oxovalerate aldolase) and ADA (acetaldehyde dehydrogenase).

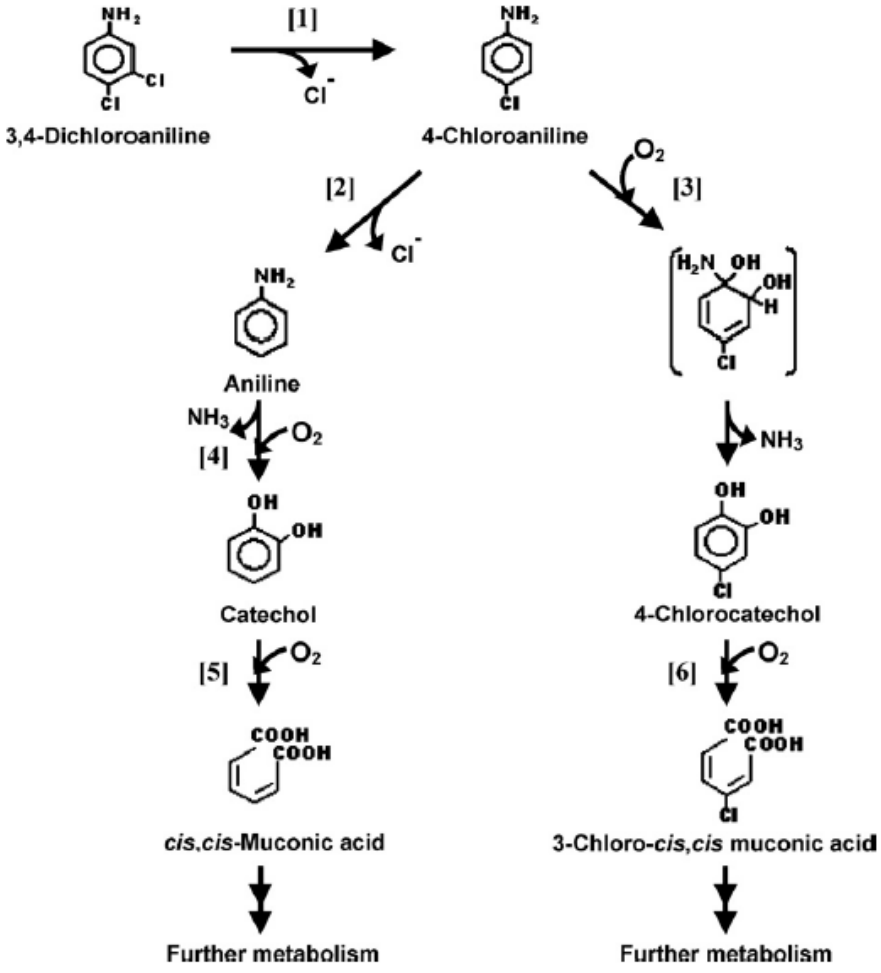


Figure 2-2. Degradation pathways for 34DCA and 4CA in *A. baylyi* strain GFJ2 (Hongsawat and Vangnai, 2011). Enzyme activity: [1], dechlorination; [2], dechlorination; [3], dioxygenation tentatively by chloroaniline dioxygenase; [4], dioxygenation tentatively by aniline dioxygenase; [5], catechol 1,2-dioxygenase; [6], chlorocatechol 1,2-dioxygenase.

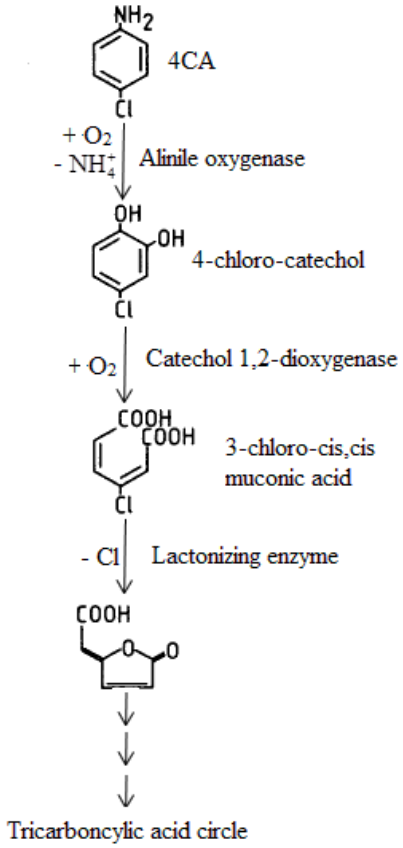


Figure 2-3. 4CA degradation pathway in *Moraxella* sp. strain G occurred *via* a modified *ortho*-cleavage pathway (Zeyer et al., 1985b).

You and Bartha (1982) reported that *P. putida* mineralized 34DCA to 4,5-dichlorocatechol as the first product by a dioxygenase, and then ring cleavage (You and Bartha, 1982b). The biodegradation pathway for 34DCA by this bacterial strain occurred through 4,5-dichlorocatechlo, 3,4-dichloromuconate, 3-chlorobutenolide and 3-chloro-4-ketadipate, and the ultimate end product was inorganic (Figure 2-4).

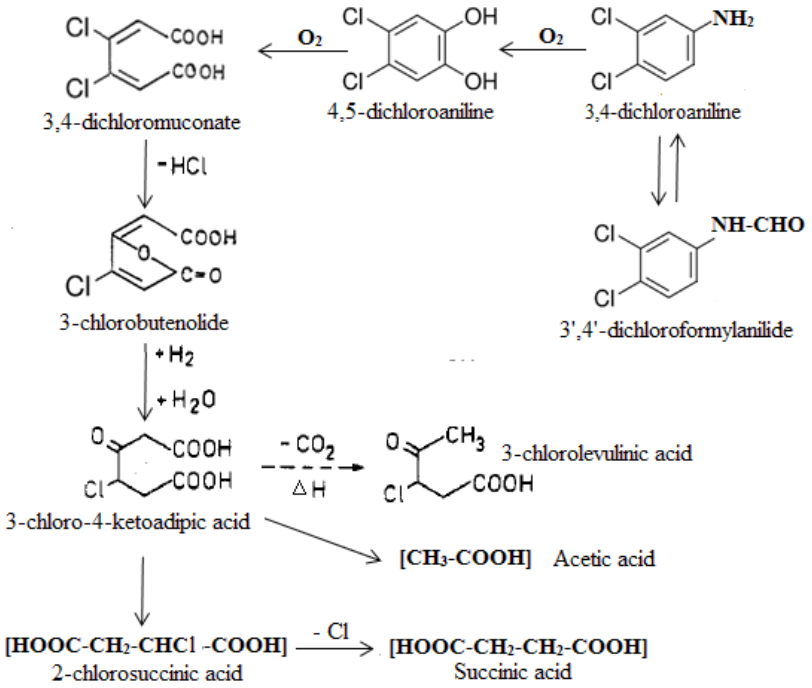


Figure 2-4. Biodegradation pathway for 34DCA in *P. putida* (You and Bartha, 1982b).

Another pathway was reported in *P. fluorescens* 26-K, where 3,4-dichloroacetanilide (formed by acylation) and 3,3-4,4-tetrachloroazoxybenzene (formed by polymerization) were detected as the intermediates in degradation of 34DCA (Travkin et al., 2003b).

2.6 Bacterial activities toward CAs degradation in anaerobic conditions

Bacteria can degrade organic compounds under anoxic and anaerobic conditions which microorganisms use inorganic ions as electron acceptors.

2.6.1 *Acinetobacter* in anoxic and anaerobic conditions

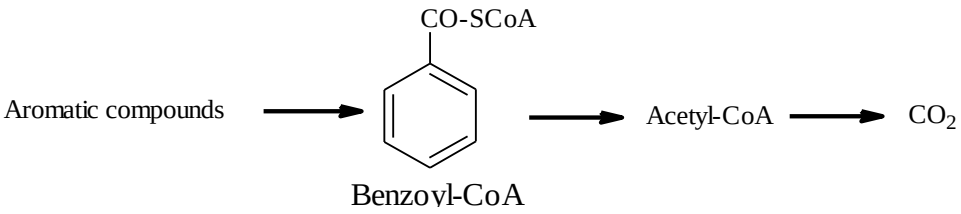
Acinetobacter is known as aerobic bacteria, but a previous study showed that *A. johnsonii* could convert nitrate to nitrite under anoxic conditions when an electron donor was provided (Boswell et al., 1999).

2.6.2 The aromatic degradation pathways under anaerobic conditions

The degradation of aromatic compounds under anaerobic conditions may take place *via* the upper or lower pathway. In the degradation process, microorganisms use inorganic compounds as electron acceptors and aromatic substrates as electron donors.

While catechol is one of the main central intermediates in the aerobic catabolism of aromatic compounds, benzoyl-CoA is known as the degradation product of aromatic compounds under anaerobic conditions.

The previous studies showed that *Alcaligenes faecalis* and *Enterobacter* species completely degraded phenol under anoxic conditions, and p-hydroxybenzoic was an intermediate product (Thomas et al., 2002). The biodegradation of Triclosan under anoxic conditions formed phenol, catechol, and 2, 4-dichlorophenol as intermediates (Gangadharan Puthiya Veetil et al., 2012). The degradation of aromatic compounds under anoxic and anaerobic conditions as following:



2.6.3 Degradation of aniline and chloroanilines in anaerobic conditions

In the anaerobic transformation of chlorinated anilines, dechlorination is common pathway. For example, pentachloroanilines were dechlorinated to dichloroanilines with the presence of sulfate as an electron acceptor (Ismail and Pavlostathis, 2010). Some anaerobic bacterial CAs- degrading strains have been reported (Table 2-5). The intermediates of anaerobic degradation toward 4CA and 34DCA were similar in estuarine sediment, methanogenic aquifer and pond sediment, which aniline and 3CA were produced (Table 2-6). The anaerobic degradation of CAs was reported with nitrate and sulfate as electron acceptors.

Table 2-5. Aniline and chlorinated anilines degradation under anaerobic conditions by pure cultures

Substrate	Bacterial strains	Electron acceptors	References
Aniline	*Delftia acidovorans* HY99	Nitrate	(Kahng et al., 2000)
Aniline	*Desuifobacterium anilini*	Sulfate	(Schnell et al., 1989)
2CA, 3CA, 4CA, 23DCA, 24DCA, 25DCA, 34DCA	*Paracoccus* sp.	Nitrate	(Bollag and Russel, 1976)
4CA	*Paracoccus* sp.	Nitrate	(Minard et al., 1977)
34DCA	*Rhodococc*us sp. 2	Nitrate	(Travkin et al., 2002)
34DCA	*E. coli*	Nitrate	(Bunce et al., 1983)

Table 2-6. Anaerobic degradation of CAs in estuarine sediment, methanogenic aquifer and pond sediment

Media	CAs	Intermediates products	References
Estuarine sediment	3CA 4CA 34DCA 2,3,4,5-tetrachloroaniline 345TCA	No detected Aniline 3CA and aniline 2,3,5TCA, 23DCA, 35DCA, 3CA 35DCA, 34DCA, 3CA	(Susarla et al., 1997)
Methanogenic aquifer	Di-, tri- and tetrachloroanilines	Tri-, di- and monochloroanilines, 3CA was an end product	(Kuhn and Suflita, 1989)
Pond sediment	34DCA	3CA and 4CA	(Struijs and Rogers, 1989)
Fermentative/methanogenic culture	Pentachloroanilines	Tetra-, tri- and dichloroanilies	(Ismail and Pavlostathis, 2010; Tas et al., 2006)

2.6.4 Electron acceptors

The anaerobic bacteria use NO_3^-, NO_2^-, SO_4^{2-}, dimethyl sulfoxide ($(CH_3)_2SO$), metal oxide (such as MnO_2), ClO_3^-, ClO_4^- or Fe^{3+} as electron acceptors. For anaerobic respiration, energy conservation can be accomplished with nitrate, ferric iron or sulfate as the electron acceptors (Heider et al., 1998; Thauer et al., 1977). However, the presence of several electron acceptors in the environment may enhance or inhibit contaminant biotransformation processes, the biodegradation of 34DCA with different electron acceptors needs to be determined.

2.6.4.1 Nitrate

Nitrate concentrations in surface water are normally low (0–18 mg/l) but can reach high levels as a result of agricultural runoff (WHO, 2011). Nitrate has been detected as a common component of municipal and industrial wastewaters. The concentration of nitrate in such wastewaters may range from less than 62 mg/1 to greater than 12.4 g/l (Boswell et al., 1999). In some places, nitrate concentrations are higher (Table 2-7).

Table 2-7. Nitrate concentration in surface water in some places.

Sources	Places	Concentrations (mg/l)	References
Metal industrial wastewater	Spain	700–1000	(Gabaldón et al., 2007)
Dairy wastewater	Germany	250	(Zayed and Winter, 1998)
Urban municipal wastewater	South Africans	0.32 – 6.5	(Odjadjare and Okoh, 2010)
River water	Swaziland	17.4 – 115	(Fadiran and Mamba, 2005)
Factory	Swaziland	72.3 – 75.7	
Drinking water	Swaziland	0.17 – 1.6	
Surface water	Luxembourg	10 – 40	(Rock and Mayer, 2002)
Surface water	Sardinia (Italy)	0.1 – 31.2	(Cidu and Biddau, 2012)
Ground water	Sardinia (Italy)	0.1 – 249	
Stream	Sardinia (Italy)	0.08 – 31.2	
River	Sardinia (Italy)	0.1 – 29.2	
Stream water of agricultural practices	Western Brittany (France)	25 – 76	(Ruiz et al., 2002)
Unconfined aquifer	Morocco	2 - 153	(Fekkoul et al., 2013)

2.6.4.2 Sulfate

Sulfate concentrations are various belonging to water sources. Extremely high sulfate concentrations in water have been recorded; for example, 1,500 mg/l in a coal mine in Pennsylvania, and 63,000 mg/l in a zinc mine in Idaho were detected (USEPA, 2003). The sulfate concentrations in some places are shown in Table 2-8.

Table 2-8. Sulfate concentration in surface water in some places.

Sources	Places	Concentration mg/l	References
Industrial wastewater	São Carlos, Brazil	200	(Sarti et al., 2012)
Urban surface water	-	20	(USEPA, 2003)
Agriculture surface water	-	25	(USEPA, 2003)
Ground urban water	-	20	(USEPA, 2003)
Ground agriculture water	-	24	(USEPA, 2003)
Public water systems	-	24 – 560	(USEPA, 2003)
Deep groundwater	Luxembourg	20 – 40	(Rock and Mayer,
Surface water		10 – 210	2002)

2.7 Environmental factors affecting biodegradation

There are a number of factors affecting biodegradation, including pH, temperature, moisture and others. Electron acceptors directly affect biodegradation of microorganisms. Oxygen is the terminal electron acceptor in aerobic processes.

The effects of chemical concentrations on CAs degradation are investigated. The increase of 4CA concentration resulted in lower growth rates of *Moraxella* sp. strain G (Zeyer et al., 1985b), lower degradation rates and growth rates of *Acinetobacter baumannii* CA2, *Pseudomonas putida* CA16 and *Klebsiella* sp. CA17 (Hongsawat and Vangnai, 2011). Similarly, the higher 34DCA concentration (> 0.6 mM) in media, the lower specific biodegradation of the substrate by *A. baylyi* GFJ2 (Hongsawat and Vangnai, 2011), and *Bacillus licheniformis* (Ding et al., 2011).

Nutrients, including nitrogen, carbon and energy sources, can be supported to enhance the degradation rates. Moreover, microorganisms play a key role in the biodegradation rate. The successful bioremediation depends on the native microbes or right augmented microbes which have a potential to degrade the pollutants. Co-substrates are nutrients sources affected on CAs degradation. For examples, the addition of YE into environment stimulated the degradation of some mono- and dichloroanilines (Zhang et al., 2010a). The addition of ammonium sulfate increased the biodegradation of 4CA by *A. baylyi* GFJ2, while the presence of sodium nitrate and urea decreased the degradation rate of bacterial strains (Hongsawat and Vangnai, 2011).

The effects of NaCl on 34DCA gradation and growth rates of *Myroides odoratimimus* LWD09 carried out (Li et al., 2012a). The strain LWD09 was moderately halophilic and showed the highest power of 34DCA degradation in media containing 5% NaCl. Moreover, other environmental factors such as temperature, pH were investigated on 4CA degradation (Ding et al., 2011) and 34DCA degradation (Li et al., 2012a).

2.8 Immobilized cells for bioremediation

Immobilization techniques are preferable for bioremediation. The carriers protect microorganisms from the toxicity of chemicals, which may improve the degrading efficiency of chemical compounds.

2.8.1 Methods of cell immobilization

The methods of immobilized cells include immobilization on the surface of a solid carrier, entrapment within a porous matrix, cell aggregation and containment behind a barrier, which are shown in Figure 2-5 (Kourkoutas et al., 2004).

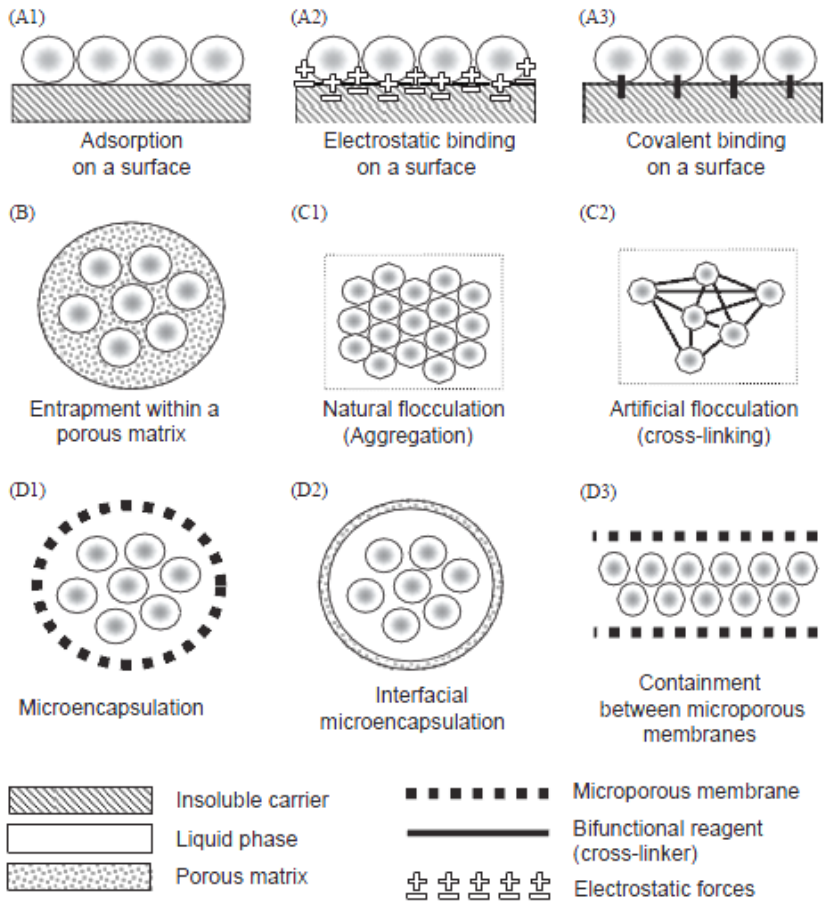


Figure 2-5. The basic methods of immobilized cells: Immobilized cells on the surface of a solid carrier (A), entrapment within a porous matrix (B), cell aggregation (flocculation) (C) and mechanical containment behind a barrier (D) (Kourkoutas et al., 2004).

2.8.2 Immobilization on surface of solid carriers

The immobilization of cells on a solid carrier by physical adsorption occurs because of electrostatic forces or by covalent binding between the cell membrane and the carrier (Kourkoutas et al., 2004). The cell attachment to an adsorption carrier depends on species, matrix types and environmental conditions (Pilkington et al.,

1998). The limitation of this method is that cells immobilized in matrices may leak because there is no barrier between cells and the surrounding medium (Pilkington et al., 1998).

2.8.2.1 Entrapment within a porous matrix

Cells penetrate into the porous matrix until their mobility is obstructed by the presence of other cells, or the porous material is formed in situ in a culture of cells (Kourkoutas et al., 2004). One of the problems of cell entrapment within a porous matrix is that the ability of cells locating on the outer layer of the beads multiplies and releases into environment (Kourkoutas et al., 2004).

2.8.2.2 Cell flocculation (aggregation)

Cell aggregation is the gathering together of units to make larger units or suspended cells adhere together to form clumps and sediment (Jin and Speers, 1998). The cell flocculation is considered as an immobilization technique applied in some types of reactors, including packed-bed, fluidized-bed and continuous stirred-tank reactors.

2.8.2.3 Cells contained behind a barrier

Cells may be retained by the porous membrane or entrapment of cells in a microcapsule or by cell immobilization onto an interaction surface of two immiscible liquids (Kourkoutas et al., 2004). The mass transfer limitations between microporous membranes and possible membrane biofouling caused by cell growth are

major disadvantages of cell immobilization between microporous membranes (Kourkoutas et al., 2004).

2.8.3 Immobilization of bacteria in biofilm

2.8.3.1 Biofilm formation process

Biofilms can be defined as communities of microorganisms attached to a surface (O'Toole et al., 2000). The biofilm formation relates to embedded process within a self-produced matrix of extracellular polymeric substance. The biofilm formation process relates to altered genetic genotype expression, physiology and signal molecule induced communication (Andersson, 2009). The process includes five steps: formation of conditioning films on the surface, initial adherence of bacterial cells, irreversible attachment of bacteria, maturation of the biofilm and detachment (Andersson, 2009).

The activities of microorganisms applied for biodegradation are limited by the environmental parameters such as temperature, pH, nutrients, oxygen, microorganisms, toxic chemicals (Vidali, 2001).

2.8.3.2 Application of biofilm for remediation

Biofilm has been applied for bioremediation in bioreactors such as Up flow Sludge Blanket, Biofilm Fluidized Bed, and other biofilm reactors. The advantages of using biofilm for bioremediation are high productivities, high cell concentration, longer reactor and economic operation (Nasib Qureshi, 2005). However, bacteria can detach, so effluent containing cells, and further treatment is required (Nasib Qureshi, 2005).

2.8.4 Immobilization of cells in alginate, PVA and mixture of PVA-alginate

2.8.4.1 Alginate

Ca-alginate is widely used for cell immobilization (Table 2-9). Alginic acid is an anionic polysaccharide produced by brown algae. Alginic acid includes a linear copolymer with homopolymeric blocks of (1-4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G) residues. The cross-linking network was formed by Calcium (Ca^{2+}) ions bonding with polyguluronic portions of the polymer (Cassidy et al., 1996). Due to cheap, easy to handle and nontoxic property, alginate has been widely used in encapsulation, including biomedical, pharmaceutical applications, bioprocess and food. However, alginate is sensitive towards chelating compounds such as phosphate, citrate and lactate, or anti-gelling cations such as Na^{+} or Mg^{2+} leading to its limitation application for wastewater treatment. The alginate structure is shown in Figure 2-6.

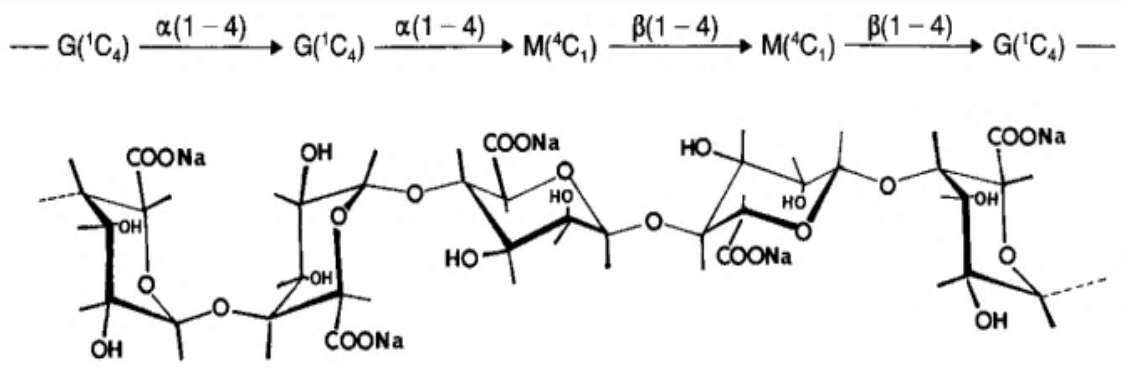


Figure 2-6. Structure chemical of calcium alginate (Smidsrød and Skjak-Break, 1990). G: Guluronic acid; M: Mannuronic acid.

With the presence of divalent cations such as Ca^{2+}, sodium alginate rapidly forms a gel structure to form a highly compacted gel network (Figure 2-7) (Kashima

et al., 2012). The spherical gel beads of calcium alginate are often applied as a carrier of immobilized living cells, enzymes, drug delivery capsules and food supplement. Some reports on immobilization ò bacteria in alginate were shown in Table 2-9.

$$\text{Sodium alginate} + CaCl_2 \longrightarrow NaCl + \text{calcium alginate}$$

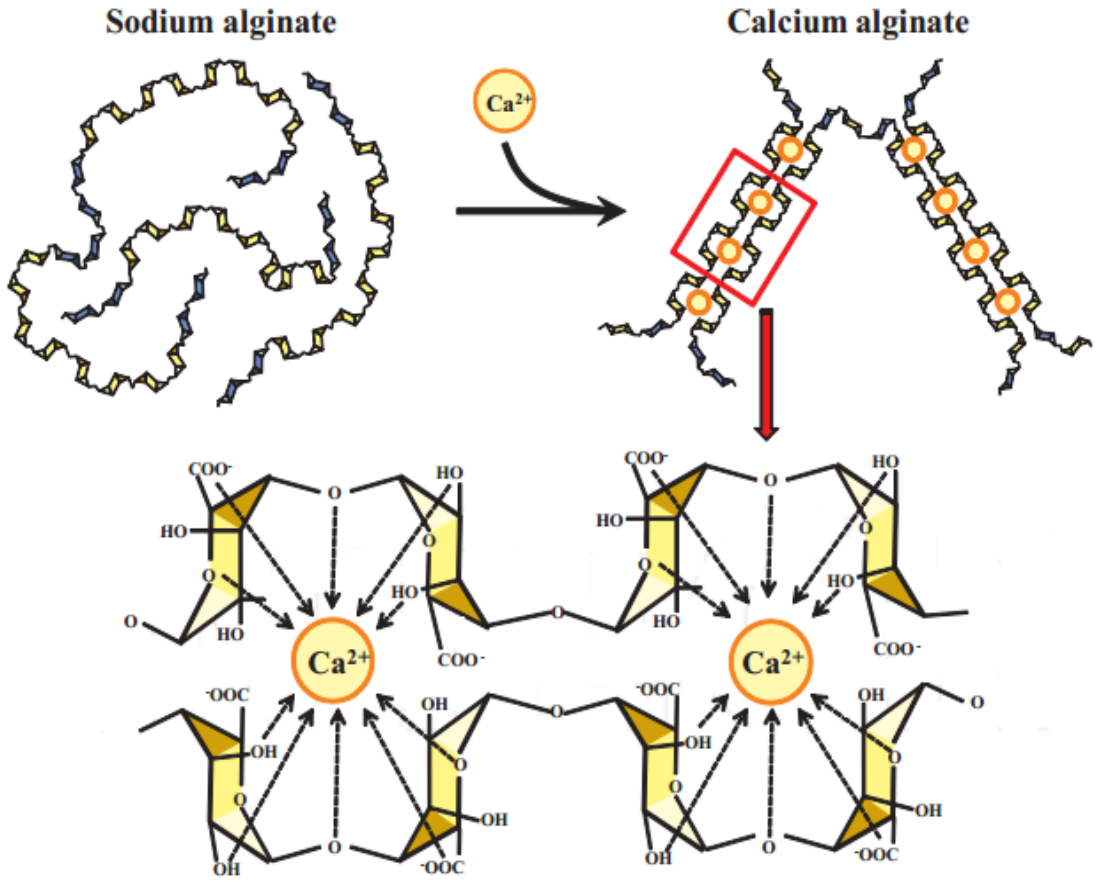


Figure 2-7. The formation of α-L-guluronic acid junction with calcium ions to form "Egg-box" model (Kashima et al., 2012).

Table 2-9. Reports on bacteria immobilized in alginate used for biodegradation

Bacteria	Chemicals	Concentration of cells	Alginate concentration (%)	Number of cell for degradation (cells/ml)	Bead diameter (mm)	References
Bacillus spp	p-nitrophenol	3×10^6 CFUs/bead	3, 4, 5	$5.4x10^6$	-	(Sreenivasulu et al., 2012)
Pseudomonas putida MK1	Pyridine	4.8 g dry weight/l	1.5	-	1, 3	(Kim et al., 2006)
Consortium	Organophosphate	1.23×10^6 CFUs/bead	4	-	2–3	(Yanez-Ocampo et al., 2009)
Bacillus subtilis strain 3C3	Diethyl phthalate	10^8 CFUs/bead	2, 3, 5	$2x10^9$	3, 9	(Sompornpailin et al., 2014)
Bacillus cereus AKG1 MTCC9817 and AKG2 MTCC 9818	Phenol	$3.5x10^9$ CFU/ml solution for immobilization	4.2	-	-	(Banerjee and Ghoshal, 2010)
Pseudomonas putida A ATCC 12633	Cationic surfactants	10^8 CFUs/bead	2.7	-	3	(Bergero and Lucchesi, 2013)
Methylibium petroleiphilum PM1	Methyl tert-butyl ether	1.2, 2.1, 4.3, 6.5 and 8.6 g dry weight/l	2, 3, 4, 5, 6	-	2.0, 2.5, 3.0, 4.0 and 5.0 mm	(Chen et al., 2008)

Nocardioides sp. NSP41	Phenol and *p*-nitrophenol	50 g wet weight/l	1.5	-	1.0–1.2	(Cho et al., 2000)
Rhodococcus rhodochrous	2-Aminobenzothiazole	10^7 CFUs/bead	4	-	3	(Chorao et al., 2009)
Bacillus sp.	Chloroform	$2x10^8$ CFUs/bead	0.5, 1.0, 1.5, 2.0, 2.5, 3.0	-	3	(Dey and Roy, 2011)
Pseudomonas putida A	Trimethylamine	$1\text{-}5x10^6$ CFUs/ ml bead	4	$6.4x10^6$	3	(Liffourrena and Lucchesi, 2014)
Bacillus sp.	di-n-butylphthalate	50 g wet weight/l	2.0, 3.0, 4.0, 5.0	10^{12}	2.5, 3.0, 3.5, 4.0, 4.5, 5.0	(Patil and Karegoudar, 2005)

2.8.4.2 PVA

PVA is a synthetic material and has the idealized formula $[CH_2CH(OH)]_n$. It is nontoxic and broadly used in cell immobilization for bioremediation. PVA has relatively good tensile stress, good biocompatibility, impact strength, high water affinity and porosity (Awad and Abuzaid, 2000; Yujian et al., 2006; Zhang et al., 2007). Due to good characteristics, PVA is preferred for cell immobilization in wastewater treatment (Zhang et al., 2007). In immobilization process, PVA was first crosslinked with boric acid to form spherical beads (Figure 2-8), which was followed by solidifying by esterification of PVA with phosphate (Chen and Lin, 1994). The immobilization by freeze-thawing method without using boric acid and phosphate is promising gel carriers for cell immobilization (Lozinsky et al.,

1997). In another method, Na_2SO_4 is applied in the immobilization process as the cross-linking agent (Zain et al., 2011). However, the agglomeration is a problem in the immobilization process. Using PVA for cell immobilization was shown in several reports (Table 2-10).

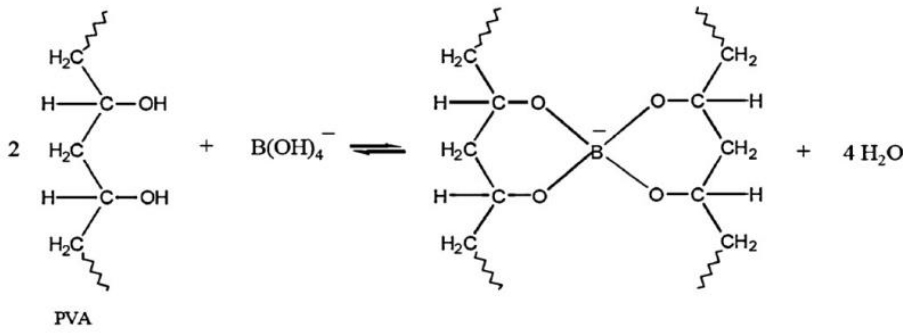


Figure 2-8. The formation of cross-liking between PVA and boric acid (Zain et al., 2011).

Table 2-10. Reports on bacteria immobilized in PVA used for biodegradation

Bacteria	Chemicals	Numbers of cells	PVA concentration (%)	Size of carrier	References
Pseudomonas putida	Phenol	0.4 g dry weight/l	30	1x1x1 cm	(Al-Zuhair and El-Naas, 2011)
Pseudomonas sp.	di-n-butyl phthalate	$2x10^7$ CFUs/ml bead	10	-	(Wang et al., 1997)
Agrobacterium radiobacter J14a	Atrazine	3.5 g dry weight/l	10	-	(Siripattanakul et al., 2009)
Pseudomonas putida	Phenol	-	10		(El-Naas et al., 2009)
Thiobacillus denitrificans	Nitrate removal	-	10	4	(Zhang et al., 2009)
Acinetobacter sp. XA05 and *Sphingomonas* sp. FG03	Phenol	25.1 g wet weight/l	7	2x2x2	(Liu et al., 2009)

2.8.4.3 Mixture of PVA-alginate

The mixture of PVA-alginate is usually applied for cell immobilization because the addition of alginate prevents agglomeration and improves the surface properties (Wu and Wisecarver, 1992). Cells immobilized in PVA-alginate enhance the cell growth inside the beads and biodegradation of chemicals (Mollaei et al., 2010). Using PVA for cell immobilization was shown in several reports (Table 2-11).

Table 2-11. Reports on bacteria immobilized in PVA-alginate beads used for biodegradation

Bacteria	Chemicals	Numbers of cells	PVA concentration (%)	Alginate concentration (%)	Bead diameter (mm)	References
Ochrobactrum sp.	Dimethylformamide	6.23× 10^{10} CFUs/g	4.5	2	2-3	(Kumar et al., 2012)
Acidithiobacillus ferrooxidans	Reduced iron content	10^9 cells/ml bead	9	0.9	4	(Yujian et al., 2006)
Burkholderia vietnamiensis C09V	crystal violet	-	10	0.3	3	(Cheng et al., 2012)
Bacillus flexus strain XJU-4	3-nitrobenzoate	$1.15x10^{11}$ CFU/bead	6	4	3	(Mulla et al., 2012)
Micrococcus sp.	2-nitrotoluene	$1.8x10^{11}$ to $2x10^{11}$ CFU/g beads	6	4	3	(Mulla et al., 2013)
Paracoccus denitrificans SD1	N,N-dimethylformamide	4.92 – $6.76x10^{10}$ CFU/g beads	4.5	2	2-3	(Sanjeevkumar et al., 2013)

2.8.5 Disadvantages of immobilized cells and enhancement of porosity and mass transfer of carriers

The poor permeability and mass transfer resistance due to the close crosslinked structure and small internal pores of carriers are major problems with PVA and PVA-alginate system (Chen et al., 1996; Park and Hoffman, 1990). Mass transfer is considered as one of the major factors affecting the activity of immobilized cells (Ha et al., 2008). To improve the permeability and mass transfer, some methods have been applied. For examples, polyethylene oxide, partially saponified PVA and soluble starch (Chen et al., 1996; Ichijo et al., 1990), sodium bicarbonate (Li et al., 2011) and PEG (Zhang and Ye, 2011) were added into carriers as porogens to improve the porosity of carriers (Table 2-12).

2.9 Roles of cryoprotectants in freeze-drying and storage of cells

Freeze-dried, entrapped cells have been applied for cell preservation in long-term storage (Kearney et al., 1990; Sompornpailin et al., 2014). However, a freeze drying process may be harmful to bacterial cells. The cell survival is influenced by some conditions such as storage temperature, moisture content, oxygen content, exposure to light and storage materials and others.

A variety of protectants, including skim milk powder, trehalose, glycerol, sucrose, glucose, lactose and polymers have been used in the freeze-drying process (Hubálek, 2003). Cryoprotectants can enhance cell survival during the freeze-drying process. The osmotic difference between the internal and external environments reduces with the accumulation of cryoprotective agents within the cells (Kets et al.,

1996). However, these compounds may become preferred carbon sources for bacteria, which may lead to inactivation or inhibition of biodegradation.

The previous study showed that using sucrose as a cryoprotectants could effectively sustain ability of the freeze-dried, entrapped cells for diethyl phthalate biodegradation after long-term storage (Sompornpailin et al., 2014). 75% rhizobacteria immobilized in alginate beads with starch as an osmoprotectant survived after one year storage at 4 ^{o}C (Schoebitz et al., 2012).

CHAPTER 3 MATERIALS AND METHODS

3.1 Laboratory equipment and chemicals

3.1.1 Equipments

Laboratory equipment	Company	Country
Autoclave HV-110	Hirayama	Japan
Autoclave NLS-3020	Sanyo Electric Co., Ltd	Japan
C18 column (Hyperclone 5u BDS C18 130A, a 250 x 4.6 mm)	Phenomenex	USA
Centrifuge (Prism R)	Labnet	USA
High Performance Liquid Chromatography (HPLC) LC-20	Shimazu	Japan
Hotplate stirrer	Lab Tech	Korea
Incubator shaker, innova 4000	New Brunswick scientific	USA
Incubator shaker, innova 4340	New Brunswick scientific	USA
Micropipette 20,100, 200, 1000 µl	Gilson	France
MiniRun Gel Electrophoresis	Bioer Technology	USA
pH meter	Mettler Toledo	USA
Protector Laboratory Hood	Science Technology	USA
Refrigerated Centrifuge, 5804R	Eppendorf	USA

Refrigerated Centrifuge, Avanti^{Tm} J-301	Beckman Coulter	USA
Spectrophotometer DU 650	Beckman	USA
Ultrasonic	Banderlin	Germany
Vortex (Touch mixer model 232)	Fisher Scientific	USA

3.1.2 Chemicals

3.1.2.1 Grade chemicals

Chemical	**Company**	**Country**
2,3-dichloroaniline (99.5% purity)	Chem Service, Inc.	USA
2,4,6-trichloroaniline	Chem Service, Inc.	USA
2-chloroaniline (99.5% purity)	Chem Service, Inc.	USA
3,4-dichloroaniline (99.5% purity)	Chem Service, Inc.	USA
3-chloroaniline (99.5% purity)	Chem Service, Inc.	USA
4-chloroaniline (99.5% purity)	Chem Service, Inc.	USA
4-chlorocatechol (99.5% purity)	Aldrish	Germany
Agar	Scharlau Chemic Microbiology	Spain
Ammonium sulfate	Bio-Rad	USA
Aniline	Merck	Germany
Bovine serum albumin (BSA)	Sigma	USA
$CaCl_2.2H_2O$	Merck	Germany
$CoCl_2.6H_2O$	Merck	Germany
$CuSO_4.5H_2O$	Scharlau Microbiology	Spain

Diazotization of sulphanilamide	Merck	Germany
$FeSO_4.7H_2O$	BDH	England
Folin-Ciocalteu's reagent	Carlo Erba Reagenti	Italy
Glycerol	Univar	Australia
H_3BO_3	Merck	Germany
H_3PO_4	Merck	Germany
Iodine crystal	BDH	England
K_2HPO_4	Riedel	Germany
KH_2PO_4	Carlo Erba Reagenti	Italy
$MgSO_4.7H_2O$	Carlo Erba Reagenti	Italy
$MnSO_4.H_2O$	Merck	Germany
MoO_3	Merck	Germany
Na_2CO_3	BDH	England
Na_2HPO_4	Fluka	Germany
Na_2SO_4	Fluka	Germany
NaCl	BDH	England
$NaNO_3$	Carlo Erba Reagenti	Italy
NaOH	Merck	Germany
Peptone	Merck	Germany
N,N-*dimethyl-p*-phenylenediamine sulfate	Merck	Germany
Poly(ethylene glycol)	Alorich	Germany
Sodium nitroprusside	Merck	Germany

Sodium salicylate	Merck	Germany
Succinic acid	Merck	Germany
Yeast extract	Scharlau Chemic Microbiology	Spain
$ZnSO_4.7H_2O$	Merck	Germany

3.1.2.2 Grade organic solvent

Chemical	**Company**	**Country**
Absolute ethanol (99.9% purity)	Merck	Germany
Acetic acid, glacial	J.T. Baker	USA
Benzene	Merck	Germany
Hexane		
Methanol	Merck	Germany
Toluene	J.T. Baker	USA

3.1.3 Culture medium and cultivation conditions

3.1.3.1 The mineral medium (MM medium)

The mineral medium was used for cultivation and degradation. The mineral medium was comprised of media and trace element (Dejonghe et al., 2003):

a) Media (g/l)

Na_2HPO_4	1.4196
KH_2PO_4	1.3609
$MgSO_4.7H_2O$	0.0985

$CaCl_2.H_2O$	0.0059

The component was dissolved in 1 liter of distilled water and adjusted pH to 7.0 by 1.0 M NaOH. The mineral medium was autoclaved at 121 ^{o}C for 15 min.

b) Trace elements (g/l):

H_3BO_4	0.116
$FeSO_4.7H_2O$	0.278
$ZnSO_4.7H_2O$	0.115
$MnSO_4.H_2O$	0.169
$CuSO_4.H_2O$	0.038
$CoCl_2.6H_2O$	0.024
MoO_3	0.010

Trace element solution was separately prepared as a stock solution. They were dissolved in 100 ml of distilled water and it was autoclaved at 121 ˚C for 15 min. Before using, 0.1% (v/v) sterile trace element was supplemented in mineral medium.

3.1.3.2 Luria-Bertani (LB)

The LB medium includes: Trytone 10 g, Yeast extracts 5 g and NaCl 10 g. The components were dissolved in 1 liter of deionized water, adjusted pH 7.0 and autoclaved at 121 ºC for 15 min.

3.2 *A. baumannii* GFJ1

3.2.1 Isolation and identification of CA-degrading bacteria

The CA-degrading bacterial strain was isolated from soil by **Dr. Worrawat Promden** with the way by enrichment culture as previously reported (Hongsawat and Vangnai, 2011). Genomic DNA samples were extracted using an InstaGene^{tm} Matrix (BIO-RAD, Hercules, USA). The 16S rRNA gene fragment was PCR amplified using the universal primers: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') at 95 ^{o}C for 2 min and then 35 cycles of 95, 55 and 72 °C for 1 min each, followed by 10 min at 72 °C. The purification of amplification products was carried out using a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing was performed using a PRISM BigDye Terminator v3.1 cycle sequencing kit. Related sequences were determined using BLAST and obtained from the GenBank database (National Center for Biotechnology Information).

The strain GFJ1, a Gram-negative, coccus-shaped bacterium, utilized a broad range of CAs as a sole carbon, nitrogen and energy source was identified. The 16S rRNA sequence had the highest degree of nucleotide identity with *A. baumannii* isolates (99% identity) of the sequences available in the NCBI GenBank database and the NJ based phylogenetic analysis placed it within the *A. baumannii* sequences in the genus *Acinetobacter* (Figure 3-1). Accordingly, this strain is referred to *A. baumannii* GFJ1 here after.

3.2.2 Phylogenetic tree

Sequences were aligned using the ClustalW algorithm and followed with the MEGA 6.0 software. The neighbor-joining (NJ) distance method and Tamura-Nei model in the MEGA 6.0 program was used to construct the phylogenetic tree. Confidence levels were generated by bootstrapping, based on 1,000 resamplings (Figure 3-1).

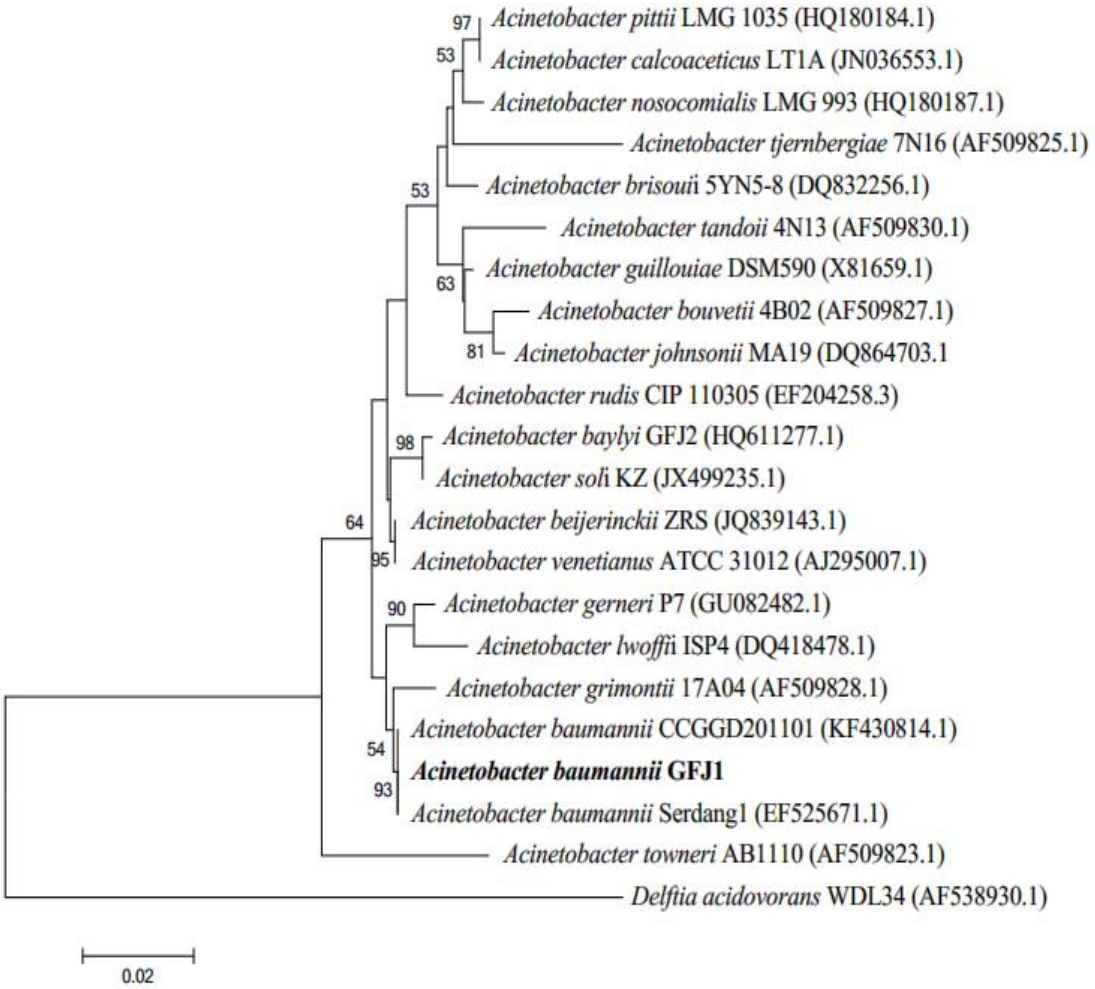


Figure 3-1. Phylogenetic tree based on 16S rRNA gene fragment shows the *A. baumannii* GFJ1 (1426 bases in 16S rRNA) position in the genus *Acinetobacter*. The

isolate GFJ1 (bold type) was identified as *A. baumannii* GFJ1 by sequence identity, and was phylogenetically placed within the genus *Acinetobacter*. The tree was constructed using the Tamura-Nei model in the MEGA version 6.0 software. The numbers at the nodes represent bootstrap values (expressed as percentages based on analysis of 1,000 resampled data sets) where greater than 50%. The accession numbers corresponding to each strain were presented in parentheses. The scale bar (0.02) shows the number of nucleotide substitutions per base.

3.3 Starter inoculum preparation

The starter inoculum was prepared as follows: One loop of bacterial colony collected from a LB agar plate was transferred into a 50-ml glass bottle containing 10 ml LB medium. The bottle was then aerobically incubated for 12 hours at room temperature (about 30 ^{o}C) with a shaking speed of 150 rpm. Cell inoculum with a turbidity of ~1.4 at 600 nm was always used at 1.0% for bacterial culture in all experiments.

3.4 Incubation condition

All of the experiments, the degradation of CAs was carried out at room temperature (about 30 ^{o}C) with a shaking speed of 150 *rpm, except degradation by biofilm (carried out at 100 rpm).*

3.5 Determination of aerobic utilization toward CAs

3.5.1 Utilization of aniline and chlorinated anilines as sources of carbon, nitrogen and energy sources

The utilization of aniline and chlorinated anilines as sources of carbon, nitrogen and energy sources was carried out in MM medium supplemented with 0.1 mM mono- or dichloroanilines. Because trichloroanilines (TCAs) were more toxic and lower water solubility than aniline, mono- and dichloroanilines, the effects of TCAs on growth and degradation rates were carried out at 0.01 and 0.05 mM.

3.5.2 Determination of aerobic utilization kinetics toward CAs

The starter inoculum was transferred into other 250 ml flasks containing 50 ml of the MM medium. One of the CAs was added at different concentrations. The bottles were then incubated and samples were taken for determination of cell growth and chemical remaining. The controls without bacteria were carried out in parallel.

The plot of the substrate concentrations versus time was used to calculate the substrate degradation rates. The non-growth Michaelis–Menten model developed by Schmidt (Schmidt et al., 1985) was used to obtain the kinetic parameters including maximum degradation rate (V_{max}) and half-maximal *rate constant* (K_s) values of each substrate derived from a linear regression fitting using the double-reciprocal plot. The inhibition constant was determined using a Dixon plot (Dixon, 1953), in which the reciprocal of the velocity ($1/v$) was plotted against the inhibition concentration (I) to obtain data.

3.5.3 Determination of effects of co-substrates on CAs degradation

The effects of co-substrates on biodegradation rates were carried out with the addition of nutrients in to MM medium, and CAs were supplemented at 0.1 mM. The effects of nutrient components included succinate, citrate and humate (carbon sources), yeast extract, sodium nitrate, ammonium chloride and ammonium sulfate (nitrogen sources) on bacterial growth and biodegradation of CAs were examined.

3.5.4 Determination of effects of NaCl on the bacterial growth rate and CAs degradation under aerobic conditions

NaCl stands for total dissolve solids (TDS) which may present in wastewater. Furthermore, saline environments are frequently contaminated with organic compounds from industrial activities (Li et al., 2012a).

For the growth rate determination, the starter inoculum was transferred to 250-ml bottle containing 50 ml of MM medium supplemented with 0.1% YE, 0.1% succinate and 0.1% ammonium sulfate (w/v) (MYSA medium). NaCl was supplemented at different concentrations and CAs were supplemented at 0.1 mM. The bottles were aerobically incubated, samples were taken for determination of cell growth and chemical remaining.

The experiment was carried out *via* resting cells**,** the starter inoculum was transferred to 500 ml-flasks containing 100 ml of MYSA medium. NaCl and CAs were supplemented at 0.5% (w/v) (8.55 mM) and 0.1 mM, respectively. After 12 hours of incubation, cells were harvested by centrifugation for 20 min at 6,000 rpm and 4^{o}C. Cells were then washed twice with NaCl solution (0.85%). Cell pellets were

transferred to 50-ml bottle containing 10 ml MYSA medium. NaCl was supplemented at various concentrations, and 4CA or 34DCA was supplemented at 0.1 mM.

The inhibition of CA degradation by NaCl was obtained mathematically by using the method described by Amor (Amor et al., 2001). The specific degradation of chroaniline (r) was dependent on NaCl concentrations (S). A plot of 1/r versus S was used for calculation K_i (NaCl inhibition constant). For reciprocal plot, the equation was written as:

$$\frac{1}{r} = \frac{1}{r_{max}} + \frac{S}{r_{max} k_i}$$

where r_{max} is maximum of NaCl concentration.

3.5.5 Determination of CAs degradation using resting cells

The resting cell samples are transferred to 50 – ml glass bottles containing 10 ml of the same medium (around $3.5x10^9$ CFU/ml). CAs are added at varies of concentration (from 0.005 to 1.0 mM). The bottles were then shaken at the same conditions. 0.5 ml of samples was collected at 3-hour intervals to determine the residual of CAs by HPLC.

3.5.6 Chemical degradation in anaerobic conditions

The anaerobic experiments were carried out using 60-ml serum vials containing 15 ml of sterile MM medium with and without supplementation with co-substrates. 4CA or 34DCA was added at 0.1 mM. Co-substrates included yeast extract (0.01%, w/v) and succinate (0.01%, w/v). The electron acceptors including nitrate ($NaNO_3$) and sulfate (Na_2SO_4) were prepared as stock solutions, and used at 50 ml/l

(0.005%) and 100 mg/l (0.01%) nitrate or sulfate (w/v) which in ranges of nitrate and sulfate detected in environment (shown in 2.4.6.1 and 2.4.6.2). The anaerobic media were prepared by boiling and then bubbling with nitrogen gas. These vials were immediately sealed with rubber septum and aluminium crimps and incubated. The vials without electron acceptor or without bacteria served as controls and were run in parallel. Syringes with needles were used for substrate addition and sample collection.

3.6 Determination of biofilm formation and degradation toward CAs by biofilm

3.6.1 Determination of bacterial biofilm formation on a 96-well microplate

The determination of biofilm formation on a 96-well polystyrene microplates was described by O'Toole and Kolte (O'toole and Kolter, 1998). To each well, 150 µL an indicated medium was added. After inoculation, the plates were covered with plastic to prevent evaporation. The microplates were aerobically incubated at 100 rpm and room temperature for 24 hours. After incubation, the plates were rinsed three times with tap water in order to remove non-adhesion bacteria, and dried by converting.

The biofilm formation was examined by staining with 150 µL of crystal violet (0.1%) per well. 300 µL of mixture ethanol and acetone (> 99%) (80 : 20, v/v) was added to dilute the stain. The solution was transferred to 1.5-m Eppendorf tubes, adjusted to 1.0 ml with distil water, and absorbance was determined using a spectrophotometer (at 600 nm) (O'toole and Kolter, 1998). *P. putida* TK2440 was

used as a positive control (Duque et al., 2012; Mohamed et al., 2007) and media without bacteria as a negative control.

3.6.2 Determination of concentrations of CAs and incubation time on bacterial biofilm formation

The effects of chemical concentrations on biofilm formation were carried out using a 96-well polystyrene microplate. Bacteria were cultured in MM medium supplemented with 0.01% YE and 0.1% $NaNO_3$. CAs were supplemented with a series of CAs concentrations (from 0.05 to 1.0 mM). The plates were incubated for 0, 12, 24, 48, 72 hours at room temperature with shaking speed of 100 rpm for biofilm formation.

3.6.3 Determination of degradation toward CAs by biofilm

For chemical degradation by biofilm, after 24 hours of incubation the medium was removed and microplates were rinsed triplicate with sterilized saline solution (0.85% NaCl). The wells were then filled with the same medium and incubated. Samples were taken after 3 hours, and the microplates were continued to incubate for 21 hours. The same operation was repeated in the following intervals of 24 hours. The experiment was carried out at chemical concentration of 0.1 mM. The immobilized cells on the well wall were determined by scrapping and counting cells detached by CFU technique.

3.7 Determination of degradation pathways

The metabolites of aerobic and anaerobic degradation were analyzed on one dimension thin layer chromatography (1D-TLC) silica gel 60 F254 plates (Merck KGaA, Darmstadt, Germany) in two mobile phase solvents: 10:20:1 (v/v/v) ratio hexane: benzene: acetone, and 9 : 1 (v/v) ratio benzene : acetic acid. The culture supernatants were harvested at the indicated time point, extracted and concentrated under vacuum prior to 1D-TLC separation. The substrates and metabolites were visualized under UV light at 254 nm, and the spots were scrapped off and extracted with methanol for mass spectrometry (MS) analysis using an LTQ Orbitrap XL (Thermo Fischer Scientific Inc., MA, USA) in the flow-injection mode.

3.8 Degradation toward CAs by *A. baumannii* GFJ1 entrapped in PVA-alginate beads

3.8.1 Immobilization methods

For the entrapped cell preparation, *A. baumannii* GFJ1 were cultured in MM medium supplemented with yeast extract, succinate and sodium nitrate (0.1% each, w/v) for 12 hours. Cells were collected by centrifugation at 6,000 rpm and 4 ^{o}C for 20 min. Cell pellet was washed twice with sterile saline (0.85% NaCl) and resuspended in 2xMM medium. The entrapment of cells in the PVA-alginate-$CaCO_3$ gel was performed according to the previous report (Bai et al., 2010) with modifications. The mixture of PVA, alginate and $CaCO_3$ (from 3% to 10% $CaCO_3$, w/v) was dissolved and blended in a boiling water bath. The solution was then cooled to room

temperature, mixed with concentrated bacteria (1:1, v/v) and gently stirred for 30 min. The mixture was dripped into a solution containing saturated boric acid and 3% $CaCl_2$ (w/v) using a syringe and stirred for 1.0 hour. After storage for 24 hours in the solution at 4^{o}C, beads were washed twice with sterile saline (0.85% NaCl). The coagulated beads were immersed in 0.1 M hydrochloric acid to dissolve calcium carbonate until no more bubbles were observed. Beads were then soaked in sterile distilled water and agitated for 30 min to remove HCl which might diffuse into the beads.

The immobilization of cells in PVA-alginate-PEG matrix was performed according to the previous report (Zhang and Ye, 2011) with modification so that the process was similar to the method with $CaCO_3$ as a porogen described above. However, beads were soaked in sterile distilled water and agitated at 150 rpm for 6 hours for removal of PEG from the beads instead of treatment with HCl. PEG is a water soluble compound, and it is diluted in water and removed.

For the entrapment of cells in PVA, the cell suspension was mixed thoroughly with a PVA solution by gently stirring to give a final concentration of 10% PVA (w/v). The mixture was dropped into a saturated boric acid solution and stored for 24 hours at 4 ^{o}C.

For the immobilization of bacteria in beads with a cryoprotectant (glycerol, sucrose, sorbitol and mannitol), a cryoprotectant was incorporated into the solution to give a final concentration of 10% or 20% (w/v) which based on the typical concentration used for bacterial cells (Hubálek, 2003). The formed beads with the average diameter were roughly 3 mm in size and contained $3.5x10^{9}$ CFUs/g wet bead

in each immobilization type, which were in in average of entrapped cells shown in Table 2-9, 2-10 and 2-11.

3.8.2 34DCA degradation rate and bacterial growth of immobilized cells

3.8.2.1 34DCA degradation by immobilized cells

The biodegradation of 34DCA by immobilized cells was carried out in sterile saline solution. 34DCA was added at 1.2 mM and served as a sole carbon, nitrogen and energy source. The final number of cells was $3.5x10^9$ CFUs/ml. Samples were taken during incubation process for determination of 34DCA remaining and cell numbers in the media.

3.8.2.2 Determination bacterial growth

The bacterial growth was determined by measuring the protein content (Miyake-Nakayama et al., 2006; Nishio et al., 1998). 1 g bead was cut into a number of pieces using a surgical knife. Beads were dissolved by heating at 95 ^oC in a solution of 1.0 M NaOH and 0.2 M NaH_2PO_4 with continuous agitation. Cell numbers were calculated based on protein content. The cell growth rate (μ) was determined according to the following equation (Pramanik and Khan, 2008):

$$\mu = \frac{\ln X_t - \ln X_0}{t}$$

where X_o is the initial cell number, and X_t is the cell number at time t.

3.8.3 Reusability of beads

Repeated batch reactions were carried out to assess the reusability of immobilized GFJ1. After each cycle of incubation (12 hours), the spent medium was decanted. Beads were then washed twice with saline solution and transferred into a new medium containing 1.2 mM 34DCA. The biodegradation was determined as described above.

3.8.4 Freeze-drying, storage and rehydration process

The freeze-drying process was carried out using a bench-scale freeze-dryer (Lab conco Co.,USA) at -58 ^{o}C and 0.1 mbar for 24 hours. The fresh and freeze-dried beads were stored in a polyethylene bag for 1 month in the dark at room temperature (about 30 ^{o}C), 4 ^{o}C and -20 ^{o}C. The rehydration process was conducted at room temperature. Beads were immersed in sterile distilled water for 30 min followed by LB medium for 30 min with a rehydration volume of 1 g bead/50 ml. The beads were then thoroughly rinsed with distilled water.

3.8.5 Characterization of beads

3.8.5.1 Determination of diffusion coefficients

The diffusion coefficients were determined as described previously (Bai et al., 2010; Zain et al., 2011; Zhang and Ye, 2011). 3.5 g bead (without cell) was equilibrated in 50 ml of 10% glucose solution (w/v). After 48 hours, beads were transferred into 50 ml of sterile distilled water and stored at 30 ^{o}C. 1 ml samples were taken at time intervals to determine the amount of glucose diffused from the beads

into the bulk solution. Diffusion of molecules in a spherical bead with radius (R) can be appropriately expressed by a following equation:

$$\frac{c_t - c_\infty}{c_0 - c_\infty} = \frac{6}{\pi^2}\sum_{n-1}^{\infty} n^{-2} \exp(-\frac{n^2\pi^2 D_e t}{R^2})$$

where c_0 and c_t and c_∞ are the concentrations of glucose at initial, at given time t and at time when diffusion reaches balance, respectively. D_e is diffusion coefficient.

For a sufficiently extended time, the expression can be simplified as follows:

$$\frac{c_t - c_\infty}{c_0 - c_\infty} \cong \frac{6}{\pi^2}\exp(-\frac{\pi^2 D_e t}{R^2}) \text{ or } \ln(\frac{c_t - c_\infty}{c_0 - c_\infty}) = -\frac{\pi^2 D_e t}{R^2} + \text{const}$$

The diffusion coefficients can be obtained by plotting the graph of $\ln(\frac{c_t - c_\infty}{c_0 - c_\infty})$ versus time t.

3.8.5.2 Physical and chemical stability tests

The physical stability tests were carried out using a beaker with 70 mm in diameter and 95 mm in height. The beaker was divided into four equal regions by baffles. Each baffle has a width of 11 mm. 1 g bead (without cell) was added to the beaker containing 200 ml distilled water. The agitation was created using a magnetic stirrer bar (3.7 cm in length), and speed was controlled at 500 rpm on a hotplate machine. After 72 hours, the beads were dried in a desiccator until no further change in weight was detected (Idris et al., 2008).

For chemical stability tests, acid and alkali solutions (pH from 1.0 to 13.0) were prepared using HCl and NaOH. 1 g bead (without cell) was soaked in each solution (10 ml) for 48 hours. The beads were then rinsed with deionized water, and

dried in a desiccator until no further change in weight was found. The stability of beads was determined based on the reduction of weight (Idris et al., 2008).

The stability of beads in phosphate buffer was conducted in saline (0.85% NaCl) supplemented with phosphate at 0.01 and 0.1 M. 1 g bead was submersed in 20 ml saline (0.85% NaCl) and kept in static condition for 12 hours. Beads were moved out, died with filter paper and weighed.

3.8.5.3 Swelling behavior

The swelling ratios and swelling rate constants were determined as described previously (Zhang and Ye, 2011). The beads were dried as described above (in 3.8.4). Dried beads were immersed in distilled water for 48 hours at room temperature. The excess of water was removed with filter paper. The swelling ratio can be determined as a function of time:

$$\text{Swelling ratio} = \frac{(W_t - W_0)\,x100\%}{W_0}$$

where W_t and W_0 denote the weight of beads with absorbed water and dried beads, respectively.

The swelling rate constants (k) were determined following the equation:

$$kt = \ln \frac{Q_e - Q_0}{Q_e - Q_t}$$

where t was swelling time; Q_t, swelling ratio at time t; Q_e, equilibrium swelling ratio.

By plotting the graph of $\ln \frac{Q_e - Q_0}{Q_e - Q_t}$ versus time, the swelling rate constants can be obtained from the slopes of the curves (Zhang and Ye, 2011).

3.8.5.4 Scanning electron microscopy (SEM)

Beads were rinsed with distilled water and fixed overnight in osmium tetroxide (OsO4) (1%, v/v in deionized water) for 1.0 hour, and washed again in distilled water. The samples were then dehydrated three times by immersion in increasing concentrations of ethanol (30%, 50%, 70%, 90%, and absolute ethanol, v/v). The specimens were then dried with CO_2 under critical conditions (Balzer CPD020, Blazers AG, Liechtenstein). The samples were mounted on metal stubs with gold-palladium, and examined using a scanning electron microscope JEOL scanning electron microscope (JSM-5410LV, Jeol, Tokyo, Japan) at 15 kV.

3.8.5.5 Determinations of density, relative pore volume and porosity

True density, pore volume and porosity determination were conducted as described previously (Bai and Li, 2006; Wan et al., 2004). Beads were dried until a constant weight using a bench-scale freeze-dryer. 1 g dried bead (W_0) was placed into a 50 ml-bottle of known weight at 30 ^{o}C, followed by filling with 16 ml cyclohexane. The bottle was stored for 24 hours at 30 ^{o}C, then filled with cyclohexane to the mark (20 ml) and weight. The density of the bead was calculated according to the following equation:

$$d = \frac{W_0}{20 - \frac{(W - W_0)}{d_c}}$$

where W is the total weight of the beads and the solvent, and W_0 is the weight of the dry beads, d_c the density of the solvent (d cyclohexane = 0.778 g/ml).

The pore volume and porosity of beads were determined by monitoring the weight gain of the beads (Greig and Sherrington, 1978). Dried bead was placed into a tube with a porous glass bottom and placed in a flask filled with cyclohexane. The flask was incubated for 48 hours at 30 ^{o}C. After removing the excess cyclohexane by centrifugation at 1,500 rpm for 1 min, the volume of cyclohexane absorbed in the beads was determined, and the amount of absorbed cyclohexane was used to estimate the relative porosity of beads. The relative porosity (ϕ) and true volume (V_o) of the beads were determined by:

$$\phi = \frac{V_p}{V_0}, V_0 = \frac{W_0}{d}$$

where V_p and W_0 are the pore volume in the beads and weight of dried beads, respectively.

3.9 Analytical methods

3.9.1 Chloroanilines residual determination

Chloroanilines residual were determined using High Performance Liquid Chromatography (HPLC). Cell samples were harvested to remove cells by centrifugation at 10,000 rpm for 5 min. The cell-free supernatant was collected, mixed with acetronitrile at ratio 3:7 (v/v), and filtered through 0.45 μm-nylon syringe filter.

The investigation of CAs biodegradation was conducted using a reverse phase HPLC equipped with a UV detector (240 nm). The separation was performed at 40 ^{o}C on C18 HPLC column (5 μm, 250mm×4.6mm; Hyperclone, Phenomenex, USA). The mixture of acetronitrile and ultrapure water (7:3, v/v) was served as the mobile phase

at a flow rate of 1.0 ml/min. The comparison the peak area of unknown peaks with those of the standard compounds with known concentration was carried to obtain qualitative and quantitative data.

3.9.2 Protein determination

Samples were centrifuged at 10,000 rpm for 5 min. The supernatant was removed and cells were washed twice with saline, and resuspended in saline. Protein was extracted from cells by heating at 95 ˚C for 10 min. The supernatant was used to determine protein concentration. The modified Lowry method (Lowry et al., 1951) was used to determine the protein concentration, using bovine serum albumen as the protein standard.

- Reagents:

- Reagent A: 2% sodium carbonate in 0.1 M sodium hydroxide containing 0.5% sodium dodecyl sulfate (SDS).
- Reagent B: 0.5% copper sulfate in 1% potassium sodium tartrate.
- Reagent C: phenol solution (Folin-Clocahen's reagent).

- Procedure:

The reagent A and B were mixed (A : B, 50 : 1, v/v). Samples were adjusted to 0.4 ml with autoclaved deionized water and put in a glass tube. 2 ml of fresh mixed solution A and B and rapidly mixed was added to the tube. The mixture was incubated at 30 ˚C for 10 min. Then 0.2 ml of solution C was added to the tube, mixed immediately, and incubated at 30 ˚C for 30 min. Finally, the quantity of protein was measured by a spectrophotometer at wavelength 730 nm and using mixture A and B as blank.

3.9.3 Determination of oxygen in liquid media

The oxygen concentration was measured using a DO probe analyzer (Oxi 3210, WTW Co., Germany). Moreover, the anaerobic condition was confirmed using the indicator rezasurine (0.4 mM).

3.9.4 Electron acceptors and intermediate transformation of electron acceptors in liquid media

3.9.4.1 Determination of nitrate concentration

Measurement of UV absorption at 220 nm enables rapid determination of NO_3^-. Because dissolved organic matter also may absorb at 220 nm and NO_3^- does not absorb at 275 nm, a second measurement at 275 nm may be used to correct the NO_3^- value. The concentration of nitrate in liquid media was the differences of UV absorption at 220 nm and 275 nm (APHA, 1992).

3.9.4.2 Determination of nitrite concentration

The determination of nitrite concentration in media was followed the International Standard (Standard, 1984). The reagents included H_3PO_4 solution, sulphanilamide (4-aminobenzene sulfonamide ($NH_2C_6H_4SO_2NH_2$)) and N-(1-naphthyl)-1,2-diaminoethane dihydrochloride ($C_{10}H_7$-NH-CH_2-CH_2-NH_2-2HCl).

The experiment was performed as following: 4.0 g 4-aminobenzene sulfonamide was dissolved in a mixture of 10 ml of H_3PO_4 (15.0 mole/l) and 50 ml DI water in a beaker. 0.25 g N-(1-naphthyl)-1,2-diaminoethane dihydrochloride ($C_{10}H_7$-

NH-CH_2-CH_2-NH_2-2HCl) was dissolved in the solution. Samples were diluted and mixed with the color reagent solution. The absorbance of this compound was measured spectrophotometrically at 520 nm and was related to the nitrite by means of a calibration curve (Standard, 1984).

In this study, diazotization of sulphanilamide (4-aminobenzene sulfonamide ($NH_2C_6H_4SO_2NH_2$) reacts with nitrite in the presence of phosphoric acid, at pH 1.9 and the subsequent formation of an azo dye with N-(1-naphthyl)-1,2-diaminoethane dihydrochloride ($C_{10}H_7$-NH-CH_2-CH_2-NH_2-2HCl).

3.9.4.3 Determination of sulfate concentration

Sulfate concentrations were measured using ion chromatography (Dionex ICS-2500, USA) and an Iopac AS19 analytical column (4 mm× 250 mm). The mobile phase was from 1 to 40 mM KOH at a flow rate of 1.2 ml/min.

3.9.4.4 Determination of sulfide concentration

Sulfide concentrations were determined by the methylene blue method (Cline, 1969). N-N-dimethyl-p-phenylenediamine sulfate and ferric chloride ($FeCl_3.6H_2O$) were dissolved in DI water (8% and 2.5%, respectively). Samples were diluted 10 times and mixed with the color reagent solution and stored for some minutes. Hydrogen sulfide reacts with N,N-*dimethyl-p*-phenylenediamine sulfate to form methylene blue. The intensity of the blue color is proportional to the sulfide concentrations. The results were obtained by spectrophotometrical measurement at 665 nm, which was calculated based on the standard compounds with known concentrations.

3.9.5 Determination of ammonium concentration

Ammonium was measured using APHA method (APHA, 1992). The reagents included NaOCl (5%, pH = 7), $MnSO_4.H_2O$ (0.3 M), Phenate reagent (2.5% NaOH and 10% phenol). Samples were dropped into $MnSO_4$ solution followed by NaOCl reagent and Phenate reagent. The mixture was vortexed, kept for 5 min and measure at 630 nm (APHA, 1992).

3.9.6 Glucose concentration determination

The concentration of glucose in liquid medium was determined using the phenol–sulfuric acid method (DuBois et al., 1956). Sugar solution (1 ml) is pipetted into a colorimetric tube following by 0.025 ml of 80% phenol. Then **2** ml of concentrated sulfuric acid is added rapidly. The tubes are allowed to stand 10 minutes, then they are shaken and placed for 10 to 20 minutes The absorbance of the characteristic yellow orange color is measured at 485 nm. Blanks are prepared by substituting distilled water for the sugar solution. The amount of sugar may then be determined by reference to a standard curve previously constructed for the particular sugar under examination.

3.9.7 Glycerol concentration determination

Glycerol concentrations were determined as described previously (Boyd, 2012). For the assay, two reagents were needed:

Reagent I: the periodate reagent, consisted of 21 mg sodium metaperiodate, 5 mL acetic acid solution (9.6%) and 5 mL of ammonium acetate solution (30.8%).

Reagent II, the acetylacetone reagent, consisted of 195 mg acetylacetone, 5 mL acetic acid solution (9.6%) and 5 mL of ammonium acetate solution (30.8%).

For preparation of standard solution, 150 mg glycerol was added into 50 ml distill water and mixed by stirring for 10 min. The solution was then diluted at specific concentrations. 0.5 ml glycerol solution was added into 0.5 ml reagent I, followed with addition of 0.5 ml reagent II. The test tube was capped, immediately vorteded for 2 – 3 min, and stored for 10 min. Samples were read in a spectrophometer set in double beam mode at 410 nm.

The immobilization experiment was carried out with 10 mg solution of bead materials immobilized in 30 ml boric acid-$CaCl_2$ solution. The removal of porogens was carried out with 10 g bead in 30 ml sterilized distill water.

CHAPTER 4 RESULTS

4.1 Degradation toward chlorinated anilines by *A. baumannii* GFJ1 under aerobic degradation

4.1.1 Growth and utilization toward aniline and chlorinated anilines of *A. baumannii* GFJ1

The determination of cell growth and utilization rates toward aniline and chlorinated anilines under aerobic conditions of *A. baumannii* GFJ1 was carried out with aniline and chlorinated anilines as sources of carbon, nitrogen and energy. Because 2,4,6-trichloaniline (246TCA) is more toxic than mono- and dichloroanilines, and lower water solubility than mono- and dichloroanilines, the determination of cell growth and degradation was carried out at lower concentrations. *A. baumannii* GFJ1 showed the highest growth and utilization rates for 4CA and 34DCA (Table 4-1), so the utilization of 4CA and 34DCA would be analyzed in other conditions in next experiments. Besides the chemicals shown in Table 4-1, the growth and utilization of other mono-, di- and trichloroanilines were carried out; however, no utilization or insignificant utilization was found. The utilization was not found in controls without bacteria.

Table 4-1. Growth and utilization rates of *A. baumannii* GFJ1 in MM medium supplemented with aniline and chlorinated anilines under the aerobic condition

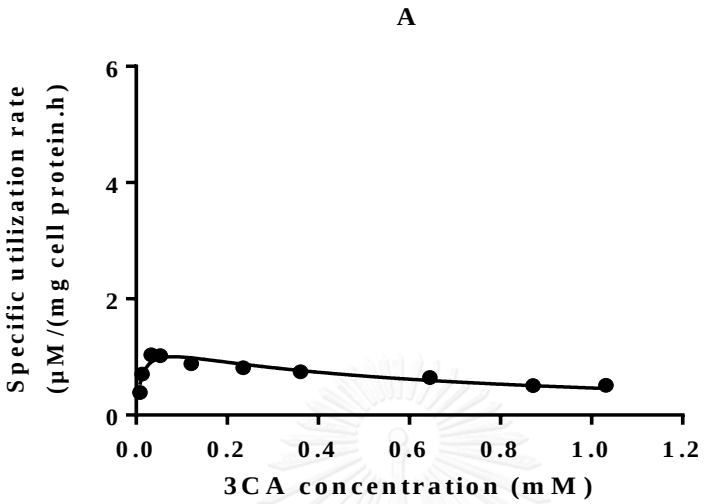
Substrate	Concentration (mM)	Growth rate (1/h)	Utilization rate (%)	Specific utilization rate (μM/mg protein.h)
Aniline	0.1	0.0032 ± 0.0007	14.58 ± 3.90*	0.747 ± 0.009
2CA	0.1	0.0049 ± 0.0002	12.96 ± 4.35*	0.307 ± 0.016
3CA	0.1	0.0054 ± 0.0004	39.06 ± 8.7*	0.885 ± 0.045
4CA	0.1	0.0251 ± 0.0001	97.10 ± 0.19**	2.879 ± 0.157
23DCA	0.1	0.0031 ± 0.0017	26.35 ± 3.90*	1.178 ± 0.221
34DCA	0.1	0.0225 ± 0.0073	99.17 ± 0.9**	2.751 ± 0.183
246TCA	0.01	0.0104 ± 0.0016	76.45 ± 10.50*	0.392 ± 0.026
246TCA	0.05	0.0089 ± 0.0008	73.04 ± 9.18*	0.202 ± 0.036

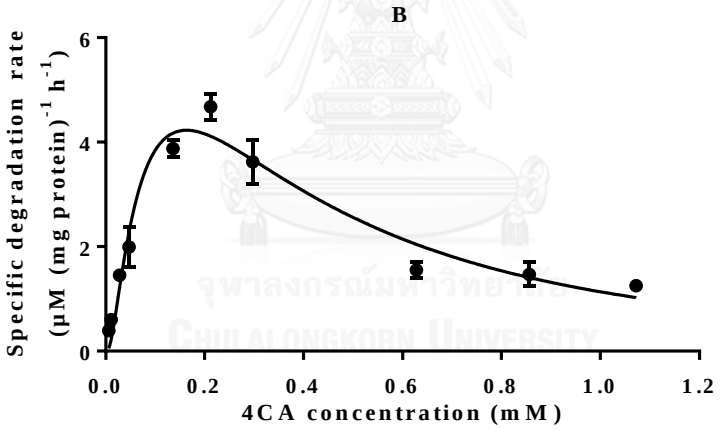
(*)Determined after 12 hours of incubation, (**)determined after 6 hours of incubation

4.1.2 Determination of utilization kinetics toward 4CA and 34DCA

The utilization of CAs by *A. baumannii* GFJ1 under aerobic conditions was investigated with 3CA, 4CA or 34DCA as a sole carbon, nitrogen and energy source. The degradation curves of 3CA, 4CA and 34DCA by *A. baumannii* GFJ1 at different concentrations (S) followed the Edward model, and substrate inhibition occurred (Figure 4-1). The degradation kinetics were calculated for various substrate concentrations and they showed that K_{max} value for 3CA was lower than for 4CA and

34DCA, K_{max} and K_s, K_i values for 4CA and 34DCA were similar (Figure 4-1, Table 4-2).





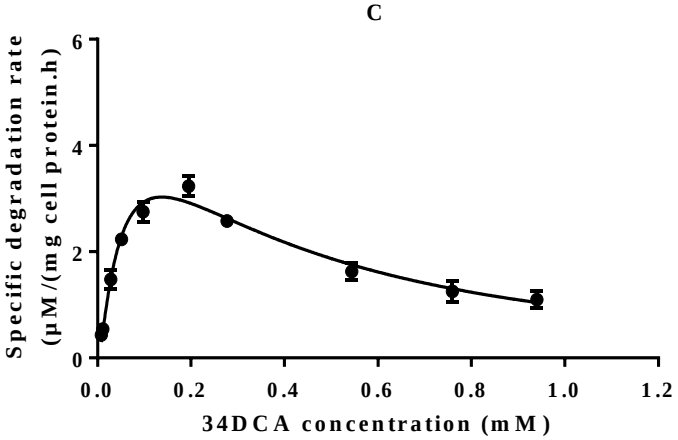


Figure 4-1. Relationship between the specific utilization rates and the concentrations of 3CA (A), 4CA (B) and 34DCA (C) of *A. baumannii* GFJ1. Cells grew and utilized CAs as sources of carbon, nitrogen and energy. The data were obtained from utilization curves and by the non-linear least-squares method. Error bars indicate standard errors.

Table 4-2. Degradation kinetic data of chloroanilines by *A. baumannii* GFJ1. Cells grew and utilized 4CA and 34DCA as sources of carbon, nitrogen and energy

Chemical	V_{max} [µM/(mg cell protein.hour)]$^{(*)}$	K_s (mM)$^{(*)}$	K_i (mM)$^{(*)}$
3CA	3.45 ± 0.33^a	0.062 ± 0.01^a	0.96 ± 0.02^b
4CA	5.53 ± 0.35^b	0.080 ± 0.01^a	0.22 ± 0.04^a
34DCA	6.10 ± 0.64^b	0.098 ± 0.02^a	0.21 ± 0.03^a

V_{max}: specific maximum the degradation rate, K_s: and half-maximal *rate constant* and K_i: inhibition coefficient.

$^{(*)}$The different letters denote significant differences ($P < 0.05$) among treatments in the same groups (within a column)

4.1.3 Effect of co-substrates on CAs degradation

The effects of co-substrates on CAs degradation were determined by supplementation with carbon and nitrogen sources. The supplementation of any co-substrate stimulated growth rates and reduced the specific degradation rates of 4CA and 34DCA. However, the degradation rate of 3CA stimulated with the presence of sodium nitrate and citrate (Table 4-3). The addition of 0.01% YE, sodium nitrate

(nitrogen sources), citrate and succinate (carbon sources) did not significantly enhance or reduce total degradation of 4CA and 34DCA in spite of reducing the specific degradation rates. However, the addition of ammonium sulfate or ammonium chloride resulted in reducing the degradation rates of 3CA and 4CA even though the presence of these compounds stimulated the cell growth (Table 4-3 and 4-4).

Besides data shown in Table 4-3, 4-4 and 4-5, the effects of co-substrates at smaller concentration (100 mg/l) were carried out. For examples, the addition of 100 mg l^{-1} of succinate, malate and citrate resulted in 97.7 ± 1.4%, 89.5 ± 4.6% and 94.9 ± 5.4% of 4CA and 96.8 ± 2.1%, 89.6 ± 0.4% and 95.9 ± 2.3 of 34DCA degraded within 6 hours. Increasing the succinate concentration ten-fold to 1,000 mg l^{-1} resulted in a decreased level of 4CA degradation, while it did not significantly change the degradation of 34DCA. However, increasing the citrate level to 1,000 mg l^{-1} did not significantly change the degradation rate of either 4CA or 34DCA.

Moreover, the addition of commercial humic acid (at 100 mg/l) acting as a dissolved organic matter which can bind to CAs to form complexes (Hsu and Bartha, 1976; Saxena and Bartha, 1983) did not stimulate or inhibit the chemical transformation. Similarly, the addition of malate did not change the degradation rate. These results showed that *A. baumannii* GFJ1 effectively utilized 4CA and 34DCA with or without any co-substrate.

Table 4-3. Exponential growth and 3CA degradation rates of *A. baumannii* GFJ1 in various growth conditions. The degradation and cell growth were determined simultaneously. 3CA was added at 0.1 mM.

Co-substrates	Exponential growth rate (h^{-1})$^{(*)}$	Degradation rate (%)$^{(*)}$	Specific degradation rate (μM.(h.mg cell protein)$^{-1}$)$^{(*)}$
None	0.004 ± 0.00^{a}	98.0 ± 0.3^{cd}	1.07 ± 0.09^{c}
0.01% YE	0.039 ± 0.00^{b}	96.8 ± 0.9^{cd}	0.35 ± 0.03^{b}
0.1% YE	0.115 ± 0.02^{e}	93.7 ± 3.9^{cd}	0.13 ± 0.00^{ab}
0.01% YE + 0.1% succinate	0.069 ± 0.01^{cd}	98.5 ± 0.1^{cd}	0.31 ± 0.06^{b}
0.01% YE + 0.1% citrate	0.064 ± 0.01^{c}	84.6 ± 1.1^{c}	1.22 ± 0.06^{c}
0.01% YE + 0.1% sodium nitrate	0.071 ± 0.01^{cd}	89.2 ± 3.5^{cd}	1.01 ± 0.22^{c}
0.01% YE + 0.1% ammonium sulfate	0.109 ± 0.0^{e}	13.1 ± 3.2^{a}	0.02 ± 0.00^{a}
0.01% YE + 0.1% ammonium chloride	0.084 ± 0.00^{d}	42.1 ± 9.9^{b}	0.04 ± 0.01^{a}

$^{(*)}$The different letters denote significant differences ($P < 0.05$) among treatments in the same groups (within a column)

Table 4-4. Exponential growth and 4CA degradation rates of *A. baumannii* GFJ1 in various growth conditions. The degradation and cell growth were determined simultaneously. 4CA was added at 0.1 mM.

Co-substrates	Exponential growth rate (1/hour)(*)	Total degradation (%)(*)	Specific utilization rate (µM/(mg cell protein.hour)(*)
None	0.03 ± 0.00^{a}	97.1 ± 0.2^{c}	2.13 ± 0.13^{c}
0.01% YE	0.06 ± 0.00^{b}	93.0 ± 2.6^{c}	1.92 ± 0.16^{c}
0.1% YE	0.11 ± 0.02^{d}	95.9 ± 1.6^{c}	0.17 ± 0.05^{a}
0.01% YE + 0.1% succinate	0.07 ± 0.00^{bc}	97.1 ± 0.7^{c}	0.26 ± 0.01^{a}
0.01% YE + 0.1% citrate	0.08 ± 0.01^{bc}	92.4 ± 0.3^{c}	1.10 ± 0.16^{b}
0.01% YE + 0.1% sodium nitrate	0.06 ± 0.00^{b}	96.1 ± 5.5^{c}	1.02 ± 0.04^{b}
0.01% YE + 0.1% ammonium sulfate	0.11 ± 0.02^{d}	45.4 ± 12.4^{b}	0.05 ± 0.00^{a}
0.01% YE + 0.1% ammonium chloride	0.09 ± 0.01^{cd}	26.6 ± 1.3^{a}	0.04 ± 0.00^{a}

(*)The different letters denote significant differences ($P < 0.05$) among treatments in the same groups (within a column)

Table 4-5. Exponential growth and 34DCA degradation rates of *A. baumannii* GFJ1 in various growth conditions. The degradation and cell growth were determined simultaneously. 34DCA was added at 0.1 mM.

Co-substrates	Exponential growth rate (1/hour)(*)	Total degradation (%)(*)	Specific utilization rate (μM/(mg cell protein.hour)(*)
None	0.02 ± 0.01^{a}	99.2 ± 0.1^{a}	2.75 ± 0.18^{e}
0.01% YE	0.06 ± 0.00^{b}	96.0 ± 0.1^{a}	2.14 ± 0.47^{d}
0.1% YE	0.12 ± 0.03^{d}	91.8 ± 1.2^{a}	0.40 ± 0.01^{ab}
0.01% YE + 0.1% succinate	0.08 ± 0.02^{bc}	92.9 ± 2.8^{a}	0.78 ± 0.03^{bc}
0.01% YE + 0.1% citrate	0.06 ± 0.00^{b}	96.6 ± 0.8^{a}	0.77 ± 0.10^{bc}
0.01% YE + 0.1% sodium nitrate	0.06 ± 0.00^{b}	98.0 ± 1.4^{a}	0.93 ± 0.09^{c}
0.01% YE + 0.1% ammonium sulfate	0.11 ± 0.02^{cd}	93.2 ± 8.8^{a}	0.16 ± 0.00^{a}
0.01% YE + 0.1% ammonium chloride	0.08 ± 0.00^{bc}	95.7 ± 0.1^{a}	0.74 ± 0.00^{bc}

(*)The different letters denote significant differences ($P < 0.05$) among treatments in the same groups (within a column)

4.1.4 Degradation toward CAs using resting cells

The determination of biodegradation toward CAs by *A. baumannii* GFJ1 using resting cells was carried out to investigate the degradation capacity for industrial application. Yeast extract, ammonium sulfate and succinate were added as other nutrient sources in the medium with condensed cells. The chemical concentrations and incubation conditions were carried out the same as the determination of utilization kinetics described above. At these concentrations, the biodegradation 4CA and 34DCA kinetics profile was followed the saturation kinetics of a Michaelis–Menten-like relationship (Figure 4-2).

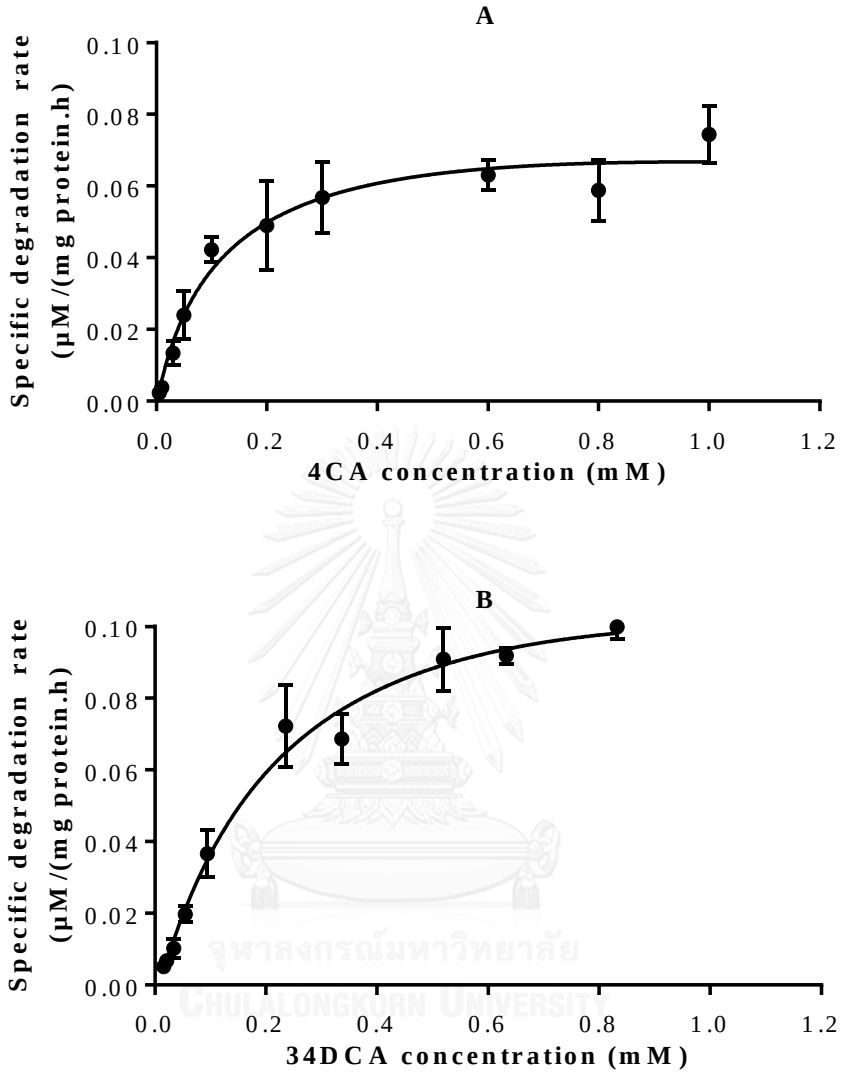


Figure 4-2. Relationship between the specific biodegradation rates and the concentrations of 4CA (A) and 34DCA (B) by *A. baumannii* GFJ1. Cells were condensed (resting cells) at $3.5x10^9$ CFUs/ml at the beginning. The data points were derived from substrate-depletion curves and fitted with Michaelis–Menten equation.

The kinetic parameters including the highest degradation velocity (V_{max}) and apparent half-saturation coefficient (K_s) values of each substrate was calculated by using a linear regression fitting shown in Table 4-6.

Table 4-6. Degradation kinetic data of 4CA and 34DCA by *A. baumannii* GFJ1. Cells were condensed (resting cells) at $3.5x10^9$ CFUs/ml at the beginning.

Chemical	V_{max} [µM/(mg cell protein.hour)]	K_s (mM)
4CA	0.23 ± 0.03	0.68 ± 0.18
34DCA	0.37 ± 0.08	1.15 ± 0.28

V_{max}: specific maximum the degradation rate, K_s: and half-maximal *rate constant.*

4.1.5 Effect of chemical concentrations on growth rates

The growth rates of *A. baumannii* GFJ1 in MYSA supplemented with CAs were carried out at different concentrations of chemicals. The results showed that the bacterial strain could grow up to 2.8 mM 3CA, 3.6 mM 4CA and 1.0 mM 34DCA (Figure 4-3). Bacteria grew with the highest exponential rates at 0.1 mM, and gradually decreased at higher concentrations of chemicals. The growth rates in medium supplemented with 0.05 – 0.4 mM were higher than in the medium without any CAs (Figure 4-3).

In comparison with MYSA medium, the exponential growth rates of the bacterial strain in MM medium were smaller; for instances, the growth rates of GFJ1 in the MM medium supplemented with 0.05, 0.1, 0.2, 0.3, 0.6, 0.8 and 1.0 mM 34DCA were 0.017 ± 0.001, 0.033 ± 0.001, 0.016 ± 0.001, 0.014 ± 0.001, 0.010 ± 0.001, 0.006 ± 0.000 and 0.001 ± 0.000 h^{-1}, respectively. The bacterial strain grew up to 0.3 and 0.1 mM 246TCA in MYSA and MM media, respectively.

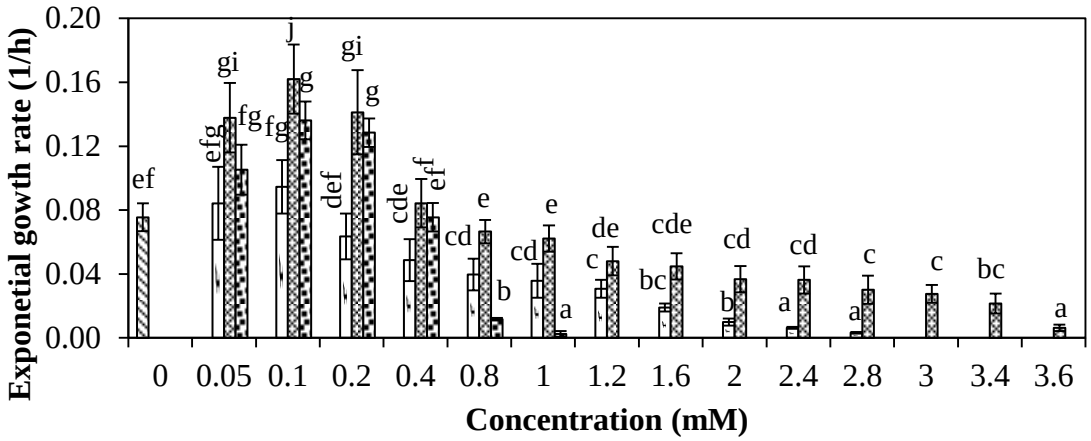


Figure 4-3. Exponential growth rates of *A. baumannii* GFJ1 in MYAS medium without CAs (▧) and supplemented with various concentrations of 3CA (□), 4CA (▨) and 34DCA (⚄). Error bars indicate standard errors. The different letters denote significant differences ($P < 0.05$) among treatments.

4.1.6 Determinations of growth rate, and 4CA and 34DCA degradation in saline medium

In MYSA supplemented with CAs (0.1 mM) and NaCl, the exponential growth rates were highest at 0.5 % NaCl, and smaller at higher NaCl concentrations (Figure 4-4). In medium supplemented with 4CA, bacteria could grow up to 4.5% NaCl. They didn't have a lag phase in medium containing at 0.5%, 1.5% and 2.5% NaCl, had 6 and 12 hours of the lag phase at 3.5% and 4.5% NaCl, respectively.

In medium supplemented with 34DCA, the numbers were smaller compared to 4CA. The maximum NaCl concentrations that bacteria could grow were 3.5% NaCl and 4.5% NaCl with supplementation of 4CA and 34DCA, respectively (Figure 4-4).

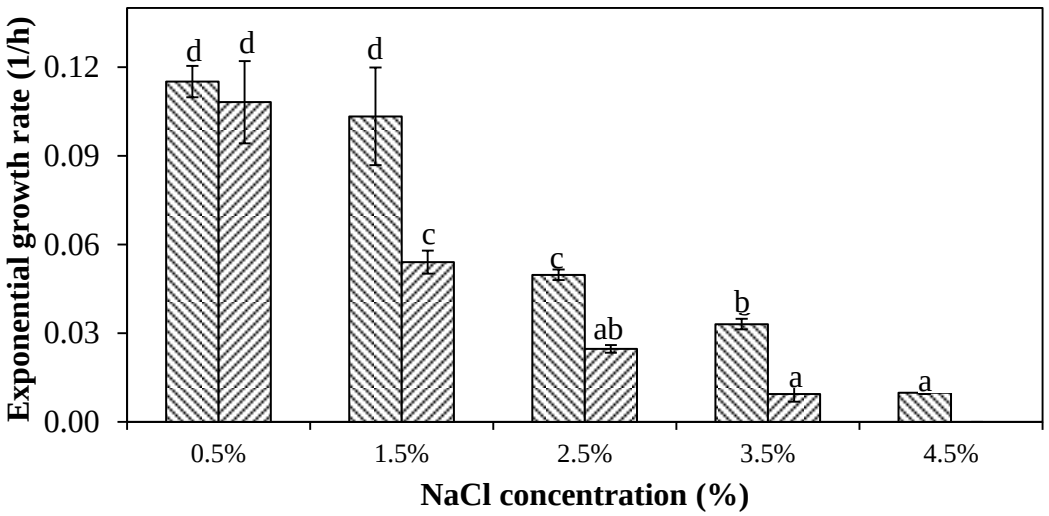


Figure 4-4. The growth rates of bacteria in MYSA medium supplemented with 4CA (▨) and 34DCA (▨) with various NaCl concentrations. Bacteria were incubated at room temperature with a shaking speed of 150 rpm. The different letters denote significant differences ($P < 0.05$) among treatments.

The more NaCl concentrations were added, the slower degradation rates of chemicals were experienced. The degradation and growth rates were slower for 4CA in comparison with 34DCA at the same chemical and NaCl concentrations even though 34DCA was toxic than 4CA (Table 4-7).

Table 4-7. 4CA and 34DCA degradation by *A. baumannii* GFJ1 in saline medium

NaCl concentration (%)	4CA		34DCA	
	Degradation rate (μM/hr)(*)	Specific degradation rate (μM/(mg cell protein.hour)(*)	Degradation rate (μM/hr)(*)	Specific degradation rate (μM/(mg cell protein.hour)(*)
0.5	4.03 ± 0.32^{d}	0.020 ± 0.001^{D}	5.37 ± 0.23^{e}	0.026 ± 0.002^{E}
1.5	2.61 ± 0.51^{ab}	0.014 ± 0.002^{BC}	3.40 ± 0.05^{c}	0.017 ± 0.001^{C}
2.5	2.59 ± 0.04^{ab}	0.012 ± 0.000^{AB}	2.91 ± 0.09^{bc}	0.015 ± 0.001^{BC}
3.5	2.12 ± 0.22^{a}	0.010 ± 0.001^{A}	2.53 ± 0.25^{ab}	0.013 ± 0.001^{AB}

(*)The different letters denote significant differences ($P < 0.05$) among treatments in the same groups

The inhibition constant (K_i) was the concentration that shows the toxicity of salt. The calculated K_i for 4CA and 34DCA was 2.66% and 2.76% NaCl, respectively. The high K_i showed NaCl concentration inhibiting the degradation.

4.1.7 Aerobic degradation pathways for 4CA and 34DCA in *A. baumannii* GFJ1

During incubation, the slightly pale yellow color appeared in liquid media containing CAs. The color might exist several hours or days belonging to chemical concentrations added at the beginning. This color was similar to the color of liquid media containing 4-chlorocatechol (Figure 4-5). In HPLC profile, an intermediate (retention time at 3.0) in 3CA and 4CA degradation, and two intermediates appearing

(retention time of 3.4 and 3.0 min) in 34DCA degradation were detected (Figure 4-7). The intermediates were then separated using TLC. The LC-MS analysis found that the intermediate at retention time of 3.0 and 3.4 min in HPLC profile was 4-chlorocatechol (*m/z* 142.99) and 3CA (*m/z* 128.03), respectively (Figure 4-6). These results showed that the degradation of 3CA, 4CA and 34DCA was occurred *via* a modified *ortho*-cleavage pathway. Accordingly, the degradation pathways for 4CA and 34DCA are proposed in Figure 4-8.

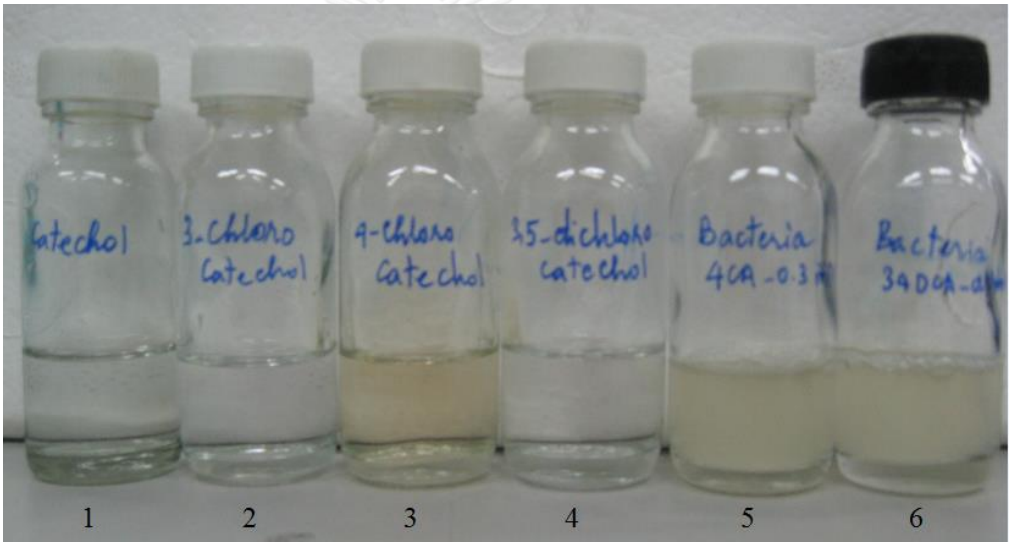


Figure 4-5. Color of the medium cultured with *A. baumannii* GFJ1 supplemented with 0.6 mM 4CA (5) and 0.6 mM 34DCA (6) was similar to color of 0.2 mM 4-chlorocatechol in distilled water (3).

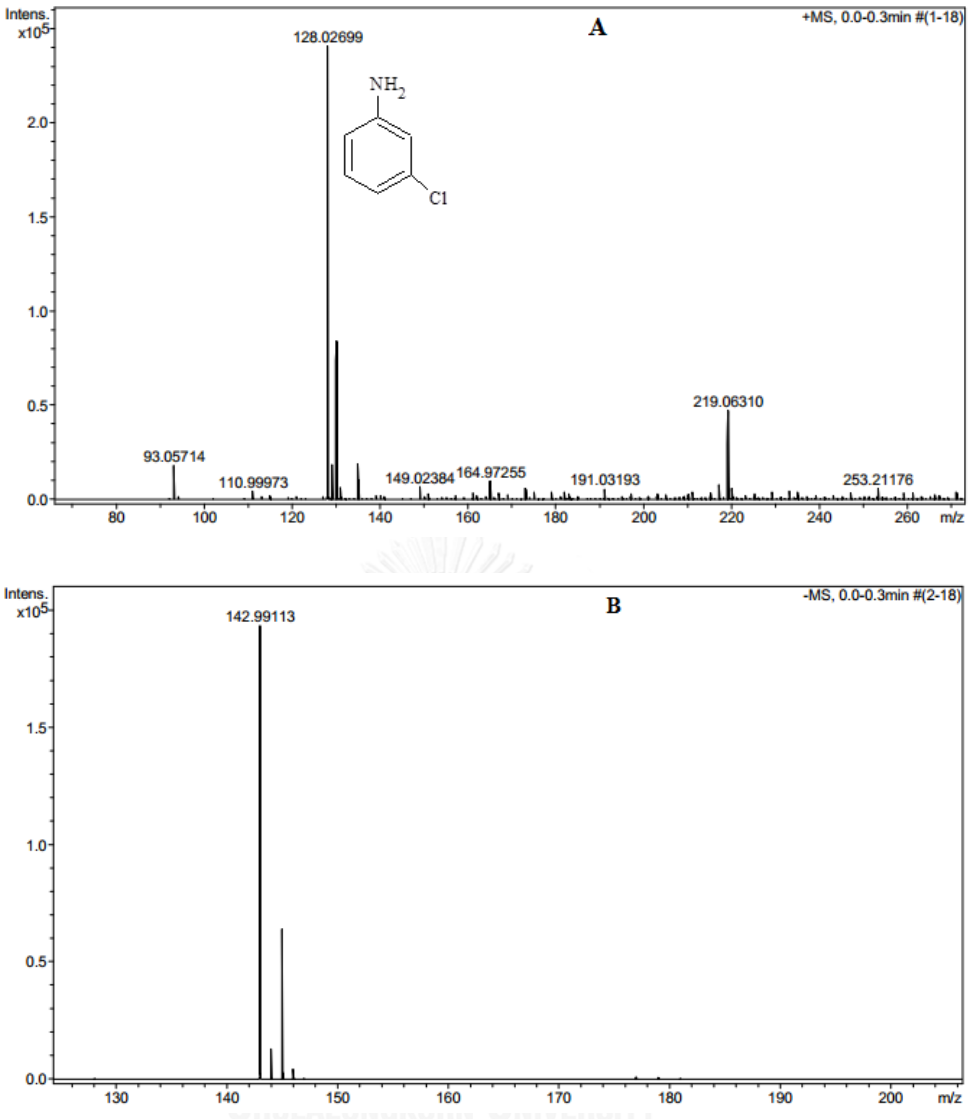


Figure 4-6. LC-MS showed the intermediate degradation products of 4CA and 34DCA under aerobic conditions: 3CA (A) and 4-chlorocatechol (B).

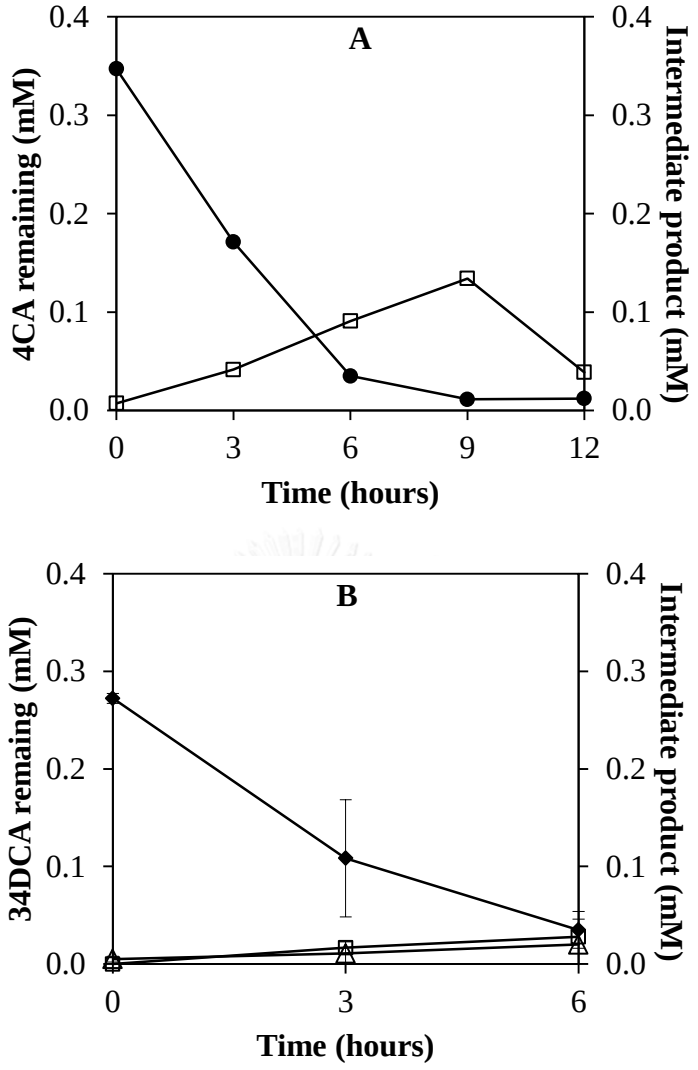


Figure 4-7. The formation of the intermediates during the biodegradation of 4CA (A) and 34DCA (B) in *A. baumannii* GFJ1. In (A), during 4CA biodegradation (●), 4-chlorocatechol (□) was produced. In (B), during 34DCA degradation (♦), 3CA (Δ) and 4-chlorocatechol (□) were produced.

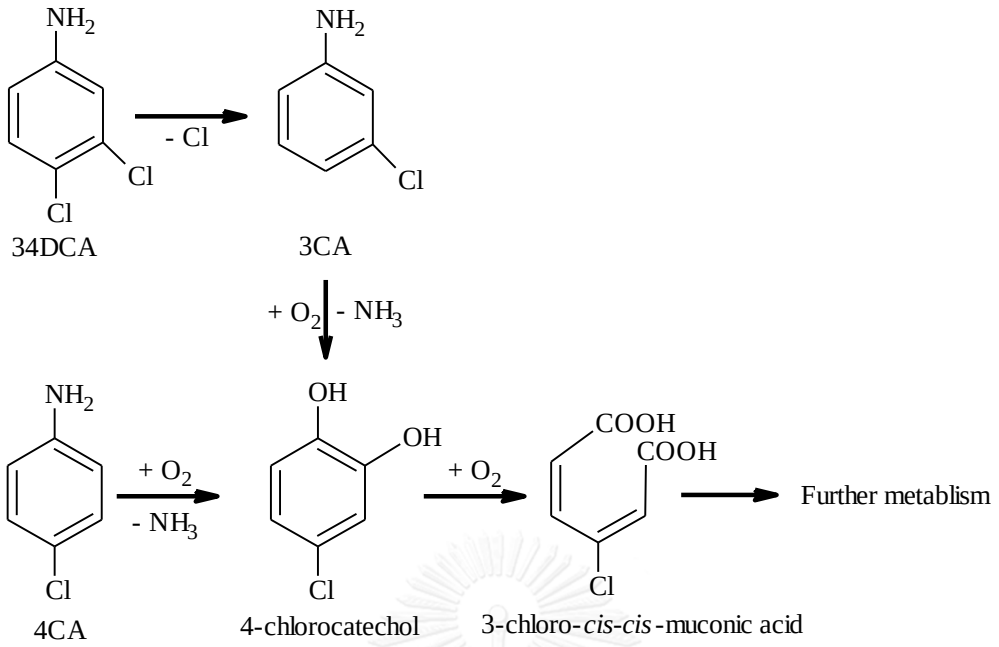


Figure 4-8. Proposed aerobic biodegradation pathways for 4CA and 34DCA in *A. baumannii* GFJ1.

4.2 Degradation rates toward CAs, cell growth and transformation of electron acceptors of *A. baumannii* GFJ1 under anaerobic conditions

4.2.1 Degradation rates toward CAs and cell growth of *A. baumannii* GFJ1 under anaerobic conditions

The determination of biodegradation toward 4CA and 34DCA in anaerobic conditions was carried out in MM medium with and without supplementation with co-substrates. In this study, *A. baumannii* GFJ1 was found to utilize 4CA and 34DCA under anaerobic conditions with nitrate or sulfate as the electron acceptors (Table 4-8). The degradation rates and growth rates of GFJ1 with nitrate as an acceptor were higher compared to sulfate. The addition of co-substrates enhanced the degradation

rates and growth rates. The twofold increase of electron acceptors in the medium resulted in increasing 4CA and 34DCA utilization.

The experiment was also carried out with Fe^{3+} as an electron acceptor. However, no chemical degradation was found when Fe^{3+} served as an electron acceptor. In all the trials here, the controls without bacteria, and anaerobic controls without any electron acceptor did not reduce the respective CA concentrations.

However, the anaerobic utilization and growth rates were slower than under aerobic conditions. Less than 75% of 4CA or 34DCA was utilized in anaerobic conditions after 20 days (Table 4-8 and 4-9). Under the same conditions, more than 90% 4CA or 34DCA was degraded within 6 hours under aerobic conditions. For example, the degradation rates of 4CA and 34DCA were 93.8 ± 1.6% and 94.7 ± 2.1% by bacteria cultured in media supplemented with YE, succinate and nitrate (100 mg/l each) within 6 hours, respectively. The exponential growth rates in this medium supplemented with 4CA and 34DCA were 0.073 ± 0.001 and 0.069 ± 0.002 1/h, respectively.

Table 4-8. Growth rates of *A. baumannii* GFJ1 and its biodegradation rates for 4CA under anaerobic conditions after 20 days in the presence of nitrate or sulfate as electron acceptors. 4CA was added at 0.1 mM.

Nutrient supplementation	NO_3^- or SO_4^{2-} (mg/l)	Nitrate(*)		Sulfate(*)	
		Utilization (%)	Growth rate (1/h)x10^{-2}	Utilization (%)	Growth rate (1/h)x10^{-2}
None	0	0	0	0	0
None	50	42.0 ± 4.5^{ab}	0.08 ± 0.00^{AB}	32.2 ± 8.5^{a}	0.07 ± 0.01^{A}
Succinate	50	56.4 ± 9.0^{bc}	0.12 ± 0.00^{DE}	39.3 ± 2.5^{ab}	0.10 ± 0.01^{BC}
YE	50	53.2 ± 2.1^{bc}	0.14 ± 0.01^{EF}	39.4 ± 11.5^{ab}	0.11 ± 0.01^{CD}
Succinate + YE	50	64.8 ± 0.0^{cd}	0.18 ± 0.02^{G}	48.6 ± 2.8^{abc}	0.15 ± 0.02^{EF}
Succinate + YE	100	74.9 ± 2.2^{e}	0.20 ± 0.01^{G}	54.8 ± 13.2^{bc}	0.16 ± 0.01^{E}

(*)The different letters denote significant differences ($P < 0.05$) among treatments in the same groups (utilization and growth rates).

Table 4-9. Growth rates of *A. baumannii* GFJ1 and its biodegradation rates for 34DCA under anaerobic conditions after 20 days in the presence of nitrate or sulfate as electron acceptors. 34DCA was added at 0.1 mM.

Nutrient supplementation (*)	NO_3^- or SO_4^{2-} (mg/l)	Nitrate(*)		Sulfate(*)	
		Utilization (%)	Growth rate (1/h)x10^{-2}	Utilization (%)	Growth rate (1/h)x10^{-2}
None	0	0	0	0	0
None	50	44.2 ± 0.2^{bc}	0.07 ± 0.01^{AB}	28.0 ± 0.3^{a}	0.06 ± 0.00^{A}
Succinate	50	48.4 ± 4.9^{cd}	0.12 ± 0.00^{CDE}	32.0 ± 2.1^{a}	0.10 ± 0.02^{ABC}
YE	50	55.9 ± 0.1^{de}	0.13 ± 0.00^{CDE}	37.7 ± 2.8^{ab}	0.10 ± 0.01^{ABC}
Succinate + YE	50	61.4 ± 4.8^{ef}	0.15 ± 0.02^{EF}	57.1 ± 10.3^{de}	0.14 ± 0.01^{DEF}
Succinate + YE	100	68.6 ± 5.6^{f}	0.19 ± 0.00^{G}	58.2 ± 0.7^{de}	0.17 ± 0.04^{FG}

(*)The different letters denote significant differences ($P < 0.05$) among treatments in the same group (utilization and growth rates)

4.2.2 The intermediate products of 4CA and 34DCA degradation under anaerobic conditions

The degradation products were elucidated from the HPLC, TLC and LC-MS analyses. 34DCA was reductive dechloronated to 3CA at the first step. 3CA and 4CA were dechloronated to aniline which then formed 4-aminobenzoic acid (*m/z* 138.05) under anaerobic condition (Figure 4-9). The anaerobic degradation of 4CA and 34DCA and intermediates of this process by *A. baumannii* GFJ1 is depicted in Figure 4-10. Accordingly, the degradation pathways of 4CA and 34DCA are proposed in Figure 4-11.

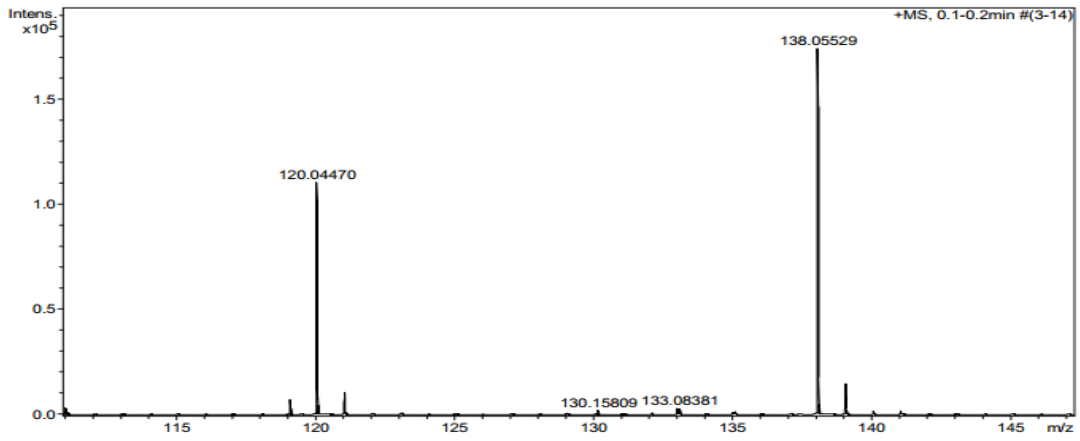


Figure 4-9. Result of LC-MS showed 4-aminobenzoate (*m/z* 138.05) as an intermediate product in 4CA and 34DCA degradation.

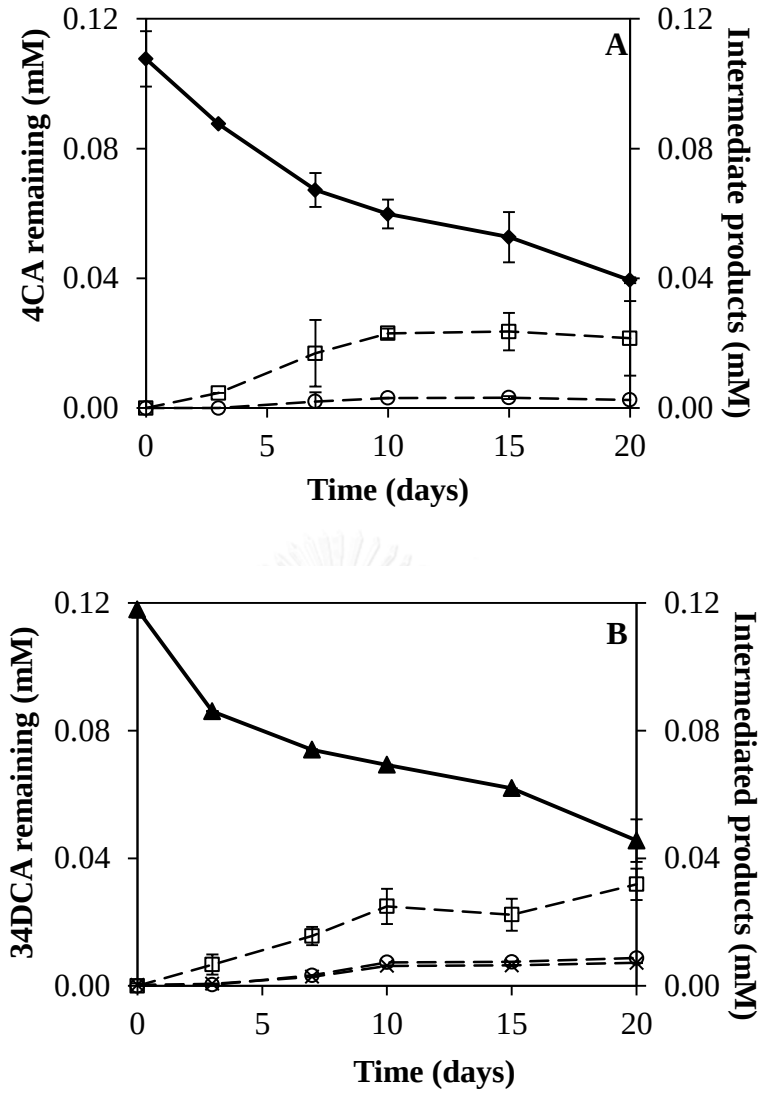


Figure 4-10. The formation of the intermediates (opened symbol, dashed line) during the biodegradation of 4CA (A) and 34DCA (B) (closed symbol, solid line) in *A. baumannii* GFJ1. In (A), during 4CA biodegradation (♦), aniline (○) and 4-aminobenzoic acid (□) were formed. In (B), during 34DCA biodegradation (▲), 3CA (x), aniline (○) and 4-aminobenzoic acid (□) were formed. Error bars indicate standard errors.

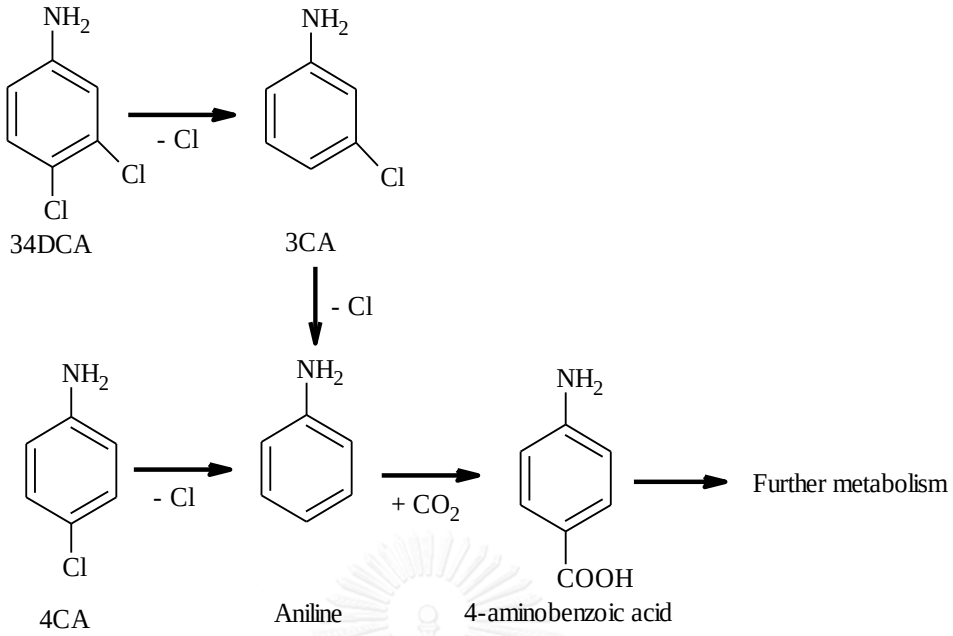


Figure 4-11. Proposed anaerobic biodegradation pathways for 4CA and 34DCA in *A. baumannii* GFJ1.

4.2.3 The nitrate consumption nitrite production under anaerobic conditions

During the degradation process, nitrate was simultaneously converted to nitrite (Figure 4-12, Tables 4-10), while no nitrate removal was found in medium without CAs or without bacteria. The theoretical stoichiometry of electron transformation was shown in the following equations:

$$C_6H_4NH_2Cl + 5.2NO_3^- + 2.4H_2O \rightarrow 6HCO_3^- + 2.6N_2 + NH_4^+ + 0.8H^+ + Cl^- \quad (1)$$

$$C_6H_4NH_2Cl + 13NO_3^- + 5H_2O \rightarrow 6HCO_3^- + 13NO_2^- + NH_4^+ + 6H^+ + Cl^- \quad (2)$$

$$C_6H_3NH_2Cl_2 + 4.8NO_3^- + 3.6H_2O \rightarrow 6HCO_3^- + 2.4N_2 + NH_4^+ + 2.2H^+ + 2Cl^- \quad (3)$$

$$C_6H_3NH_2Cl_2 + 12NO_3^- + 6H_2O \rightarrow 6HCO_3^- + 12NO_2^- + NH_4^+ + 7H^+ + 2Cl^- \quad (4)$$

The theoretical molar ratios of nitrate consumption to 4CA degradation based on equation (1) and (2) were 5.2 and 13, and 34DCA based on equation (1) and (2) were 4.8 and 12.0. The theoretical nitrate consumption was calculated based on 4CA and 34DCA consumption was shown in Table 4-10. It could be observed in this table that the consumption of nitrate was higher than the theoretical values if nitrate was completely reduced to nitrogen gas, but it was lower than nitrate reduced to nitrite. These results suggested that nitrate was transformed into both nitrite and nitrogen gas.

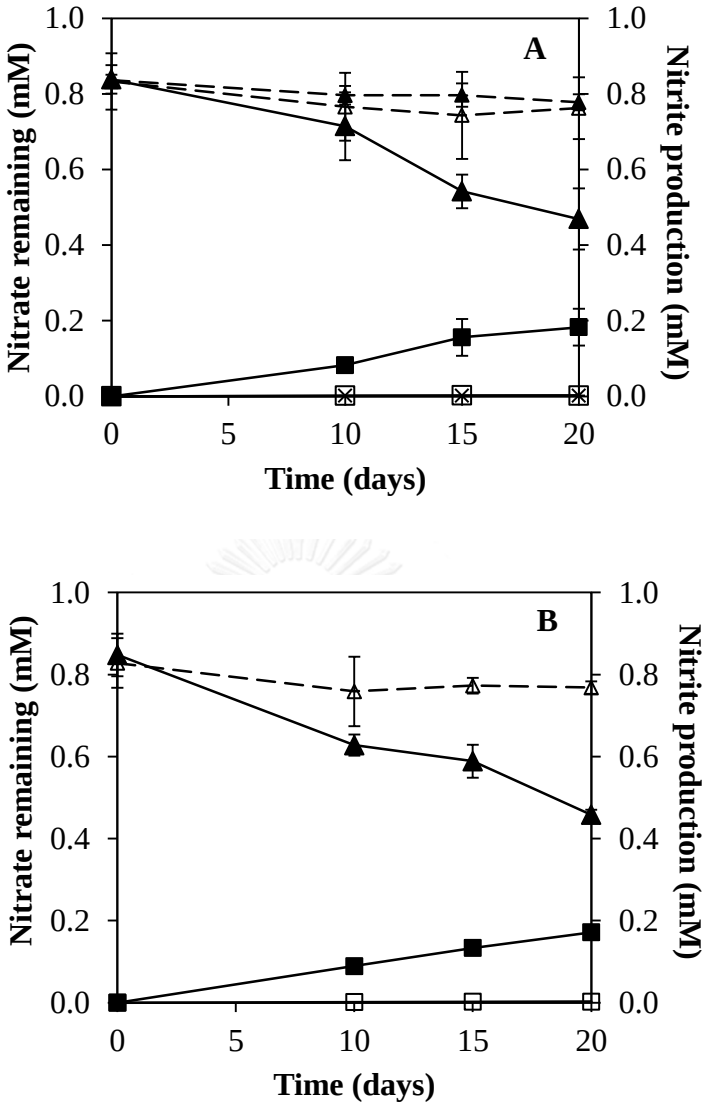


Figure 4-12. Nitrate consumption (solid line, ▲), nitrite production (solid line, ■) in anaerobic transformation of 4CA (A) and 34DCA (B), nitrate concentrations in the controls without bacteria (dashed line, Δ) and the controls without electron acceptor (dashed line, ▲); nitrite concentrations in controls without bacteria (solid line, □) and controls without electron acceptor (solid line, x). 4CA and 34DCA were added at 0.1 mM. The experiments were carried out in MM medium without co-substrate.

Table 4-10. Nitrate consumption and nitrite production during the degradation of 4CA and 34DCA by *A. baumannii* GFJ1.

Substrates	Degradation (mM)	Nitrate transformation (mM)			Nitrite production (mM)
		Theoretical values if all nitrate transformed to nitrogen gas	Theoretical values if all nitrate transformed to nitrite	Measured values	
4CA	0.049 ± 0.009	0.256 ± 0.020	0.641 ± 0.050	0.369 ± 0.043	0.183 ± 0.019
34DCA	0.058 ± 0.008	0.278 ± 0.038	0.695 ± 0.096	0.390 ± 0.064	0.172 ± 0.012

4.2.4 Sulfate consumption, sulfide and ammonium production under anaerobic conditions

During anaerobic degradation, sulfate was transformed to sulfide. The reduction of sulfate was always lower than expected (Table 4-11). In the sulfate transformation process, sulfide produced was lower than sulfate consumed. Similarly, ammonium production was significantly smaller than 4CA and 34DCA consumed based on equation (1) and (2) (Table 4-12). There was no sulfate reduction and sulfide production in controls without bacteria. The degradation equations of 4CA and 34DCA could be proposed:

$$C_6H_4NH_2Cl + 3.25SO_4^{2-} + 5H_2O \rightarrow 6HCO_3^- + 3.25HS^- + NH_4^+ + 2.75H^+ + Cl^- \quad (5)$$

$$C_6H_3NH_2Cl_2 + 3.0SO_4^{2-} + 6H_2O \rightarrow 6HCO_3^- + 3.0HS^- + NH_4^+ + 4H^+ + 2Cl^- \quad (6)$$

Table 4-11. Sulfate consumption and sulfide production during degradation of CAs by *A. baumannii* GFJ1.

Substrates	Degradation (mM)	Sulfate transformation (mM)		Sulfide production (mM)
		Theoretical values	Measured values	
4CA	0.033 ± 0.009	0.106 ± 0.028	0.075 ± 0.014	0.041 ± 0.006
34DCA	0.054 ± 0.004	0.161 ± 0.024	0.074 ± 0.017	0.042 ± 0.006

Table 4-12. Ammonia produced during anaerobic degradation toward 4CA and 34DCA

Electron acceptor	Time (days)	4CA degradation (mM)	34DCA degradation (mM)	Ammonia production (mM)	
				4CA	34DCA
Nitrate	0	0	0	0	0
	10	0.032 ± 0.003	0.044 ± 0.002	0.0019 ± 0.0000	0.0064 ± 0.0008
	15	0.040 ± 0.001	0.043 ± 0.003	0.0116 ± 0.0000	0.0091 ± 0.0026
	20	0.049 ± 0.004	0.058 ± 0.008	0.0116 ± 0.0000	0.0090 ± 0.0023
Sulfate	0	0	0	0	0
	10	0.020 ± 0.014	0.020 ± 0.011	0.0068 ± 0.0000	0.0019 ± 0.0000
	15	0.025 ± 0.016	0.032 ± 0.013	0.0116 ± 0.0000	0.0092 ± 0.0000
	20	0.031 ± 0.007	0.036 ± 0.016	0.0092 ± 0.0034	0.0068 ± 0.0000

4.3 Determination of biofilm formation and degradation toward CAs by biofilm of *A. baumannii* GFJ1

4.3.1 Biofilm formation on a 96-well microplate of *A. baumannii* GFJ1

The biofilm formation was analyzed by using 96-well polystyrene microplates. The cells adhered on the plate were stained with crystal violet (Figure 4-13). The absorbance data were used as the biofilm formation levels. Biofilm formation with high levels was in MM medium supplemented nitrogen sources: YE (0.1%), ammonium sulfate and ammonium chloride. However, carbon sources, including succinate citrate did not significantly affect the biofilm formation (Figure 4-14).

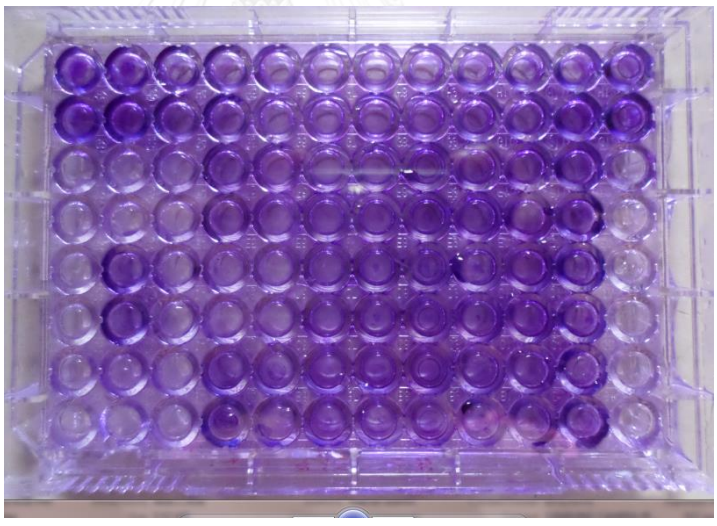


Figure 4-13. Biofilm formation on 96-well polystyrene microplates. Bacteria were cultured for 24 hours and the biofilm was stained with 0.1% crystal violet. The wells without bacteria did not stain with color.

The presence of $NaNO_3$ in medium showed the intermediated biofilm formation, and this compound did not inhibit degradation. Therefore, MM medium containing 0.01% YE and 0.1% $NaNO_3$ (MYNa medium) was used to test in next experiments of biofilm.

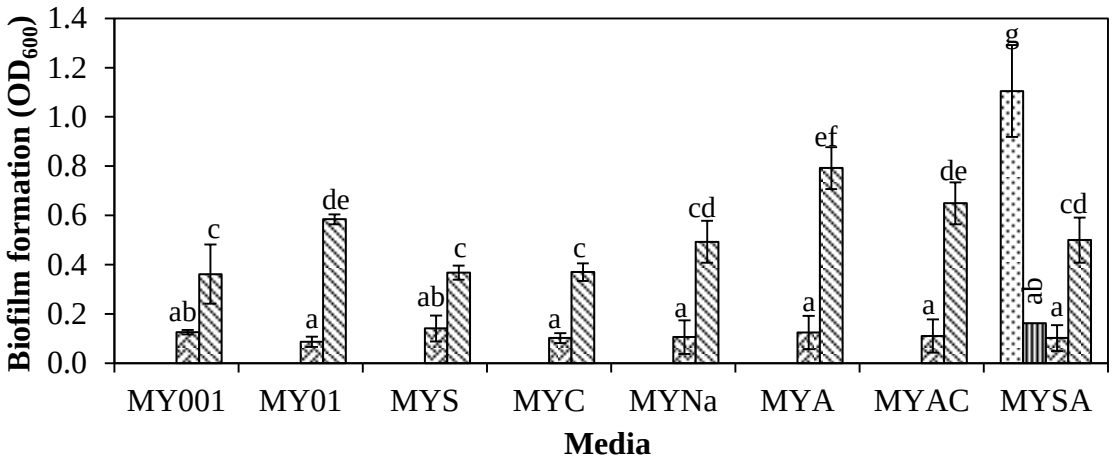


Figure 4-14. The biofilm formation on microplates in MM medium supplemented with 0.01% YE (MY001), 0.1% YE (MY01), 0.01% YE and 0.1% succinate (MYS), 0.01% YE and 0.1% citrate (MYC), 0.01%YE and 0.1% sodium nitrate (MYNa), 0.01%YE and 0.1% ammonium sulfate (MYA), 0.01%YE and 0.1% ammonium chloride (MYAC), and 0.01% YE, 0.1% succinate and 0.1% ammonium sulfate (MYSA). *P. putida* KT2400 (⬚) served as a positive control. The negative control without bacteria was carried out in parallel (▨). *E. coli* DH5α (▥) showed low biofilm formation. All assays were performed at least four times, and mean standard deviation was shown. The different letters denote significant differences ($P < 0.05$) among treatments.

4.3.2 Effects CAs concentrations on biofilm formation on 96-well polystyrene microplates of *A. baumannii* GFJ1

The biofilm formation is affected by environmental conditions, including chemical concentrations. At chemical concentrations of 0.0, 0.05 and 0.1 mM 3CA, 4CA and 34DCA, the biofilm levels were not significantly different (Figure 4-15). Biofilm formation reached at peak within 24 hours of incubation, and did not significantly change in the second and third day. At higher chemical concentrations (0.3, 0.6 and 1.0 mM), the maximum of biofilm levels was after 48 hours, and was not significantly different among the media (after 48 hours) with and without supplementation with CAs (except 34DCA at 1.0 mM) (Figure 4-15).

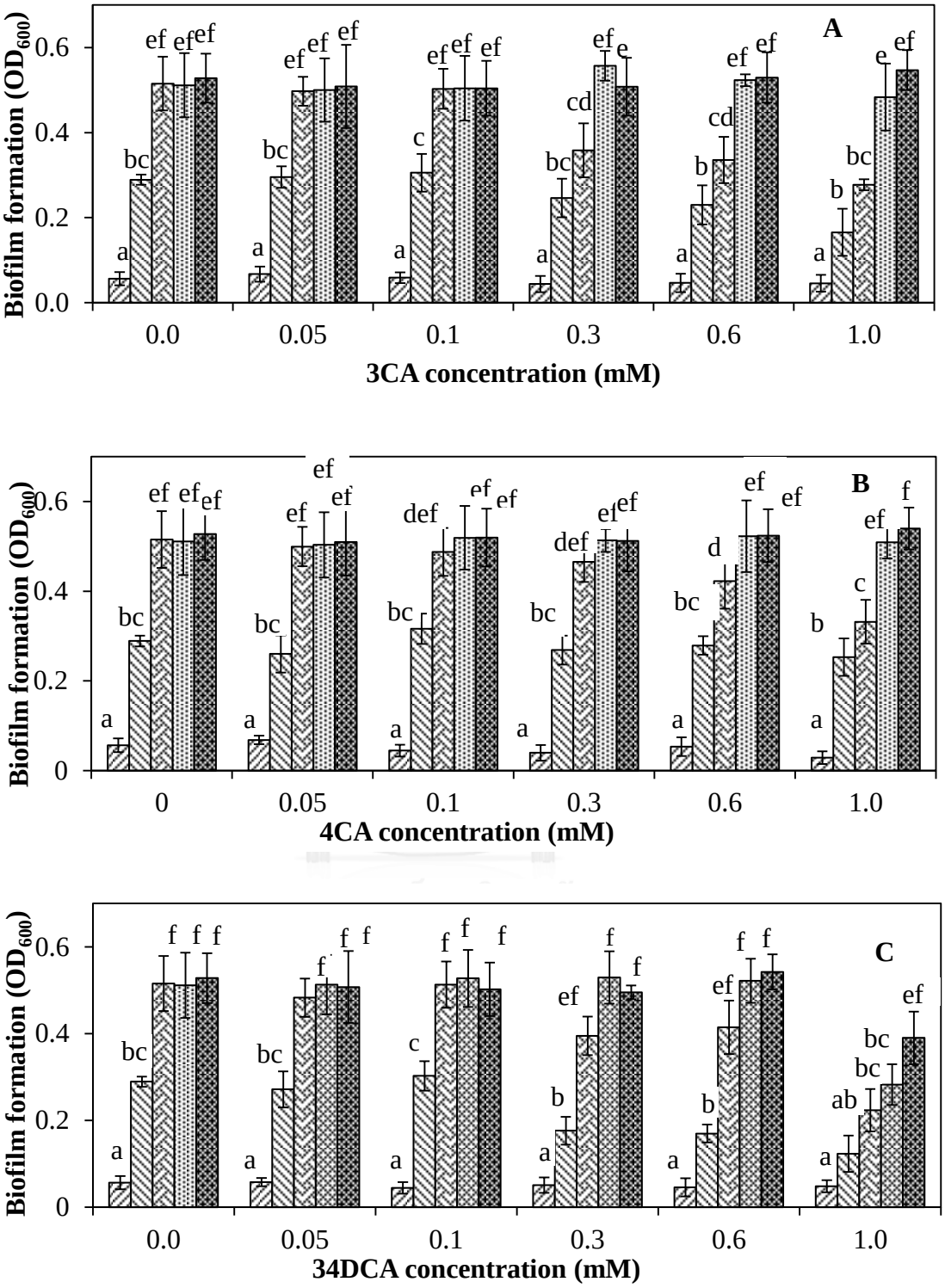


Figure 4-15. The biofilm formation in MYNa medium supplemented with 3CA (A), 4CA (B) and 34DCA (C) on the 96 well-microplate. Data were obtained after staining

biofilm with crystal violet, then diluting the stain with a mixture of absolute ethanol and acetone (80 : 20, v/v) and determination at 600 nm using a spectrophotometer. The incubation time was 0 hour (▨), 12 hours (▧), 24 hours (▩), 48 hours (▦) and 72 hours (▩).All assays were performed at least four times, and mean standard deviation was shown. The different letters denote significant differences ($P < 0.05$) among treatments.

4.3.3 CAs degradation by biofilm

The biofilm formation increased almost twice and thrice at the second and the third interval compared to the first interval on 96-well polystyrene microplates. Interestingly, the refresh of the medium resulted in the increase of biofilm formation at following intervals, while biofilm levels did not increase in the same medium after 2 and 3 days of incubation compared to the first day. The chemical degradation by biofilm at the first interval time was 35% for 3CA, almost 75% for 4CA and 34DCA after 3 hours, respectively. At the fourth interval, degradation rates of 3CA, 4CA and 34DCA were 95.3%, 92.6% and 100%, respectively (Figure 4-16).

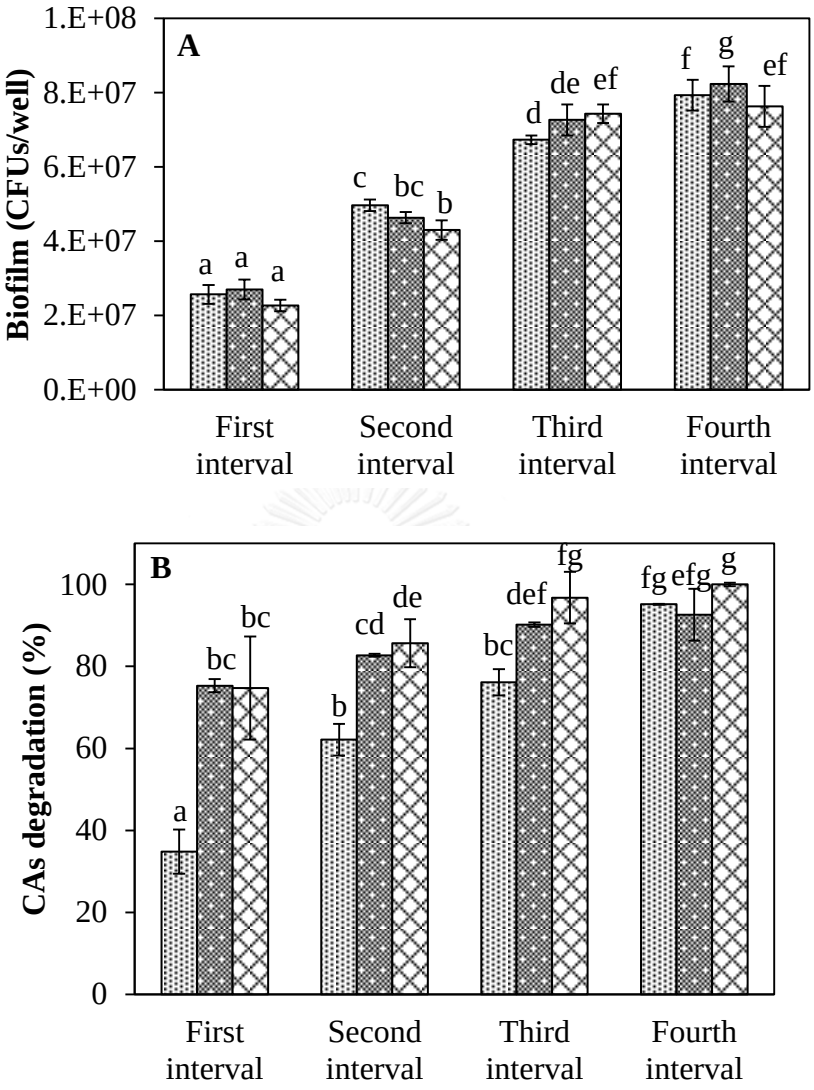


Figure 4-16. The biofilm formation (A) and chloroaniline degradation (B) by biofilm. The experiments were carried out in MYNa medium supplemented with 3CA (▨), 4CA (▩) and 34DCA (▩) at 0.1 mM, using microplates. Each interval lasted 24 hours and samples were taken after 3 hours of incubation for determination of biodegradation. The different letters denote significant differences ($P < 0.05$) among treatments in a figure.

4.4. Degradation toward 34DCA by *A. baumannii* GFJ1 immobilized in PVA-alginate beads

4.4.1 34DCA degradation rate and bacterial growth of freely suspended and cells immobilized in PVA and PVA-alginate beads

The degradation rates and growth rates of freely suspended cells and entrapped counterparts were determined in batch culture. The 34DCA degradation rate of freely suspended cells was lower than immobilized cells (Figure 4-17). Table 4-13 illustrates that the growth rates of entrapped cells during incubation were higher than freely suspended cells, and the beads with higher alginate concentrations reduced cell leakage.

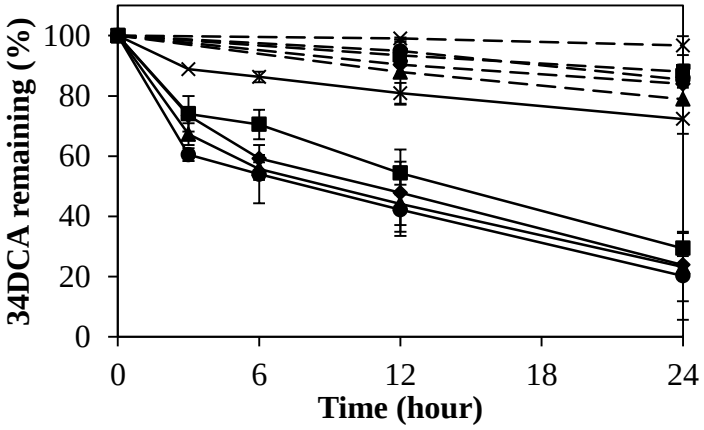


Figure 4-17. 34DCA degradation (solid lines) by freely suspended cells (x), immobilized cells in 10% PVA (♦), 10% PVA and 1% alginate (●), 10% PVA and 2% alginate (▲), and 10% PVA and 3% alginate (■). Abiotic controls without cells (dashed lines) were carried out to show the absorption of 34DCA into beads and autolysis of the chemical.

Table 4-13. Cell growth and cell leakage during degradation of entrapped cells and freely suspended cells of *A. baumannii* GFJ1. Data were obtained after 12 hours of incubation.

Type of cells	PVA (%)	Alginate (%)	Cell growth (1/h)(*)	Cell leakage ($x10^6$ CFUs/g bead)(*)
Entrapped cells	10	0	0.033 ± 0.003^c	5.17 ± 0.58^c
	10	1	0.033 ± 0.003^c	4.50 ± 0.50^c
	10	2	0.031 ± 0.004^c	2.67 ± 0.29^b
	10	3	0.021 ± 0.002^b	1.83 ± 0.29^a
Freely suspended cells	-	-	0.011 ± 0.001^a	-

(*)The different letters denote significant differences ($P < 0.05$) among treatments in the same groups (within a column)

4.4.2 34DCA degradation rate, cell growth and cell leakage of GFJ1 immobilized in PVA-alginate-porogen beads

After immobilization, the removal of porogens is necessary. Cell leakage during removal of PEG was significantly higher than during removal of $CaCO_3$ (Table 4-14). The beads with higher porogens, the cell leakage increased during removal of porogens and degradation process.

The addition of $CaCO_3$ as a pore-forming agent resulted in the changes of 34DCA degradation, cell growth and cell leakage (Table 4-15). The biodegradation

and cell growth during the incubation process were directly proportional, while cell leakage during incubation increased with the higher concentrations of $CaCO_3$, and decreased with higher concentrations of alginate. The activities of entrapped cells in beads with PEG as a porogen were determined to compare with beads with $CaCO_3$. The highest degradation and cell growth of entrapped cells in bead with 1% alginate, 2% alginate and 3% alginate were 3%, 5% and 7% $CaCO_3$, respectively (Table 4-15). With the same amount of the porogen, the degradation rates of cells immobilized in beads with PEG were smaller than beads with $CaCO_3$ (except 10% porogens) even though the growth rates of cells were insignificantly different. For example, the activities of entrapped cells in beads with 10% PVA, 2% alginate and 3 – 10% PEG are shown in Table 4-16. Bead with these components, the degradation of entrapped cell achieved with 5% porogen. The degradation rates, cell leakage and cell growth in beads with the same amount of PEG1000 and PEG6000 were insignificantly different. The cell leakage and cell growth of beads containing the same amount of $CaCO_3$ and PEG as porogens during incubation were also insignificantly different (Table 4-15 and 4-16).

Among the treatments, the cells entrapped in the bead with 10% PVA, 2% alginate and 5% $CaCO_3$ achieved the highest degradation rate (96.3 ± 2.8%). Moreover, the bead with these components was quite physically and chemically stable (presented in 4.5.7), so it was selected for the next experiments.

Table 4-14. *A. baumannii* GFJ1 leakage during removal of porogens from gel beads. Bead materials included 10% PVA, 2% alginate and 0 – 10% porogens.

Porogen	Concentration (%)	Cell leakage during immobilization and removal of porogens ($x10^6$ CFU/g bead)(*)
$CaCO_3$	3	4.3 ± 0.3^{a}
	5	5.0 ± 0.2^{a}
	7	6.0 ± 0.2^{a}
	10	6.9 ± 0.7^{a}
PEG1000	3	233.3 ± 33.3^{b}
	5	321.7 ± 24.7^{c}
	7	375.0 ± 21.8^{cd}
	10	443.3 ± 33.9^{e}
PEG6000	3	246.7 ± 7.6^{b}
	5	353.3 ± 47.5^{c}
	7	410.0 ± 52.7^{de}
	10	461.7 ± 50.0^{e}

(*)Different superscript letters indicate statistically significant differences ($p < 0.05$) among treatments using the oneway ANOVA with Duncan's test in SPSS software version 22.0.

Table 4-15. Effects of alginate and $CaCO_3$ concentrations on 34DCA degradation rates and cell leakage of immobilized *A. baumannii* GFJ1. The beads materials included 10% PVA, 1 – 3% alginate and 3 – 10% $CaCO_3$. Data were obtained after 12 hours of incubation.

Alginate (%)	$CaCO_3$ (%)	34DCA degradation (%)(*)	34DCA degradation rate (µM/h)(*)	Growth rate (1/h)(*)	Cell detected in the medium ($x10^6$ CFUs/ml)(*)
1	3	66.8 ± 10.7^{ab}	55.4 ± 6.3^{ab}	0.038 ± 0.004^{ab}	6.3 ± 0.3^{d}
	5	60.9 ± 5.0^{a}	46.5 ± 8.9^{a}	0.034 ± 0.004^{a}	8.8 ± 0.6^{f}
2	3	74.7 ± 2.5^{abc}	72.4 ± 4.3^{bcd}	0.042 ± 0.003^{abc}	3.8 ± 0.3^{b}
	5	96.3 ± 2.8^{d}	93.5 ± 9.3^{d}	0.054 ± 0.005^{d}	5.3 ± 0.3^{c}
	7	80.8 ± 9.5^{bc}	79.2 ± 5.2^{bcd}	0.042 ± 0.005^{abc}	7.0 ± 0.9^{de}
	10	67.0 ± 3.8^{ab}	63.8 ± 4.9^{abc}	0.035 ± 0.004^{a}	8.5 ± 0.9^{f}
3	3	76.2 ± 9.7^{bc}	70.4 ± 20.1^{abcd}	0.041 ± 0.006^{abc}	2.5 ± 0.0^{a}
	5	79.2 ± 7.2^{bc}	74.0 ± 18.0^{bcd}	0.045 ± 0.007^{bcd}	3.8 ± 0.3^{b}
	7	88.8 ± 0.5^{cd}	80.7 ± 6.1^{cd}	0.050 ± 0.005^{cd}	5.3 ± 0.6^{c}
	10	73.0 ± 0.3^{ab}	64.1 ± 5.4^{abc}	0.035 ± 0.003^{a}	7.8 ± 0.6^{ef}

(*)Different superscript letters indicate statistically significant differences ($p < 0.05$) among treatments within a column using the one-way ANOVA with Duncan's test in SPSS software version 22.0

Table 4-16. Effects of PEG concentrations on 34DCA degradation rates, growth rates and cell leakage of immobilized *A. baumannii* GFJ1. Bead materials included 10% PVA, 2% alginate and 3 – 10% PEG. Data were obtained after 12 hours of incubation.

PEG type	Concentration of PEG (%)	34DCA degradation (%)(*)	34DCA degradation rate (µM/h)(*)	Growth rate (1/h)(*)	Cell detected in the medium ($x10^6$ CFUs/ml)(*)
PEG1000	3	62.3 ± 1.4^{ab}	55.0 ± 0.9^{a}	0.040 ± 0.006^{a}	3.3 ± 0.3^{a}
	5	77.0 ± 7.6^{cd}	74.8 ± 5.6^{bc}	0.052 ± 0.006^{bc}	4.8 ± 0.4^{bc}
	7	62.1 ± 8.7^{c}	62.5 ± 8.8^{ab}	0.042 ± 0.005^{ab}	6.2 ± 0.3^{de}
	10	57.5 ± 4.6^{ab}	55.2 ± 6.6^{a}	0.036 ± 0.002^{a}	7.7 ± 0.6^{fg}
PEG6000	3	69.3 ± 4.9^{bc}	73.7 ± 4.8^{bc}	0.046 ± 0.007^{abc}	3.9 ± 0.6^{ab}
	5	88.0 ± 0.7^{d}	83.2 ± 5.6^{c}	0.055 ± 0.007^{c}	5.5 ± 0.8^{cd}
	7	69.3 ± 3.1^{bc}	69.2 ± 2.3^{b}	0.046 ± 0.005^{abc}	7.0 ± 0.9^{ef}
	10	53.8 ± 2.4^{a}	50.5 ± 0.8^{a}	0.038 ± 0.005^{a}	8.3 ± 0.8^{g}

(*)Different superscript letters indicate statistically significant differences ($p < 0.05$) among treatments within a column using the one-way ANOVA with Duncan's test in SPSS software version 22.0

4.4.3 Effect of cryoprotective agents on 34DCA biodegradation of the fresh and freeze-dried, entrapped cells

The bead with 10% PVA, 2% alginate and 5% $CaCO_3$ used for cell entrapment achieving the highest degradation rate was selected for this experiment. The degradation rates of entrapped cells in beads with 10% and 20% cryoprotectants before and after freeze-drying were insignificantly different. After the process, the biodegradation rates of entrapped cells in beads without any cryoprotectant showed the highest reduction (Table 4-17).

Table 4-17. 34DCA degradation by immobilized *A. baumannii* GFJ1. The freeze-dried beads were rehydrated before determination of biodegradation. Bead materials included 10% PVA, 2% alginate, 5% $CaCO_3$ and cryoprotectants. Data were obtained after 12 hours of incubation. 34DCA was added at 1.2 mM.

Cryoprotectant		Fresh bead(*)		Freeze dried bead(*)	
Cryoprotectant	%	Degradation (%)	Degradation rate (μM/h)	Degradation (%)	Degradation rate (μM/h)
None	0	96.3 ± 2.8^{e}	93.5 ± 9.3^{BC}	52.9 ± 5.2^{a}	53.6 ± 7.9^{A}
Glycerol	10	91.2 ± 8.0^{de}	102.6 ± 24.0^{C}	81.9 ± 9.5^{bcde}	83.3 ± 9.0^{BC}
	20	82.4 ± 1.1^{bcde}	95.6 ± 15.2^{BC}	80.7 ± 7.3^{bcde}	85.2 ± 6.5^{BC}
Sucrose	10	87.5 ± 1.9^{cde}	94.9 ± 1.8^{BC}	76.0 ± 6.9^{bcd}	81.3 ± 12.5^{AB}
	20	80.2 ± 3.1^{bcde}	89.8 ± 18.7^{BC}	72.8 ± 3.9^{bc}	78.4 ± 0.7^{ABC}
Sorbitol	10	86.9 ± 9.9^{cde}	90.4 ± 12.2^{BC}	66.5 ± 9.8^{ab}	71.2 ± 10.4^{AB}
	20	79.2 ± 10.9^{bcd}	81.9 ± 2.1^{BC}	72.3 ± 5.0^{bc}	75.5 ± 10.1^{AB}
Mannitol	10	80.2 ± 13.6^{bcde}	84.7 ± 14.7^{BC}	71.1 ± 11.8^{bc}	78.1 ± 12.2^{ABC}
	20	73.4 ± 9.0^{bc}	81.3 ± 16.1^{BC}	66.2 ± 5.0^{ab}	71.3 ± 0.5^{AB}

(*)The different letters denote significant differences ($P < 0.05$) of comparison among treatments in the same groups with different cryoprotectants, and before and after freeze-drying process.

4.4.4 Effect of cryoprotective agents on 34DCA biodegradation of the freeze-dried, entrapped cells in storage conditions

The biodegradation by entrapped cells after 1-month storage is depicted in Figure 4-18. The degradation by fresh and freeze-dried, entrapped cells did not significantly change before and after storage at 4 or -20 ^{o}C. Among cryoporotectants, glycerol incorporation showed the highest effective maintenance for 34DCA degradation at room temperature (around 30 ^{o}C). After 1-month storage at room temperature, cells immobilized in fresh beads without cryoprotectant showed the highest decrease of 34DCA degradation (reduced by 23.5%), followed by freeze-dried beads without cryoprotectant (reduced by 17.9%).

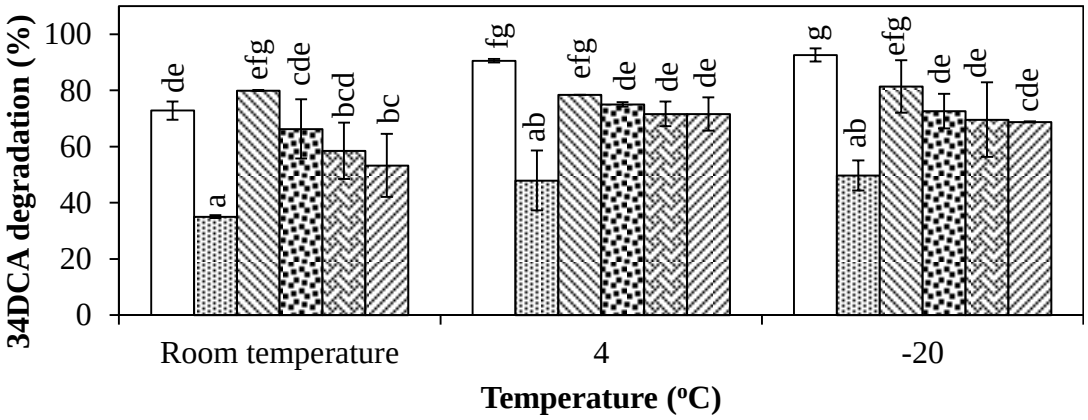


Figure 4-18. 34DCA degradation by the entrapped *A. baumannii* GFJ1 in PVA-alginate-$CaCO_3$ gels with and without cryoprotectant incorporation after 1-month storage at various temperatures. Beads included fresh bead without any cryoprotectant (□), freeze-dried bead without any cryoprotectant (▨), freeze-dried bead with 10% glycerol (▧), freeze-dried bead with 10% sucrose (▩), freeze-dried bead with 10%

sorbitol (▨) and freeze-dried bead with 10% mannitol (▨). Freeze-dried beads were rehydrated before determination of biodegradation. 34DCA was added at 1.2 mM. The different letters denote significant differences ($P < 0.05$) among treatments.

4.4.5 Glycerol diffusion from gel beads during immobilization and removal of porogen

The beads with glycerol showed the effectively to enhance the tolerance during freeze drying process and long-term storage. During the immobilization and removal of porogens processes, glycerol was leaked from beads. It can be seen in table 4-18 that glycerol leakage from beads with PEG as a porogen was higher than beads with $CaCO_3$. Data were obtained when ratio volume of gel bead solution to boric acid-$CaCl_2$ solution was 3.0. However, the diffusion of glycerol depended on volume of liquid used for immobilization and removal of porogens. For example, glycerol diffusion was 41.2% ± 5.9% when ratio volume of gel bead solution to boric acid-$CaCl_2$ solution was 1.0.

Table 4-18. Glycerol leakage during immobilization and removal of porogens. The experiments were carried out using beads with 10% PVA, 2% alginate and 0-10% porogens and 10% glycerol.

Porogen		Glycerol leakage (%)(*)	
Types of porogens	(%)	Glycerol leaked during immobilization	Glycerol leaked during removal of porogens
$CaCO_3$	0	67.4 ± 8.0^{a}	-
	3	66.4 ± 4.3^{a}	6.2 ± 0.6^{a}
	5	66.8 ± 6.6^{a}	7.0 ± 0.6^{a}
	7	68.5 ± 4.7^{a}	9.8 ± 1.3^{b}
	10	67.5 ± 7.1^{a}	12.9 ± 1.1^{c}
PEG1000	3	66.6 ± 6.2^{a}	14.2 ± 1.3^{de}
	5	70.0 ± 5.8^{a}	16.2 ± 1.2^{def}
	7	71.8 ± 6.2^{a}	17.1 ± 0.9^{ef}
	10	72.3 ± 5.9^{a}	18.0 ± 1.5^{f}
PEG6000	3	68.7 ± 7.2^{a}	15.0 ± 1.1^{cde}
	5	71.4 ± 3.4^{a}	16.8 ± 2.0^{ef}
	7	72.7 ± 4.3^{a}	18.1 ± 1.5^{f}
	10	73.3 ± 5.1^{a}	18.6 ± 2.2^{f}

(*)The different letters denote significant differences ($P < 0.05$) among treatments in the same groups (within a column).

4.4.6 Reusability of entrapped cells

The long-term stability of 34DCA degradation by immobilized cells was established by repeating batch degradation. The degradation efficiency maintained stably for 6 cycles (Figure 4-19). At the following cycles, the degradation rates of entrapped cells in both fresh and freeze-dried beads started reducing, beads became weakened, reduction of weight and higher cell leakage.

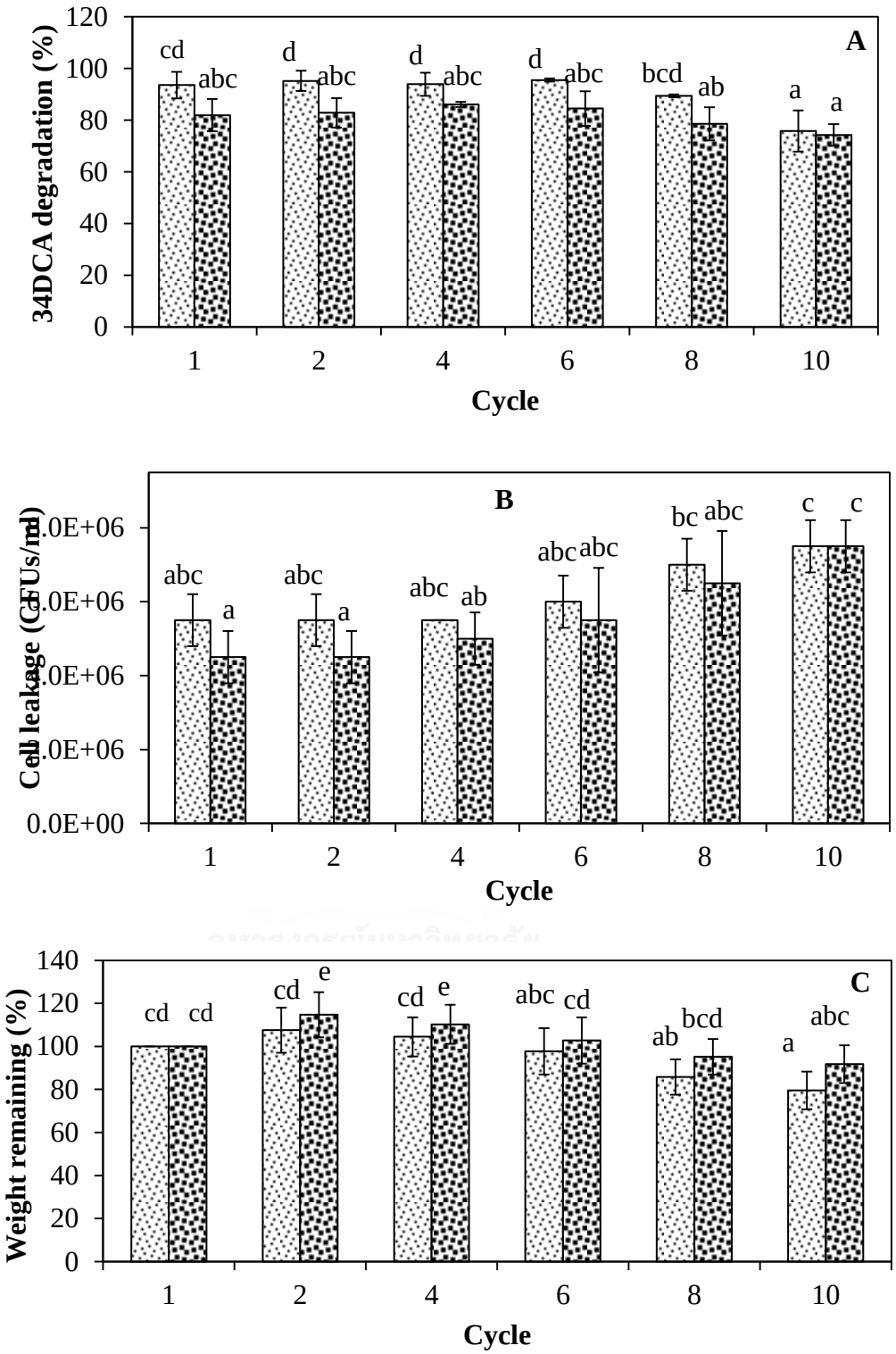


Figure 4-19. Repeat 34DCA degradation (A) by entrapped cells, cell leakage (B) and weight losses (C) of fresh beads with 10% PVA-2% alginate-5% $CaCO_3$ (▨) and

freeze-dried bead with 10% PVA-2% alginate-5% $CaCO_3$-10% glycerol (▨). 34DCA was added at 1.2 mM**.** The different letters denote significant differences ($P < 0.05$) among treatments in within a figure.

4.4.7 Characterization of bead

4.4.7.1 Diffusion coefficient determination

Analyzing effective diffusion coefficient of substrate molecules within a gel matrix plays an important role to characterize its kinetic behavior and optimize the conditions for cell immobilization (Ha et al., 2008). The diffusion coefficients of glucose were calculated based on the plot of $-\ln(C_t - C_\infty)/C_0 - C_\infty)$ versus diffusion time shown in Figure 4-20, and the results were shown in Table 4-19. The values c_∞ were determined after 72 hours, which further change of glucose concentration was not found. The diffusing rates of glucose from beads were directly proportional to the amount of $CaCO_3$ and PEG, indicating the increase of permeability of the gel beads. The diffusion coefficients of beads with $CaCO_3$ and PEG6000 were insignificantly different and higher than beads with PEG1000 in some treatments (Table 4-19).

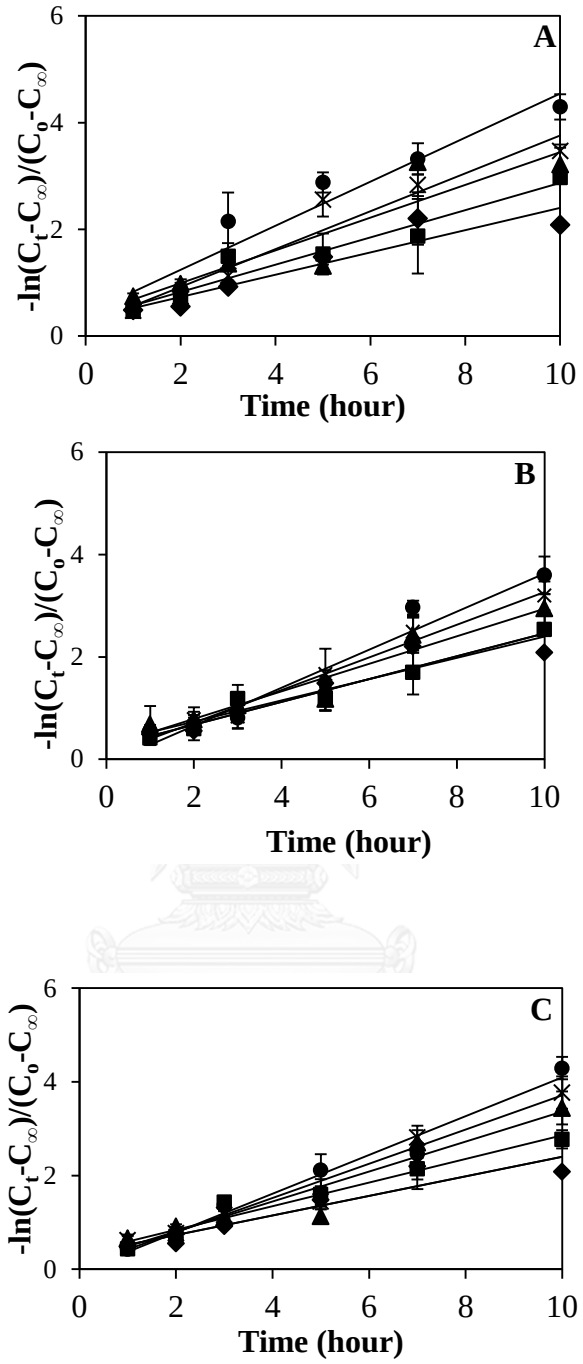


Figure 4-20. Plot of $-\ln(C_t - C_\infty)/C_0 - C_\infty)$ versus diffusion time of beads with different concentrations of $CaCO_3$ (A), PEG1000 (B) and PEG6000 (C) as a function of diffusion time t. The immobilization matrices included 10% PVA and 2% alginate and 0 % (♦), 3% (■), 5% (▲), 7% (x) and 10% porogens (●).

Table 4-19. The diffusion coefficients of glucose inside the beads without bacteria. Bead materials included 10% PVA, 2% alginate and 0 – 10% porogens. The experiment was carried out at 30 ^{o}C.

Porogen	Concentration of porogen (%)	Diffusion coefficient (x10^{-5} cm^2/s)$^{(*)}$
None	0	2.9 ± 0.3^{a}
$CaCO_3$	3	3.6 ± 0.3^{b}
	5	4.3 ± 0.5^{cd}
	7	5.0 ± 0.3^{ef}
	10	5.8 ± 0.6^{g}
PEG1000	3	3.1 ± 0.2^{ab}
	5	3.8 ± 0.3^{bc}
	7	4.5 ± 0.2^{de}
	10	5.2 ± 0.5^{fg}
PEG6000	3	3.6 ± 0.2^{b}
	5	4.5 ± 0.5^{de}
	7	5.1 ± 0.3^{ef}
	10	5.8 ± 0.2^{g}

$^{(*)}$The different letters denote significant differences ($P < 0.05$) among treatments

4.4.7.2 Physical and chemical stability tests

The stability of a polymer matrix is necessary for cell immobilization and its application in remediation of industrial wastewaters. Beads were broken or

some parts of carriers were dissolved in the physical test (Figure 4-21). It can be seen that the increase of alginate enhanced the physical stability, while weight remaining reduced when porogen contents were increased (Figure 4-22). There was no significant difference of weight losses among beads with the same concentrations of $CaCO_3$, PEG1000 and PEG6000.

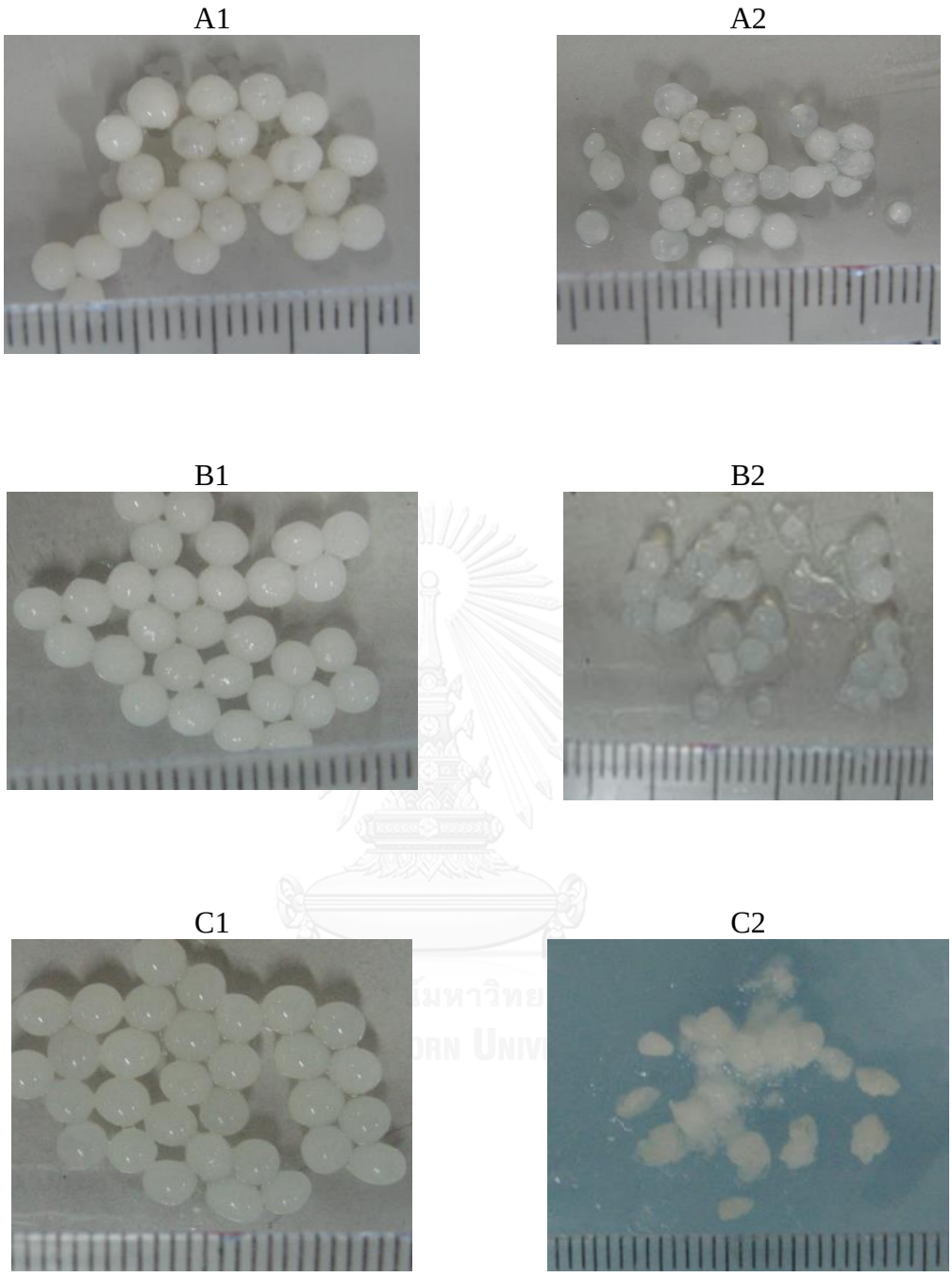


Figure 4-21. Beads before (1) and after (2) the physical test. Beads included 10% PVA- 2% alginate-0% $CaCO_3$ (A), 10% PVA-2% alginate-10% $CaCO_3$ (B) and 10% PVA-2% alginate-10% PEG6000 (C).

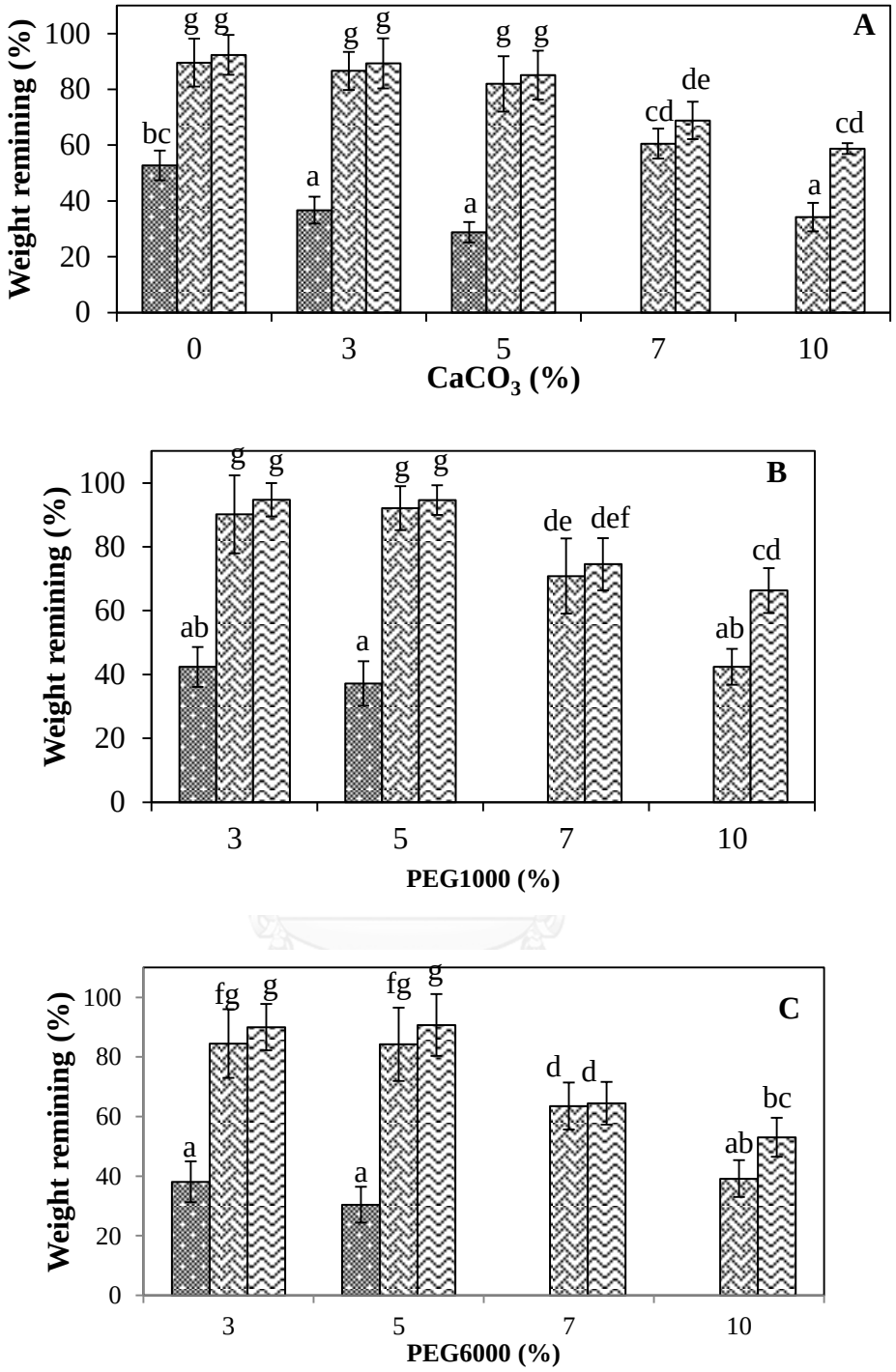


Figure 4-22. Physical stability tests for PVA-alginate-$CaCO_3$ (A), PVA-alginate-PEG1000 (B) and PVA-alginate-PEG6000 (C) matrices. The bead materials included 10% PVA, 1% alginate (▩), 2% alginate (▩), 3% alginate (▩) and 0% - 10%

porogens. The different letters denote significant differences ($P < 0.05$) among treatments in all figures.

For the chemical stability tests of the beads with the same materials, the weight losses at pH < 7 were smaller than at pH > 7. For example, no more than 20% weight of beads with 10% PVA, 2% alginate and 0 – 10% $CaCO_3$ was lost at pH < 7, while the significant weight was lost at pH values greater than 8 (Figure 4-23 and 4-24).

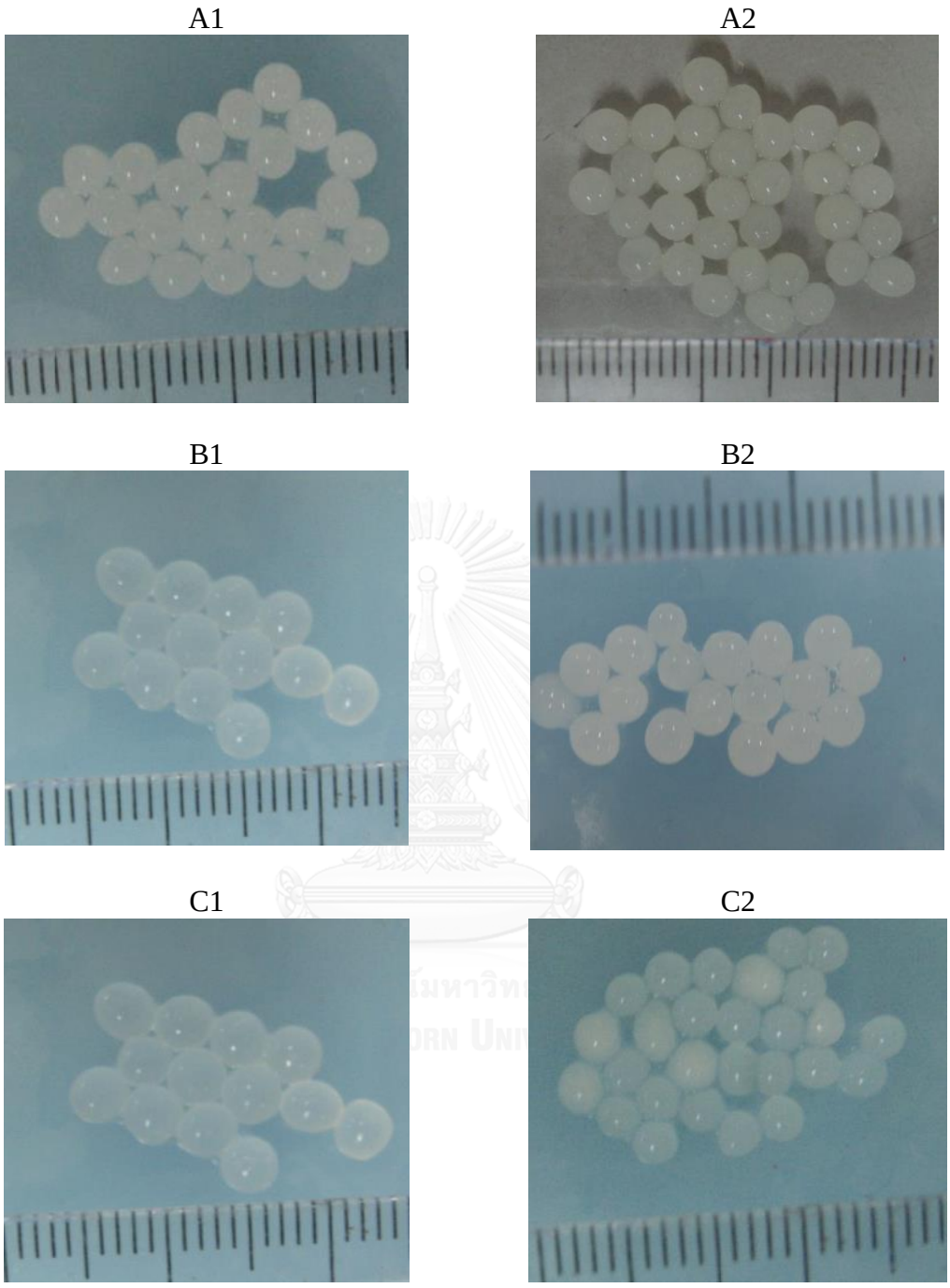


Figure 4-23. Beads before (1) and after (2) the chemical tests at pH = 13. Beads included 10% PVA-2% alginate-0% $CaCO_3$ (A), 10% PVA-2% alginate-10% $CaCO_3$ (B) and 10% PVA-2% alginate-10% PEG6000 (C).

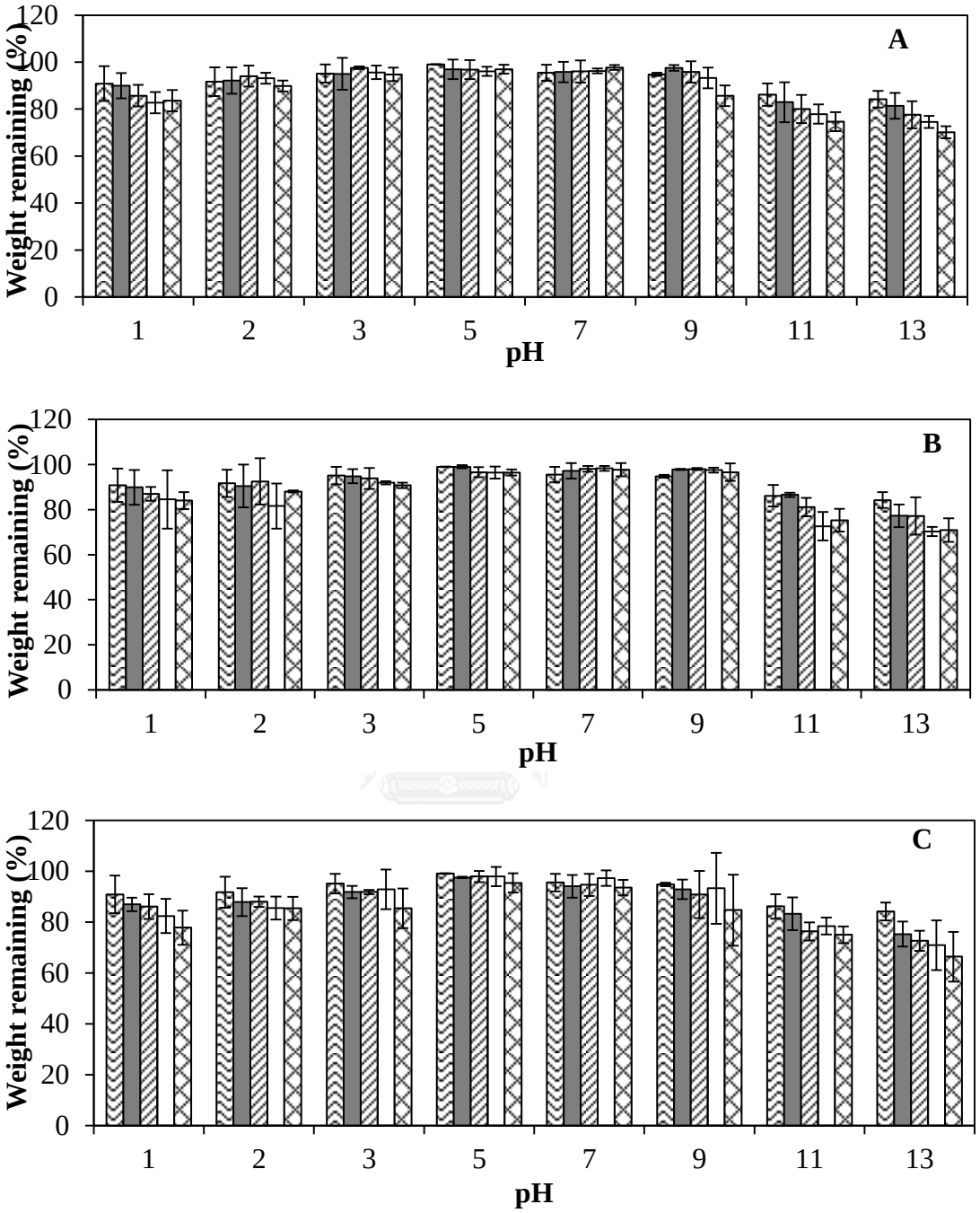


Figure 4-24. Weight remaining in chemical stability tests for PVA-alginate-$CaCO_3$ (A), PVA-alginate-PEG1000 (B) and PVA-alginate-PEG6000 (C) matrices. The bead materials included 10% PVA, 2% alginate and 0% porogen (▨), 3% porogens (■), 5% aporogen (▨), 7% porogens (□) and 10% porogens (▩). The experiment was carried

out using beads without cell. The different letters denote significant differences ($P < 0.05$) among treatments in all figures.

4.4.7.3 Stability of beads in phosphate buffer

Beads were submersed in phosphate buffer with different concentrations (0.01 and 0.1 M phosphate). In medium containing 0.01 M phosphate, weight of beads slightly increased. In this medium, beads were stable, did not break or change the shape after 12 hours. On the other hand, beads become soft after 6 hours, viscous after 12 hours and a part of beads was diluted in medium containing 0.1 M phosphate (Table 20).

Table 4-20. Characteristics of beads after treatment in phosphate buffer. The experiment was carried out at static condition.

Cryoprotectants	After 6 hours				After 12 hours			
	Fresh beads		Freeze dried beads		Fresh beads		Freeze dried beads	
	Weigh remaining (%)	Characteristics of beads(*)	Weigh increasing (%)	Characteristics of beads(*)	Weigh increasing (%)	Characteristics of beads(*)	Weigh increasing (%)	Characteristics of beads(*)
	0.01 M phosphate							
None	105 ± 4.4	Stable	108.6 ± 5.3	Stable	100 ± 5.9	Stable	106.7 ± 2.3	Stable
10% glycerol	110 ± 8.8	Stable	107.0 ± 6.2	Stable	103 ± 5.1	Stable	110.4 ± 7.7	Stable
10% sucrose	108 ± 6.1	Stable	104.8 ± 5.7	Stable	105 ± 6.4	Stable	109.8 ± 2.9	Stable
10% sorbitol	106 ± 5.2	Stable	112.4 ± 10.4	Stable	107 ± 8.6	Stable	108.9 ± 7.5	Stable
10% mannnitol	110 ± 11.1	Stable	110.2 ± 11.4	Stable	103 ± 3.3	Stable	109.9 ± 6.0	Stable
	0. 1 M phosphate							
None	142.3 ± 11.0	Soft	168.1 ± 18.0	Soft	-	Viscous	-	Viscous
10% glycerol	146.2 ± 14.2	Soft	167.2 ± 12.2	Soft	-	Viscous	-	Viscous
10% sucrose	142.4 ± 11.7	Soft	173.1 ± 14.1	Soft	-	Viscous	-	Viscous
10% sorbitol	157.2 ± 12.3	Soft	179.2 ± 11.5	Soft	-	Viscous	-	Viscous
10% mannnitol	160.3 ± 15.5	Soft	166.6 ± 11.6	Soft	-	Viscous	-	Viscous

(*)Visual inspection of the immobilized beads

4.4.7.4 Determination of swelling ratio and swelling rate constant

After the freeze-drying process, the bead diameter was smaller (Figure 4-25). Bead diameter was around 3.0 and 2.5 – 2.7 mm before and after the process, respectively. After absorption of water, weight and diameter of beads increased (Figure 4-26).

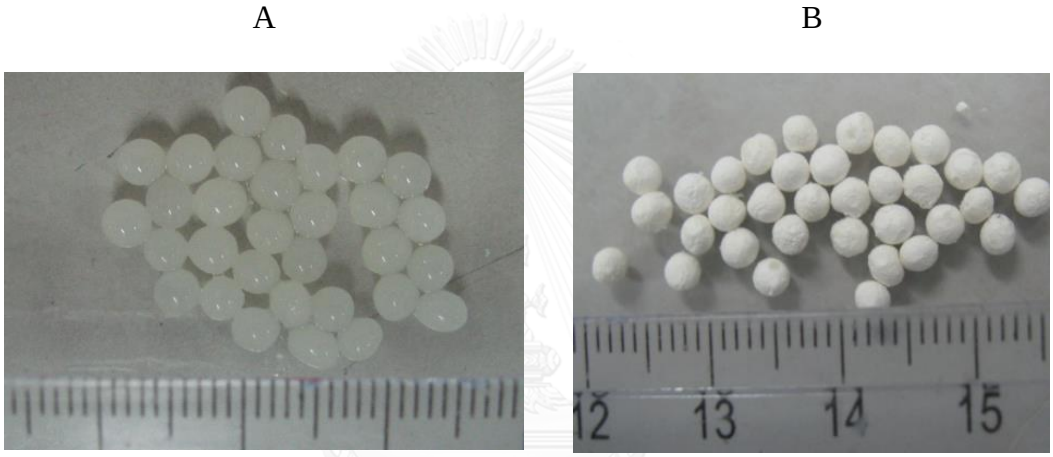


Figure 4-25. PVA-alginate beads before (A) and after freeze drying (B).

Figure 4-26. Dried beads before (A) and after rehydration (B). The bead materials included 10% PVA, 2% alginate and without porogen (1), 10% PVA, 2% alginate and 5% $CaCO_3$ (2), and 10% PVA, 2% alginate and 10% $CaCO_3$ (3).

Swelling of a carrier shows water absorption capacity. It can be seen in Figure 4-27 that beads absorbed water rapidly in the initial swelling stage, and increased as a function of time. Beads with higher porogen concentrations could absorb water better than low porogen concentrations (Figure 4-27). Swelling ratios and rate constants directly depended on the porogen contents in the beads, the water absorption capacity increased with the beads containing higher porogens.

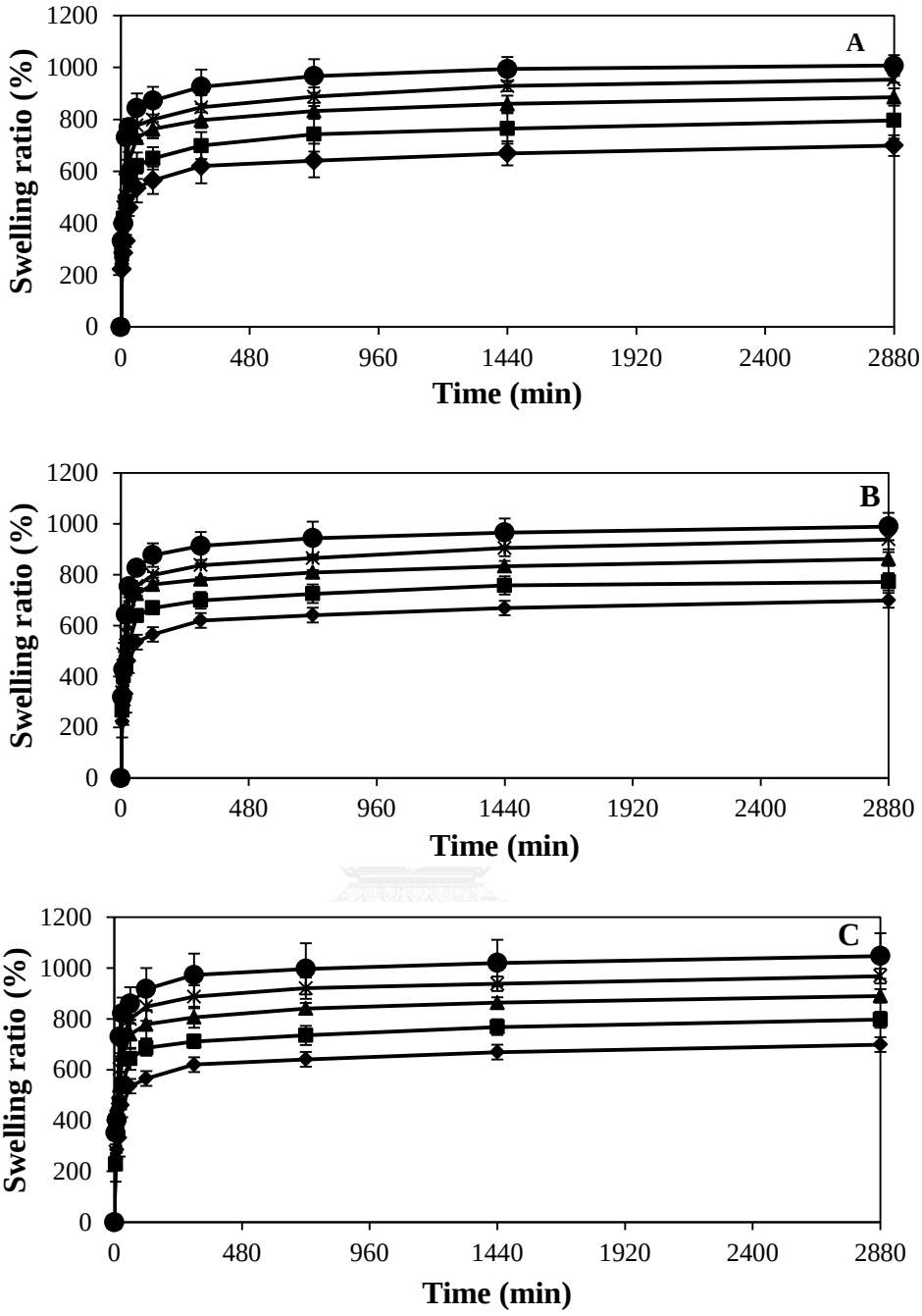


Figure 4-27. Swelling ratio curves of beads as a function of time and $CaCO_3$ (A), PEG1000 (B) and PEG6000 (C) concentrations. The bead materials included 10% PVA, 2% alginate and 0 % (♦), 3% (■), 5% (▲), 7% (x) and 10% porogens (●).

The swelling rate constants of beads were calculated based on slope of $\ln(Q_e-Q_0)/(Q_e-Q_t)$ versus time (t) (Figure 4-28). The swelling constants of beads containing $CaCO_3$ and PEG were not significantly different (Table 4-21).

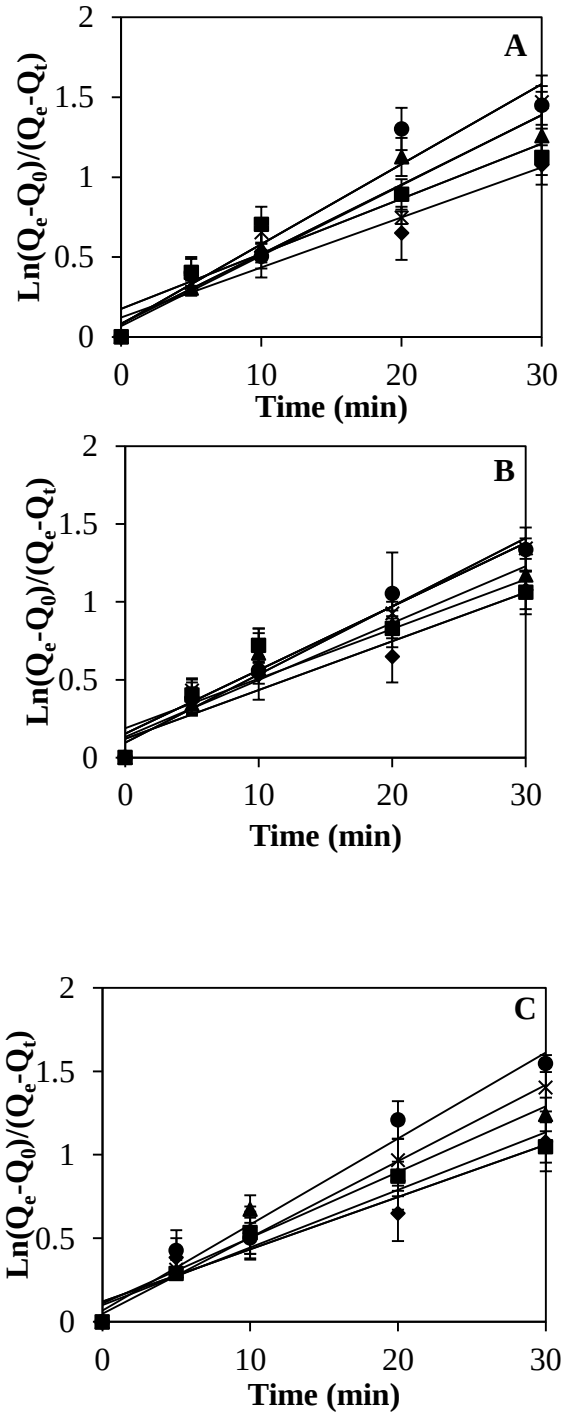


Figure 4-28. $Ln(Q_e-Q_0)/(Q_e-Q_t)$ versus swelling time of PVA-alginate-$CaCO_3$ (A), PVA-alginate-PEG1000 (B) and PVA-alginate-PEG6000 (A) matrices with various porogen concentrations. Bead materials included 10% PVA, 2% alginate and 0 % (♦), 3% (■), 5% (▲), 7% (x) and 10% porogens (●).

Table 4-21. The swelling rate constants of beads without bacteria. Bead materials included 10% PVA, 2% alginate and 0 – 10% porogens.

Porogen	Concentration (%)	Swelling rate constant k (1/h)(*)
None	0	1.88 ± 0.23^{a}
$CaCO_3$	3	2.35 ± 0.12^{bcd}
	5	2.62 ± 0.24^{cdef}
	7	2.77 ± 0.30^{def}
	10	3.00 ± 0.31^{ef}
PEG1000	3	2.02 ± 0.18^{ab}
	5	2.49 ± 0.27^{cd}
	7	2.52 ± 0.20^{cd}
	10	2.82 ± 0.20^{def}
PEG6000	3	2.20 ± 0.23^{abc}
	5	2.55 ± 0.27^{cde}
	7	2.71 ± 0.25^{def}
	10	3.07 ± 0.30^{f}

(*)The different letters denote significant differences ($P < 0.05$) among treatments

4.4.7.5 Structure of beads

The real density, porosity and pore volume of beads directly enhanced with the amount of porogens (Table 4-22). These parameters were not significantly different with the same amount of the $CaCO_3$, PEG1000 and PEG6000 in beads. The SEM images of bead cross-sections proved such phenomena. It can be seen in Figure 4-29A that the beads in the absence of any porogen showed a dense cross-section, which leaded to resist the mass transfer. The increase of porogens resulted in increasingly large of pore sizes (Figure 4-29B, C, D), and thus facilitated mass transfer inside the gel carriers. It can see in the Figure that bacteria embedded within hydrogel-matrix layers of the fresh beads and beads after freeze-drying, storage and rehydration process (Figure 4-29 and 4-30).

Table 4-22. Density, pore volume and relative porosity beads. Bead materials included 10% PVA, 2% alginate and 0 – 10% $CaCO_3$ or PEG.

Porogen	% porogen	Density (mg/cm^3)(*)	Pore volume (ml/g)(*)	Relative porosity(*)
None	0	1.48 ± 0.04^{a}	0.10 ± 0.01^{a}	0.15 ± 0.01^{a}
$CaCO_3$	3	1.36 ± 0.06^{a}	0.38 ± 0.02^{b}	0.52 ± 0.01^{b}
	5	1.39 ± 0.04^{a}	0.73 ± 0.05^{c}	1.01 ± 0.03^{c}
	7	1.38 ± 0.14^{a}	1.02 ± 0.06^{e}	1.41 ± 0.20^{d}
	10	1.44 ± 0.14^{a}	1.33 ± 0.03^{g}	1.91 ± 0.23^{f}
PEG1000	3	1.35 ± 0.09^{a}	0.32 ± 0.03^{b}	0.43 ± 0.04^{b}
	5	1.40 ± 0.10^{a}	0.67 ± 0.05^{c}	0.94 ± 0.13^{c}
	7	1.44 ± 0.16^{a}	0.92 ± 0.12^{d}	1.32 ± 0.12^{d}
	10	1.35 ± 0.07^{a}	1.23 ± 0.06^{f}	1.66 ± 0.01^{e}
PEG6000	3	1.43 ± 0.13^{a}	0.33 ± 0.01^{b}	0.46 ± 0.04^{b}
	5	1.45 ± 0.14^{a}	0.71 ± 0.03^{c}	1.03 ± 0.11^{c}
	7	1.36 ± 0.10^{a}	1.03 ± 0.07^{e}	1.40 ± 0.19^{d}
	10	1.35 ± 0.14^{a}	1.37 ± 0.03^{g}	1.86 ± 0.19^{ef}

(*)The different letters denote significant differences ($P < 0.05$) among treatments in the same groups (within a column)

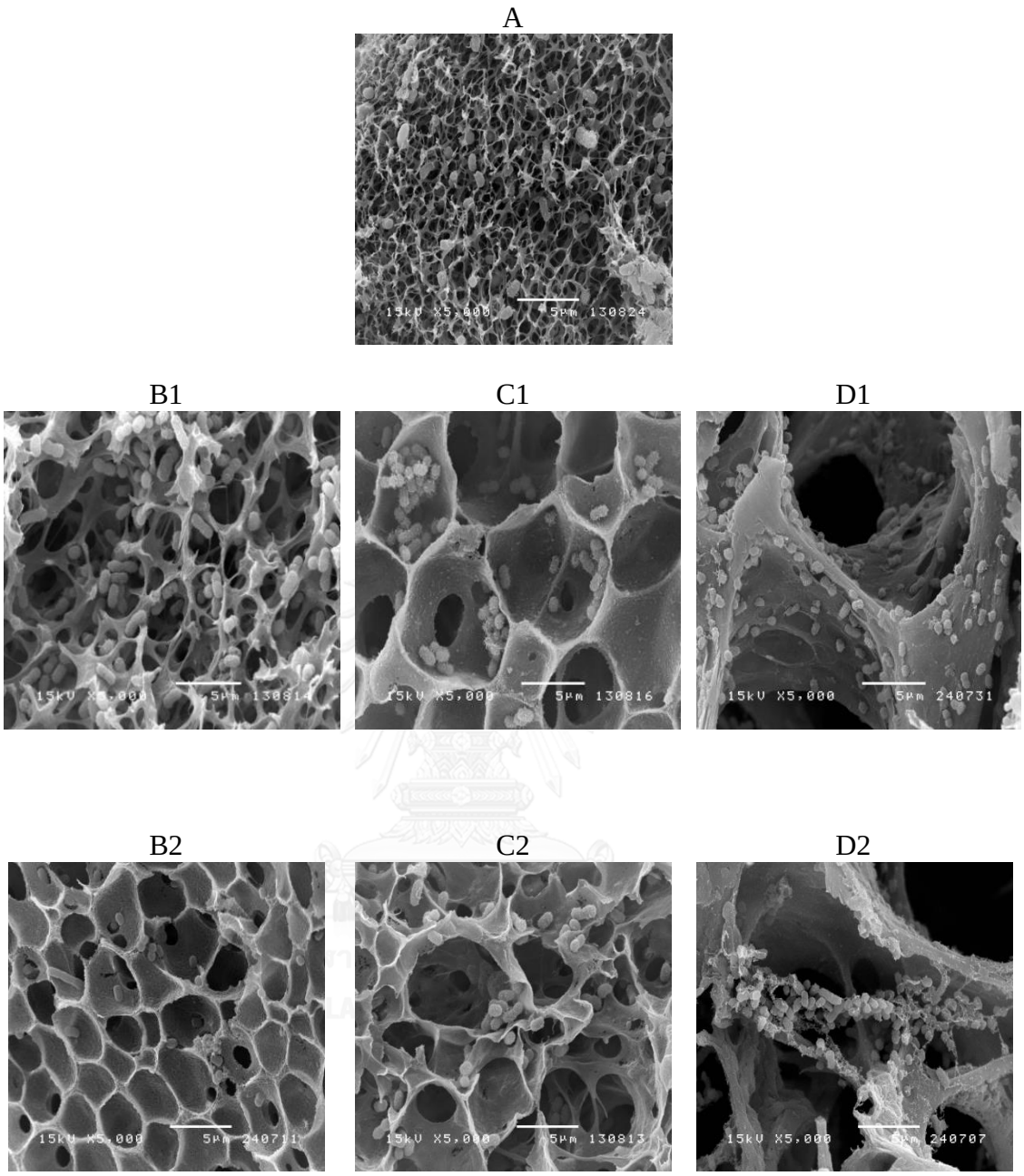


Figure 4-29. Scanning electron micrographs of fresh beads with 10% PVA-2% alginate without the porogen (A) and 3% (B), 5% (C) and 10% (D) porogens. The porogens included $CaCO_3$ (1) and PEG6000 (2). The magnification was 5000x.

After the entrapped cell beads were freeze-dried, stored and rehydrated, the bead with glycerol was larger pore size compared to the bead without any cryoprotectant (Figure 4-31).

A B

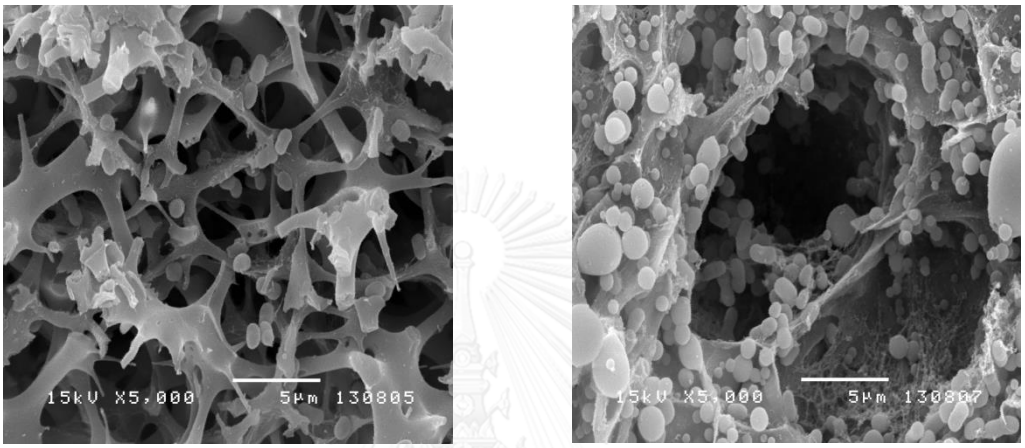


Figure 4-30. Scanning electron micrographs of freeze-dry-rehydration beads with 10% PVA-2% alginate-5% $CaCO_3$ without glycerol (A) and 10% glycerol (B). The magnification was 5000x.

CHAPTER 5 DISCUSSION

5.1 Degradation of CAs under aerobic conditions by *A. baumannii* GFJ1

5.1.1 Utilization of CAs as sources of carbon, nitrogen and energy

A. baumannii GFJ1 could utilize CAs as sources of carbon, nitrogen and energy under both aerobic and anaerobic conditions. The degradation of CAs belongs to bacterial strains. Moreover, the ability to degrade chlorinated compounds depends on structure, number of chlorine substituents and position of chlorine in the molecules (Bhatt et al., 2007). The extent of degradation of a chemical also depends on bacterial strains. Aniline is considered to be less toxic and less recalcitrant than any CAs. Previous reports showed that the aerobic biodegradation rate for aniline was higher than for chlorinated anilines (Loidl et al., 1990; Reber et al., 1979; Schukat et al., 1983; Zeyer et al., 1985b; Zhang et al., 2010a). The aniline-adapted cells of *P. multivorans* An1 degraded CAs in the order of 4CA < 2CA < 3CA, while this strain insignificantly degraded 34DCA. On the other hand, the utilization of aniline and other chlorinated anilines by *A. baumannii* GFJ1 was significantly lower than the utilization of 4CA and 34DCA. The properties of chemicals such as low values of van der Waals radius and bulky substituents did not apparently affect the biodegradation by *A. baumannii* GFJ1.

There are a number of reports describing the utilization of CAs as sources of carbon, nitrogen and energy, including monochloranilines (Ferschl et al., 1991;

González et al., 2014; Loidl et al., 1990; Vangnai and Petchkroh, 2007; Zeyer and Kearney, 1982; Zhang et al., 2010b) and dichloroanilines (Li et al., 2012a; Travkin and Golovleva, 2003; Yao et al., 2011b). However, there are only some bacterial strains which could degrade both mono- and dichloroaniline. For instance, *P. diminuta* was described to be able to utilize 3CA, 4CA and 34DCA (up to 0.3 mM) as growth substrates (Surovtseva et al., 1985). *Delftia tsuruhatensis* H1 was another strain that could utilize monochloroanilines and 34DCA. Moreover, most bacterial strains showed low rates of 34DCA utilization. For examples, the aerobic utilization of 34DCA by *Bacillus megaterium* IMT21 was about 75% at initial of 50 mg/l after 20 days (Yao et al., 2011b), or *Myroides odoratimimus* LWD09 could utilize 74% of 34DCA with initial concentration of 30 mg/l after 24 hours (Li et al., 2012a) (Table 5-1). In contrast, it took 24 hours for *A. baumannii* GFJ1 to utilize 34DCA nearly completely as a sole carbon, nitrogen and energy source at an initial concentration of 97 mg/l.

Although a number of studies have investigated the degradation of CAs, there is only one report describing the utilization of mono-, di- and trichloroanilines as growth substrates by a bacterial mixture (Lu et al., 2009). However, this bacterial complex required 8 days of lag phase and degraded less than 50% of the 246TCA after 12 days from an initial concentration of 0.05 mM (Lu et al., 2009). 246TCA is considered to have a lower toxicity than the other TCAs (Gruzdev et al., 2011). ***A. baumannii* GFJ1 is the first pure culture showing the ability of degradation toward aniline, mono-, di- and trichloroaniline.** These results showed that *A. baumannii* GFJ1 could degrade a broad range of CAs, probably it was high adaption capacity with CAs.

The determination of chemical utilization during cell growth (low cell numbers at the beginning) shows the adaptation, tolerance of bacteria with chemicals, which illustrates the utilization capacity. The analysis of the degradation kinetics showed that the inhibition of 3CA, 4CA and 34DCA utilization occurred at high substrate concentrations ($\geq$ 0.3 mM). At these concentrations, the degradation rate was reduced due to the toxicity of chemicals to bacteria. Similarly, the degradation of 34DCA by *A. baylyi* GFJ2 (Hongsawat and Vangnai, 2011), and 4CA and 34DCA by aerobic granules (Zhu et al., 2012; Zhu et al., 2011a) was inhibited at high substrate concentrations. In batch cultivation with a low initial cell number, cells grew exponentially and the kinetic models of the degradation of organic compounds depended upon the concentration of chemicals (Schmidt et al., 1985). A previous study showed that the maximum specific degradation rates of 4CA and 34DCA by *A. baylyi* GFJ2 were smaller than *A. baumannii* GFJ1, and the half-saturation coefficient was higher than *A. baumannii* GFJ1 (Hongsawat and Vangnai, 2011) (Table 5-1). The maximum degradation rate indicates the biodegradation efficiency and shows the advantage for further treatment of CAs. A smaller K_s indicates the degradation rate approaching K_{max} at lower concentration of chemicals, and bacteria have a low threshold of the concentration at which bacteria degrade the chemicals. Thus, overall these data show that *A. baumannii* GFJ1 utilized 4CA and 34DCA effectively, especially at low concentrations.

The utilization rates toward 4CA and 34DCA by *A. baumannii* GFJ1 during cell growth with low cell numbers at the beginning were higher than high cell numbers (resting cells). The reasons for this phenomenon were probably because of the shortage of inorganic minerals in the medium with condensed cells. Another

reason was that the presence of ammonium sulfate in the medium inhibited the degradation rate (shown in 4.1.3). The determination of chemical degradation *via* resting cells shows the real degradation efficiency.

The effects of co-substrates on CAs degradation were determined by supplementation with carbon and nitrogen sources. The indicated factors may be useful information for further application in bioremediation. The supplementation of co-substrates in culture media stimulated cell growth. However, the addition of co-substrates reduced the specific degradation. Bacteria used co-substrates, CAs as nutrient sources resulting in higher cell growth as well as protein content. Carbon sources (succinate, citrate) did not inhibit the degradation rates (Table 4-3, 4-4 and 4-5). On the other hand, the addition of ammonium sulfate and ammonium chloride as nitrogen sources inhibited the degradation rates of 3CA and 4CA, while degradations rate (% degradation) of 34DCA did not reduce, which indicated that the effects of nutrients on biodegradation toward 3CA, 4CA and 34DCA were not similar. Moreover, the presence of sodium nitrate as a nitrogen source did not reduce the total of degradation rates of CAs. These results were in contrast to the degradation of 4CA by *A. baumannii* CA2, *P. putida* CA16 and *Klebsiella* sp. CA17. These bacterial strains reduced the degradation rates with the addition of sodium nitrate (Vangnai and Petchkroh, 2007). The degradation of 4CA by *A. baylyi* GFJ2 increased with the addition of ammonium sulfate and decreased with the addition of sodium nitrate, respectively (Hongsawat and Vangnai, 2011). The utilization of CAs by *A. baumannii* GFJ1 (at 0.1 mM) did not enhance with the supplementation of any co-substrate (except the addition of citrate and nitrate in degradation 3CA) even though they stimulated cell growth because bacteria had more nutrients. The presence of co-

substrates, bacteria preferred to use these compounds for growth. These results were different from previous reports that the degradation of CAs increased with the presence of other nutrients (Travkin et al., 2003b; Zhang et al., 2010a). With these results, *A. baumannii* GFJ1 can be applied for bioremediation of 3CA, 4CA and 34DCA unnecessary addition of any nutrient source.

5.1.2 Effects of CAs concentrations on growth of *A. baumannii* GFJ1

GFJ1 could grow up to 2.8 mM 3CA, 3.6 mM 4CA and 1.0 mM 34DCA, while it could not grow at > 0.3 mM 246TCA. The growth of bacteria belonged to toxicity of the chemicals. Obviously, 246TCA is more toxic than 34DCA, and 34DCA is more toxic than 3CA and 4CA. The previous study showed that *A. baumannii* CA2, *P. putida* CA16 and *Klebsiella* sp. CA17 were inhibited to grow at higher 1.2 mM 4CA (Vangnai and Petchkroh, 2007). These numbers showed that *A. baumannii* GFJ1 had a broad range of chemical concentrations, which showed the potential of application in bioremediation. The supplementation with 4CA and 34DCA at 0.05 – 0.4 mM increased growth rates because bacteria had more carbon and nitrogen sources. However, the growth was inhibited at higher chemical concentrations due to the increase of toxicity.

A. baumannii GFJ1 could grow in medium containing high concentration of CAs and could degrade a wide range CAs with high rates compared to others (Table 5-1), which can make *A. baumannii* GFJ1 as a potent bacterial strain for bioremediation toward 3CA in polluted areas.

Table 5-1. Comparison of the degradation rates and growth of *A. baumannii* GFJ1 and several previous bacterial strains under aerobic condition

Bacteria strain	Chemical	Degradation condition	Characteristic degradation	Reference
A. baumannii CA2, *P putida* CA16 and *Klebsiella* sp. CA17.	4CA	No strain could grow in medium containing more than 1.6 mM	Degradation rates in medium supplemented with 0.1% yeast extract of *A. baumannii* CA2 and *P. putida* CA16 were 8.7 ± 1.60, 13.6 ± 2.50 and 19.0 ± 2.33 nmol min^{-1} (mg cell $protein)^{-1}$, respectively.	(Vangnai and Petchkron, 2007)
A. baylyi strain GFJ2	4CA and 34DCA	Various concentrations	Degradation CAs with the presence of co-substrates. Vmax of 4CA = 0.17 ± 0.01 and 34DCA = 0.36 ± 0.02 μmol(h.mg cell $protein)^{-1}$; K_s of 4CA = 0.79 ± 0.12, and 34DCA = 0.70 ± 0.02	(Hongsawat and Vangnai, 2011)
Bacillus megaterium IMT21	34DCA	0.3 mM	The strain utilized 34DCA as a sole carbon, nitrogen source with about 70% after 20 days.	(Yao et al., 2011)
Myroides odoratimimus LWD09	34DCA	Various concentrations, degradation in saline condition	LWD09 utilized 74% of 34DCA with initial concentration of 30 mg/l after 24 hours	(Li et al., 2012a)
Mixture of bacteria	2CA, 3CA,	20 mg/l and 10mg/l	Bacteria had a lag phase in degradation of CAs, and	(Lu et al., 2009)

	4CA, 24DCA, 246TCA		degraded not over 50% of 246TCA at initial concentration of 10 mg/l after 16 days.	
A. baumannii GFJ1	Aniline, mono-, di- and trichloroanilines	Various concentrations	The strain utilized 4CA and 34DCA as a sole carbon, nitrogen source with 93.0 ± 1.5% after 12 hours and 86.7 ± 12.8% after 6 hours at initial concentration of 0.3 mM, respectively.	This study
A. baumannii GFJ1	Aniline, mono-, di- and trichloroanilines	Bacteria could grow in medium with maximum concentrations of 3CA, 4CA and 34DCA were 2.8, 3.6 and 1.0 mM, respectively.	Utilization of CAs as sole carbon, nitrogen and energy sources with maximum degradation rates were 3.45 ± 0.33, 5.53 ± 0.35 and 6.10 ± 0.64 μmol(h.mg cell protein)$^{-1}$.	This study

5.1.3 CAs degradation rates and cell growth of *A. baumannii* GFJ1 in the saline condition

The abilities of CAs degradation in various environmental conditions such as in saline medium cannot be ignored (Li et al., 2012a) because organic compounds are frequently contaminated in saline environments as a result of industrial activities (Oren et al., 1992). In medium supplemented with 4CA, GFJ1 could grow at higher NaCl concentration than medium supplemented with 34DCA (4.5% and 3.5% of

NaCl, respectively). Obviously, 34DCA is more toxic than 4CA, so bacteria were inhibited by salt at a lower concentration than in medium supplemented with 4CA. Previous reports showed that moderately halophilic *Myroides odoratimimus* LWD09 could degrade 34DCA with highest concentration of 5% NaCl (Li et al., 2012a). Two 4CA-degrading bacteria *A. denitrificans* strain PA2 and *Cellulomonas* sp. strain PA1 could grow up to 5% NaCl, but no report on degradation of 4CA with the presence of NaCl (Fashola et al., 2013). **To my knowledge, *A. baumannii* GFJ1 is the first reported halophilic degradation of both 4CA and 34DCA.** The determination of NaCl concentration affecting on chemical degradation was useful to clean up CAs contaminated in saline water, or in wastewater with high salt or total dissolved solid concentration.

5.1.4 Aerobic degradation pathways for 4CA and 34DCA

During degradation of 4CA and 34DCA, the transient accumulation of the corresponding intermediates was found. The reductive dechlorination of 34DCA to form 3CA was the first step of 34DCA degradation. The degradation toward both 4CA and 34DCA by *A. baumannii* GFJ1 occurred *via* the modified *ortho*-cleavage pathway. Similarly, the 4CA degradation pathway of *Moraxella* sp. strain G occurred *via* modified *ortho*-cleavage pathway (Zeyer et al., 1985b). The previous report shows that the dechlorination of 34DCA by *A. baylyi* GFJ2 also occurred to form 4CA. The further degradation of this bacterial strain was *via* both *ortho*-cleavage pathway and modified *ortho*-cleavage pathway (Hongsawat and Vangnai, 2011). The widely known 34DCA biodegradation pathway of *P. putida* by a dioxygenase formed 4,5-dichlorocatechol as the first intermediate (You and Bartha, 1982b). Previous reports

showed that monochloroanilines were degraded *via* a modified *ortho*-cleavage pathway (Hinteregger et al., 1992; Zeyer et al., 1985b), *ortho*-cleavage pathway (Latorre et al.), and both *ortho*-cleavage and modified *ortho*-cleavage (Zhang et al., 2010a). In another report, on the other hand, *Comamonas testosteroni* I2 mineralized 3CA *via* the distal *meta*-cleavage of chlorocatechol (Boon et al., 2000). The oxidation of 3CA and 4CA in *P. acidovorans* formed 4-chlorocatechol, 4-chlorocatechol was then transformed *via meta*-cleavage pathway, and the degradation was not completed (Loidl et al., 1990). To my knowledge, *A. baumannii* GFJ1 was the first strain described to reductively transform 34DCA into 3CA under aerobic media.

5.2 Cell activities toward CAs under anaerobic conditions

5.2.1 Biodegradation and growth rates of *A. baumannii* GFJ1 under anaerobic conditions

Within the environment, CAs have been detected in anoxic and anaerobic environments, such as in sediments (Boehncke et al., 2003; Bureau, 2006). In these media, anaerobic microbial degradation plays an important role. In the same conditions, the anaerobic utilization rates and cell growth rates were significantly lower than aerobic ones. Similarly, *Bacillus licheniformis* strain ycsd02 degraded 4CA under aerobic with much higher rates than in anaerobic conditions (Ding et al., 2011). The lower biomass in the anaerobic experiments might be responsible for the lower degradation rates (Huang et al., 2005). The utilization rates and growth rates with nitrate as the electron acceptor were higher than with sulfate (Table 4-8 and 4-9).

For *A. baumannii* GFJ1, oxygen was the most favorable electron acceptor, followed by nitrate and sulfate, while it was not favorable for Fe^{3+}.

The supplementation of yeast extract and succinate into media did not enhance the utilization rates of CAs under aerobic conditions, but these co-substrates increased the anaerobic transformation rates (Table 4-8 and 4-9). In anaerobic utilization with low energy gained from nitrate and sulfate reduction, yeast extract and succinate were electron donors which stimulated the transformation of chemicals and cell division. Similarly, yeast extract and succinate act as electron donors and play an important role in the anaerobic dechlorination and degradation of chlorinated phenols (Field and Sierra-Alvarez, 2008).

The first pure culture, *Paracoccus* sp., had the capacity to degrade both mono- and dichloroanilines under aerobic and anaerobic conditions with the simultaneous reduction of nitrate to nitrite (Bollag and Russel, 1976). In another report, *Paracoccus* isolated from soil converted 4CA with nitrate as an electron acceptor (Minard et al., 1977) (Table 5-2). *Rhodococcus* sp. strain 2 transformed 34DCA into some intermediates under nitrate reducing conditions, but no data for aerobic degradation were reported (Travkin et al., 2002). However, the degradation of CAs by these bacterial strains carried out in media containing co-substrates. *A. baumannii* GFJ1, on the other hand, could utilize 4CA and 34DCA in both aerobic and anaerobic media as the sole carbon and nitrogen sources, probably it was high adaption capacity with different conditions.

It can be observed in Table 4-10 that the consumption of nitrate was higher than the theoretical values of complete reduction of nitrate to nitrogen gas, but it was lower than nitrate reduced to nitrite. Nitrite produced was lower than nitrate

consumed. These results suggested that nitrate was transformed into nitrite, but not all nitrite was transformed into nitrogen gas.

The reduction of sulfate was lower than expected (Table 4-11), which could be because 4CA and 34DCA were transformed into intermediate products and not completely degraded to CO_2, or/and a part of the respective CAs was converted into bacterial materials. The level of sulfide produced was lower than that of sulfate consumed. This result agrees with previous reports on the anaerobic degradation of other aromatic compounds (Dou et al., 2008; Hu et al., 2007), where sulfate was probably transformed to H_2S and transformed into gas phase (Dou et al., 2008).

In anaerobic degradation, ammonium ion was released from the amino nitrogen. However, ammonium concentration was significantly lower than the amount of CAs degradation (Table 4-12), which might be explained that ammonium produced was used by bacteria as a nitrogen source. In previous studies, the anaerobic deamination of aniline by *Desulfobacterium anilini* (Schnell et al., 1989) and *Delftia acidovorans* HY99 (Huang et al., 2005) to form ammonia was reported.

5.2.2 Pathways for anaerobic degradation of 4CA and 34DCA

Although a number of studies have described the degradation pathways of CAs under aerobic conditions by pure cultures (Boon et al., 2000; Hongsawat and Vangnai, 2011; You and Bartha, 1982b; Zeyer et al., 1985b; Zhang et al., 2010b), not many publications investigated the anaerobic degradation pathways. Previous studies stated that anaerobic reductive dechlorination of 34DCA in sediments or aquifer

slurries occurred at the *para* position to form 3CA (Kuhn and Suflita, 1989; Kuhn et al., 1990; Struijs and Rogers, 1989), or at both the *para* and *ortho* positions to form 3CA and 4CA (Struijs and Rogers, 1989). However, further degradation of these products was not observed. Similar to the dechlorination by *A. baumannii* GFJ1, 4CA was transformed to aniline in sediments (Susarla et al., 1997; Susarla et al., 1998). Aniline was than transformed into 4-amonobenzoic acid under anaerobic media (Kahng et al., 2000; Schnell and Schink, 1991). The degradation pathways of CAs by pure culture with different intermediates were shown in Table 5-2.

Table 5-2. Degradation toward CAs in several bacterial strains and *A. baumannii* GFJ1 under anaerobic condition

Substrate	Bacterial strains	Condition	Media	Degradation intermediates (in anaerobic condition)	References
Mono- (0.16 mM) and dichloroanilines (0.12 mM)	*Paracoccus* sp	Anaerobic and aerobic	Czapek-Dox broth (containing co-substrate)	Unknown volatile organic compounds	(Bollag and Russel, 1976)
4CA	*Paracoccus* sp	Anaerobic	Czapek-Dox broth (containing co-substrate)	Triazene	(Minard et al., 1977)
34DCA (0.6 mM)	*Rhodococc*us sp. strain 2	Anaerobic	Medium with co-substrates	3,4-dichloroacetanilide, 3,4-dichloro-N-(3,4-dichlorophenyl) benzamide and 1,2-dichlorobenzene	(Travkin et al., 2002)
4CA and 34DCA (0.1 mM each)	*A. baumannii* GFJ1	Anaerobic and aerobic	Mineral medium with or without co-substrate	Under anaerobic condition, 34DCA was dechlorinated to 3CA, and then aniline. 4CA was transformed to aniline. Aniline then continued to degrade to other compounds	This study

5.3 Biofilm development and degradation of CAs by biofilm

5.3.1 Biofilm development of *A. baumannii* GFJ1

The determination of 3CA degradation by biofilm may play an important role for future application in using biofilm for bioremediation. Biofilm is a cell living form that microorganisms may enhance the endurance of environmental stresses. *A. baumannii* GFJ1 formed biofilm with different levels belonging to nutrients and chemical supplemented. Ammonium sulfate and ammonium chloride inhibited 3CA degradation; however, they stimulated the biofilm formation. These results suggested that there was a special interaction between bacteria and these co-substrates. Previous reports showed that the biofilm formation is influenced by nutrients presenting in the bulk fluid and environmental conditions, especially during initial attachment stages (Donlan, 2002). The roles of nitrogen sources have mentioned as an important nutrient for biofilm formation (Fujishige et al., 2006). In another report, the increase of nitrogen concentration enhanced the rate and extent of biofilm accumulation of *P. putida* isolated from a paper machine (Rochex and Lebeault, 2007). The biofilm formation of GFJ1 was lower than *P. putida* KT2440 and higher than *E. coli* DH5α cultured in MM medium supplemented with succinate and ammonium sulfate (Figure 4-14).

The biofilm formation depends on bacterial growth. At CAs concentration of 0.0, 0.05 and 0.1 mM, bacteria grew without lag phase and reached to maximum within one day, while it took two days for bacteria to grow to the maximum at 0.6 and 1.0 mM of 4CA. For 34DCA, bacteria had a lag phase and grew to maximum level within 2 days at 0.3 and 0.6 mM, while it inhibited the growth of bacteria at 1.0 mM,

resulted in the low biofilm formation (34DCA is more toxic than 3CA and 4CA). The numbers showed that the time bacteria required forming biofilm depending on chemical concentrations. At high chemical concentrations (≥ 0.3 mM), medium was more toxic, so bacteria required more time to grow and develop biofilm. The increase of cell numbers in the exponential growth phase resulted in the higher contact with material surface to stimulate the adhesion. In previous reports showed that cells detached from biofilm in following time due to the factors like nutrient depletion, increased concentration of detrimental metabolites and changes in culture conditions (Delaquis et al., 1989; Nisha et al., 2015; Wang et al., 2016). On the other hand, the biofilm of *A. baumannii* GFJ1 was stable after reaching the maximum biofilm formation, which showed the potential application in bioremediation of the toxicant.

5.3.2 Degradation toward CAs by biofilm

After 24 h, the biofilm formation was consistent in the same medium (MYNa medium) with 0.1 mM CAs described above. However, biofilm increased with the supply of fresh medium because bacteria had more nutrients. Once anchored at the surface, cell division and recruitment of planktonic bacteria resulted in growth and development of the biofilm community (Andersson, 2009). Cells required nutrients in the conditioning film and the aqueous bulk to grow and produce more extracellular polymeric substance (*EPS*) resulting in the formation of microcolonies to extend the biofilm thickness (Kumar and Anand, 1998).

At the following intervals, the degradation rates of 3CA increased because of the increase of cell numbers in biofilm. The application of biofilm on biodegradation is highly desirable, especially for waste water treatment. In this experiment, CAs

degradation was carried out by cells in biofilm and probably by cells detached from biofilm. The increase of biofilm formation and degradation rates of chemicals with the supply of fresh medium had a significant role for further application in 3CA treatment by using a continuous biofilm reactor. These results indicated that there was a relation of nutrients, biofilm and biodegradation.

The drawback to apply biofilm for biodegradation in this study is that bacteria form high levels of biofilm with the presence of ammonium sulfate and ammonium chloride. However, these substrates inhibited the biodegradation toward CAs. Moreover, bacteria could not grow and form biofilm in media with higher 1.0 mM 34DCA. Using entrapped cell in other carriers such as PVA and alginate materials for degradation 34DCA should be selected to degrade CAs to overcome the problem.

5.4 Degradation toward 34DCA by *A. baumannii* GFJ1 immobilized in PVA-alginate matrix

The highest concentration of 4CA detected was 0.12 mM, while the concentrations for 34DCA were 1.2 mM and 1.6 mM (shown in 2.1.4). *A. baumannii* GFJ1 (as freely suspended cells) utilized 0.12 mM 4CA within several hours; however, they were inhibited to grow and degrade 34DCA at 1.2 mM. The entrapped cells improved degradation of 34DCA and could degrade 34DCA at 1.2 mM. Therefore, the degradation toward 34DCA by entrapped cells was investigated.

5.4.1 34DCA degradation rates and bacterial growth of freely suspended and cells immobilized in PVA and PVA-alginate

The entrapped cells showed better performances than freely suspended cells probably because the carrier materials protected cells from the surrounding environment, which was attributed to the increased tolerance of the immobilized cells to 34DCA over freely suspended cells (Bergero and Lucchesi, 2013; Keweloh et al., 1989; Tallur et al., 2009). In this study, PVA concentration was used at 10% which is suitable for cell immobilization (Cheng et al., 2012; Nunes et al., 2010; Zhan et al., 2013). At the same PVA concentration, the degradation rates by entrapped cells in PVA-alginate beads were not significantly different at 1% and 2% alginate (Figure 4-17). It is the fact that more densely cross-linked gel structure is probably formed when sodium alginate concentration is increased resulting in more diffusion resistance (Idris and Suzana, 2006). Although immobilized bacteria in the gel beads achieved higher degradation rates of 34DCA than freely suspended cells, the enhancement of degradation rate by modification of the carrier is the target in this study.

The activities of entrapped cells depend on materials of carriers, relative porosity and methods to immobilize cells (Cassidy et al., 1996; Scott, 1987). The nominal pore size of PVA gels governing the diffusion of substrates and oxygen to the entrapped cells might be responsible for bacterial activities (Lozinsky and Plieva, 1998). Moreover, the presence of microorganisms in the beads can significantly reduce the pore volume (Tsai et al., 2013), and can cause mass transfer resistances (Mazzer et al., 2008). Furthermore, high concentrations of PVA and alginate are required to form a stable carrier. As a consequence, substrates diffusing to immobilized cells and metabolites leaving the carrier may be obstructed. A previous

report illustrated that there was a high effect of diffusion resistance on the biodegradation rate (Chen et al., 2013). Microenvironments with suitable pore sizes can promote cell seeding and growth, which affects the biodegradation. The modification of carriers to improve the permeability and facilitate mass transfer may increase bioactivities of bacteria entrapped in the beads.

5.4.2 Effects of porogens on characterization of beads

The stability of carriers, including physical and chemical strength and swelling of beads, is the key parameters for the potential application in industries. The addition of porogens enhanced porosity of the carriers (Table 4-22) resulting in the increased diffusion (Table 4-19). The porosity, pore volume, diffusion and swelling ratios of beads with $CaCO_3$ and PEG6000 as porogens were insignificantly different. In some experiments, these data were higher than beads with PEG1000. The previous study confirmed that higher molecular weight of PEG could facilitate to form the relatively large pores in PVA gel beads, diffusion and swelling of PVA gel beads (Zhang and Ye, 2011). The increase of alginate in beads enhanced the physical stability of beads. Alginate formed cross-linking in beads resulted in lower porosity and enhancement of bead structure. However, the increase of porogens reduced the physical stability probably due to high porosity. Similarly, the addition of sodium bicarbonate as a porogen enhanced porosity and reduced the physical property of PVA-alginate beads (Li et al., 2011). Because of poor physical strength, the determination of 34DCA by immobilized cells in the carrier with 10%PVA, 1% alginate and 7% or 10% $CaCO_3$ was not carried out.

Although the increase of porogens reduced physical strength, the weight remaining in the chemical stability test was not significantly reduced with the addition of porogens. At high pH, the cross-linking of carriers might be destroyed by excess OH^- ions presenting in the solution, so a part of bead was diluted, which resulted in higher weight losses at high pH than at low pH (Idris et al., 2008). The increase of carrier porosity also increased diffusion rates and swelling ratios of the beads. Beads with high porosity increased to absorb water resulting in enhancement of swelling ratios. The key factors controlling the swelling are porosity and cross-linking density (Gupta et al., 2011). Such phenomena have been reported in other publications (Bai et al., 2010; Li et al., 2011; Zhang and Ye, 2011).

5.4.3 Effects of porogens on biodegradation, cell leakage and growth rates of entrapped cells

The addition of porogens enhanced porosity of the carriers. The bacterial activities in such immobilized gels were likely influenced by mass transfer. The beads with more pores and more channels, bacteria had more opportunities to contact oxygen and 34DCA. With 1%, 2% and 3% alginate in matrix, the highest degradation rates by entrapped cells in the matrices were 0%, 5% and 7% $CaCO_3$, respectively. Cells entrapped in the beads with 10% PVA, 2% alginate and 5% $CaCO_3$ showed the highest degradation rate, so the bead with these materials was selected for other experiments. The results showed that bacterial activities were optimally in a specific porosity of beads. At high concentration of porogen (such as 10%), the growth and degradation rates were reduced probably because beads were soft and poor mechanical strength resulting in high cell leakage and toxicity of 34DCA to cells.

Cell growth and cell leakage of PVA-alginate-$CaCO_3$ and PVA-alginate-PEG beads during the biodegradation process was similar. However, the degradation rates of cells immobilized in PVA-alginate-PEG beads were lower than in PVA-alginate-$CaCO_3$ beads. PEG is a water-soluble polymer, and cells were probably suspended in PEG or incorporated with PEG in solution leading to high cell leakage during immobilization and removal of PEG, which resulted in reducing biodegradation rates. PEG1000 and PEG6000 were diluted in liquid media resulting high cell leakage. Moreover, cell leakage during removal of PEG was higher than during removal $CaCO_3$ probably due to the toxicity of HCl outside the beads which reduced cell leakage. The degradation rates by entrapped cells in beads with PEG1000 were smaller than PEG6000 from 7 - 11% except the beads with 10% PEG, which were probably due to the lower porosity and diffusion of beads with PEG1000.

In previous reports, porogens were added into beads to increase the porosity, pore volume, swelling ratio (Table 5-3). **In my knowledge, this study is the first report showed the increase of toxic chemicals by modification of the carrier using porogens.**

Table 5-3. Using porogens in immobilization in previous reports

Porogen	Concentration (%, w/v)	Bead material	Application and characterization of carriers	References
Soluble starch	2 - 10	PVA	No improvement towards the gas permeability of the beads, no increase of the nitrate reduction activity	(Chen et al., 1996) (Chen et al., 1996)
Partially saponified PVA	1, 3, 5	PVA	Many pores inside were created, but no increase of the nitrate reduction activity	
Sodium bicarbonate	0.2 – 0.6	PVA-alginate	The addition of sodium bicarbonate increased porosity, pore size, swelling but degreased physical property	(Li et al., 2011)
$CaCO_3$	10%	PVA-alginate	The addition of $CaCO_3$ increased porosity, pore size swelling ratio	(Bai et al., 2010)
PEG with molecule	1 – 8	PVA-alginate	The addition of PEG increased porosity, pore	Zhang and Ye, 2011)

weights from 1,000 to 10,000			size, swelling ratio	
$CaCO_3$	3 – 10	PVA-alginate	The addition of $CaCO_3$ increased porosity, pore size, swelling ration but degrease physical property. The addition of $CaCO_3$ at 3%, 5% and 7% increased the degradation and growth rate of entrapped cells. However, cell leakage increased with the increase of $CaCO_3$ concentration.	This study
PEG with molecule weights of 1,000 and 6,000	3 – 10	PVA-alginate	The same as the addition of $CaCO_3$	This study

5.4.4 Stability of beads in phosphate solution

When the concentration of phosphate in solution was 0.01 M, beads were stable because the amount of phosphate was not enough to disrupt cross-linking in beads. However, when phosphate increased to 0.1 M, beads absorbed more water, broken and become viscous. Phosphate serves as a chelating compound which disrupted the cross-linking of alginate inside the beads resulting in breaking the beads even though phosphate esterifies PVA.

5.4.5 Reusability of entrapped cells

One of the important factors for practical application of the immobilized cell system is the stability during long-term operation. The reuse of beads reduces time and cost. In this study, the degradation of entrapped cells in both fresh and freeze-dry-rehydration beads was stable for 6 cycles. The degradation rates were declined in later batches, probably due to increase of cell leakage. The previous reports showed that the biodegradation of entrapped cells was reduced in following cycles; for example, the degradation of p-nitrophenol by *Rhodococcus* sp. Y-1 immobilized in PVA-alginate was stable for 8 cycles.

5.4.6 Degradation toward 34DCA by entrapped cells with the supplemented with cryoprotective agents

The beads with PEG were not selected in this experiment because of lower degradation rates, higher glycerol diffusion and cell leakage during removal of porogen (shown in 4.4.5 and discussed in 5.4.7). The bead with 10% PVA, 2%

alginate and 5% $CaCO_3$ was used in this experiment because it showed the highest degradation rate.

Freeze-dried, entrapped cells have been applied for cell preservation for long-term storage (Kearney et al., 1990; Sompornpailin et al., 2014). Among cryoprotectants, bacteria entrapped in beads containing glycerol did not reduce the degradation rate of entrapped cells after freeze-dry process. Moreover, glycerol incorporation did not reduce the degradation rate of entrapped cells in long term storage. Because the determination of viability of GFJ1 immobilized in beads after lyophilization and rehydration was impossible, the examination of these substances on biodegradation before and after these processes was necessary to select an appropriate cryoprotectant.

The 34DCA degradation rate of entrapped cells in the bead without any protective agent was sharply reduced after freeze-drying probably due to low cell survival. With the presence of cryoprotectants inside cells, the osmotic difference with the external environment is reduced, or cold tolerance is improved (Kets et al., 1996). In this study, cryoprotectants offered protection for cells not only in freeze-drying, but also during storage. The supplementation of glycerol showed effectively in biodegradation rates after 1-month storage at room temperature. The biodegradation ability of entrapped cells was reduced after 1-month of storage (except beads with glycerol incorporation) at room temperature probably due to higher cell mortality. Biochemical reactions may be accelerated at high temperature, which resulted in lower cell survival (Champagne et al., 1996).

Previous reports showed that the survival of entrapped microorganisms was enhanced with the addition of glycerol (Cui et al., 2006; Kearney et al., 1990; Wang

et al., 2012; Zohar-Perez et al., 2002). Zohar-Perez showed that the survival of *Pantoae agglomerans* IC1270 entrapped in alginate beads with 30% glycerol was higher than in beads with 10% or 50% glycerol after lyophilization (Zohar-Perez et al., 2002). In this study, the degradation rates of entrapped cells in beads with 10% and 20% glycerol after lyophilization were insignificantly different, which suggested that the survival of cells in these beads was similar. Glycerol could prevent ice-crystal formation after penetration into the cells (Madigan et al., 1997). Dry beads effectively preserved the entrapped cells as a result of incorporation with glycerol, forming unique structures in the dried matrix (Zohar-Perez et al., 2002). The addition of glycerol protected the microorganism, increased pore size in beads, and controls the structure of the dried macrocapsules (Zohar-Perez et al., 2002).

Freeze-dried beads sustained the biodegradation ability better than the fresh beads, which indicate that freeze-dried beads have a potential application for long term storage.

5.4.7 Glycerol leakage from beads

Glycerol played an important role in preserving the degradation rates in this study. However, not all glycerol added was remained in bead and activated entrapped cells. The glycerol remaining in bead is useful for bacteria. Glycerol is a water soluble compound. A part of glycerol was diffused during immobilization, removal of porogens and incubation process. The leakage of cryoprotectants might affect the tolerance of bacteria. The amount of glycerol diffusing during immobilization process from beads with PEG and $CaCO_3$ as porogens was similar. However, glycerol diffusing during removal PEG was higher than during removal of $CaCO_3$ because the

removal PEG required longer time and processing in shaking condition (the removal of $CaCO_3$ required 1.0 – 2.0 hours depending on $CaCO_3$ concentrations) (Table 4-18). The previous report showed that glycerol diffusing from alginate beads was 65%-85% during immobilization (Zohar-Perez et al., 2002). The time that glycerol diffusion through cell-free calcium alginate beads reached the equilibrium ratio belonged to alginate concentrations (Garbayo et al., 2002).

CHAPTER 6 CONCLUSION

6.1 Degradation of CAs by freely suspended *A. baumannii* GFJ1

A. baumannii GFJ1 utilized several mono-, di- and trichloroanilines as growth substrates. To our knowledge, *A. baumannii* GFJ1 is the first strain that could utilize organic compounds (CAs) as sole carbon, nitrogen and energy sources in both aerobic and anaerobic conditions. Moreover, *A. baumannii* GFJ1 is the first pure culture that could utilize mono-, di- and trichloroanilines as sources of carbon and nitrogen.

Under aerobic conditions, the addition of co-substrates stimulated growth rates, but did not enhance degradation rates of CAs. The kinetic analysis showed that the aerobic utilization of both 4CA and 34DCA followed the Edward model during exponential growth. The highest degradation velocity of 3CA, 4CA and 34DCA was 3.45 ± 0.33, 5.53 ± 0.35 and 5.53 ± 0.35 μM/(mg cell protein.hour), respectively. 34DCA was transformed into 3CA in the first step of aerobic biodegradation by *A. baumannii* GFJ1. 3CA and 4CA were aerobically degraded *via* a modified *ortho*-cleavage pathway.

Under anaerobic conditions, *A. baumannii* GFJ1 utilized CAs with nitrate and sulfate as electron acceptors *via* reductive dechlorination to aniline, benzoate as intermediate products. However, the growth and degradation rates under anaerobic conditions were significantly lower than under aerobic conditions. The addition of co-substrates (YE and succinate) enhanced degradation rates and growth rates in anaerobic media. 4CA and 34DCA were dechlorination to 4-aminobenzoic acid during the anaerobic degradation. The degradation results suggest that *A. baumannii*

GFJ1 has the potential to be widely applied for bioremediation of these toxic compounds.

However, *A. baumannii* GFJ1 could not grow and utilize 34DCA in media with higher 1.0 mM. In this case, using immobilized cell could solve the problem.

6.1 Degradation of 34DCA by entrapped *A. baumannii* GFJ1

Freely suspended *A. baumannii* GFJ1 showed a low degradation of 34DCA at high concentration (1.2 mM). Bacteria entrapped in beads showed higher degradation rates. The degradation toward 34DCA by entrapped *A. baumannii* GFJ1 in PVA and PVA-alginate beads was higher than freely suspended cells. The modifications of gel beads used for cell immobilization were carried out to enhance the degradation of entrapped cells. The addition of pore-forming agents, including $CaCO_3$ and PEG into the carrier enhanced the porosity and mass transfer of PVA-alginate beads, which resulted in changes of cell activities. The beads with 10% PVA, 2% alginate and 5% porogen achieved the highest biodegradation rates and growth rates in the tests with different $CaCO_3$ and PEG concentrations.

The presence of cryoprotectants inside the beads reduced the adverse effects of the freeze-dry process. Among cryoprotectants, the incorporation of glycerol showed effective preservation of biodegradation during freeze-drying and storage. After 1-month storage at 4 ^{o}C, and -20 ^{o}C, there was not significant reduction of biodegradation rates of cells entrapped in freeze-dried or fresh beads, with or without cryoprotectants; however, the degradation rates were decreased when beads were stored at room temperature except beads containing glycerol. Cryoprotectant incorporation reduced the adverse effects in freeze-drying process and during storage.

The development of formulas for cell entrapment to enhance the biodegradation rate by the addition of porogens, to reduce the adverse effects during freeze-drying and storage by using glycerol as a cryoprotectant showed the potential for practical applications, for examples glycerol can be added in carriers if the carriers must be freeze-dried and stored.

In conclusion, this study investigated the CAs degradation by *A. baumannii* GFJ1. The determination of effects of conditions (chemical concentrations, nutrients) on biodegradation rates provided the characterizations of bacteria, which is useful for further application. The modification of bead for entrapped cells applied in biodegradation, which suggested a new method to increase the degradation efficiency.

CHAPTER 7 RECOMMEMDATION FOR FUTURE WORK

The 34DCA degradation efficiency of *A. baumannii* GFJ entrapped in PVA-alginate with modification was carried out. The future work should be determination of degradation toward other chloroaniline such as 4CA and 246TCA.

The entrapped cells in beads with modification and containing glycerol have high efficiency to degrade 34DCA after 1-month storage at room temperature, 4 ^{o}C and minus 20 ^{o}C. The investigation the degradation after longer time storage and maximum storage time using for commercialization should be carried out.

In a previous report, genes relating to aerobic degradation pathways toward CAs in *Diaphorobacter* sp. PCA039 were investigated (Zhang et al., 2010). However, the degradation pathway in *A. baumannii* GFJ1 was different from *Diaphorobacter* sp. PCA039. Therefore, genes involving in CAs degradation in GFJ1 should be determined.

CAs were contaminated with high concentration in wastewater (Livingston and Willancy, 1991; Brookes and Livingston, 1993). Using reactor with cells entrapped in alginate beads for biodegradation of 3CA was determined (Ferschl et al., 1991). The bioremediation using a bioreactor and entrapped cells should be investigated for bioremediation of other CAs contaminated in wastewater. The investigation of CAs degradation using entrapped in a bioreactor cells first carried out using artificial wastewater and real waste water.

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APPENDIX A:
CAs standard curve

Standard curve was built based on HPLC results with specific concentrations of chemicals. Standard curves used to calculate the chemical concentration in liquid media. The standard of CAs concentration was prepared in acetonitrile as a stock solution at 1.0 mM. The stock was then diluted to smaller concentrations. The concentrations of CAs were analyzed using a reverse phase HPLC, and separation was performed on a C18 HPLC column.

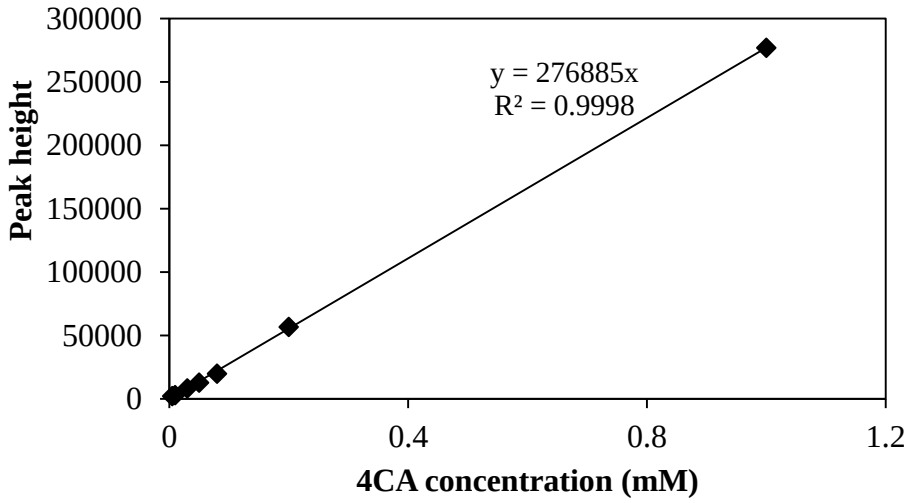


Figure A1. 4CA standard curve

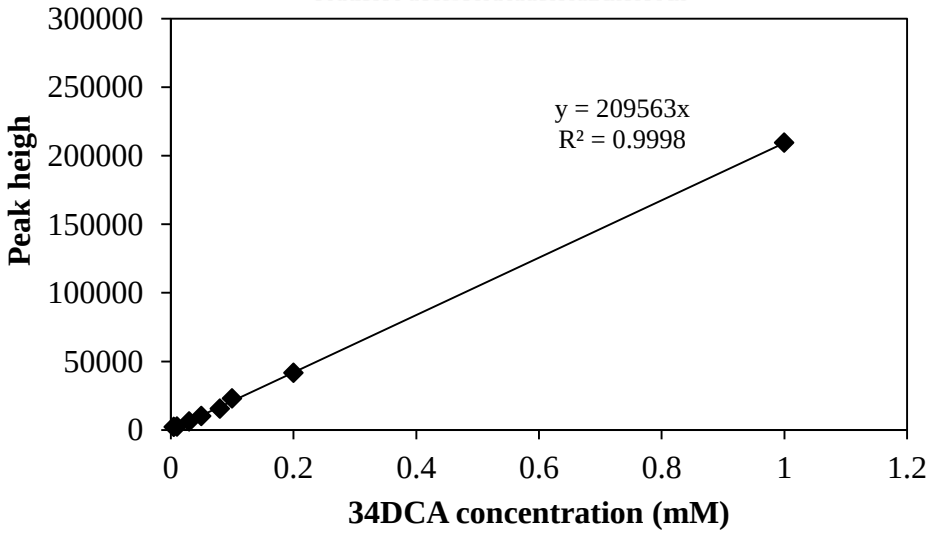


Figure A2. 34DCA standard curve

APPENDIX B:
Calculation of chloroaniline concentration

The equation of linear trend line is y = ax + b
where a is the slope and b is constant value

$$\text{The concentration of CAs} = \frac{\text{Peak height}}{\text{slope}}$$

Calculation of chloroaniline residual

Data of chloroaniline were obtained by comparing the peak area of unknown peaks with those of the standard compounds with known concentration.

$$\text{Remaining } (\%) \text{ of chloroaniline at specific time } (t) = \frac{\text{Peak height at t(hours)}}{\text{Peak height at 0 hour}}$$

Degradation (%) after specific time (t) = 100% - remaining (%) at this time

APPENDIX C:
Protein calibration curve

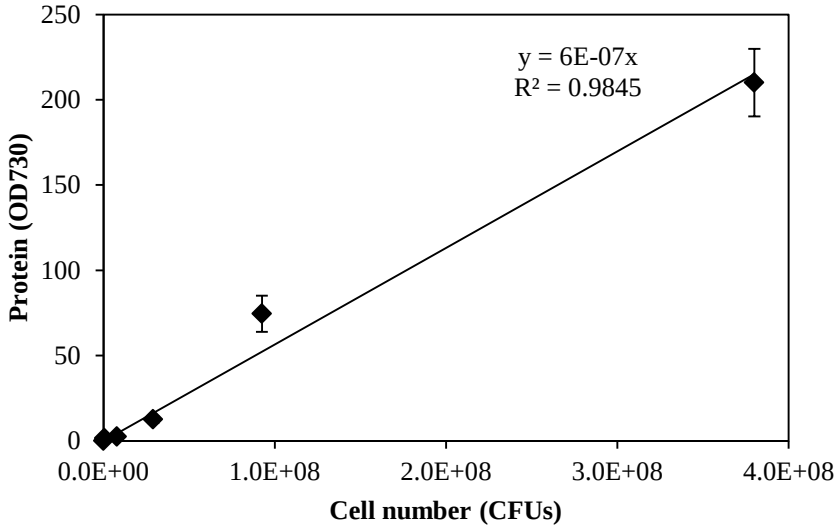
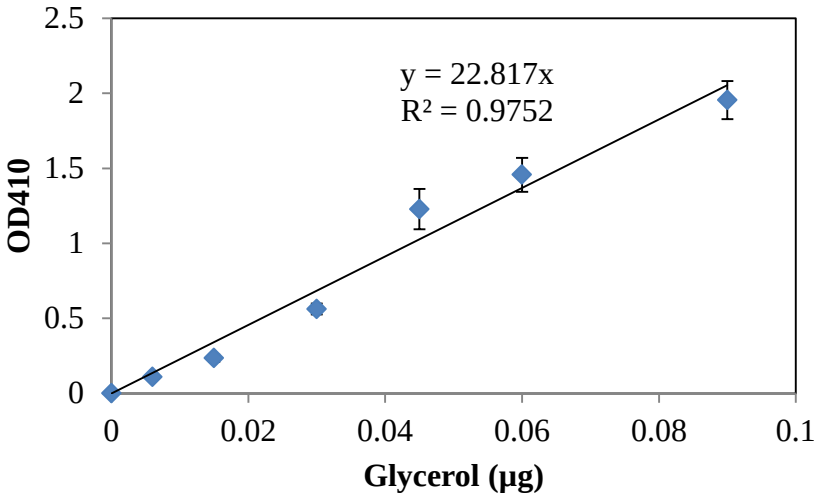


Figure C2. Protein-cell number curve

APPENDIX D:
Glycerol calibration curve



APPENDIX E:
Retention time of CAs

The retention time of 4CA and 34DCA in HPLC profiles were 3.44 and 3.91 min, respectively (Figure E1 and E2).

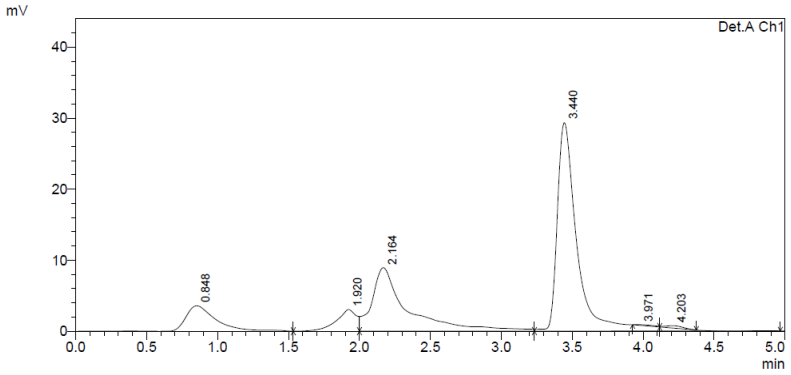


Figure E1. HPLC of 4CA at 0.1 mM (retention time 3.44 min).

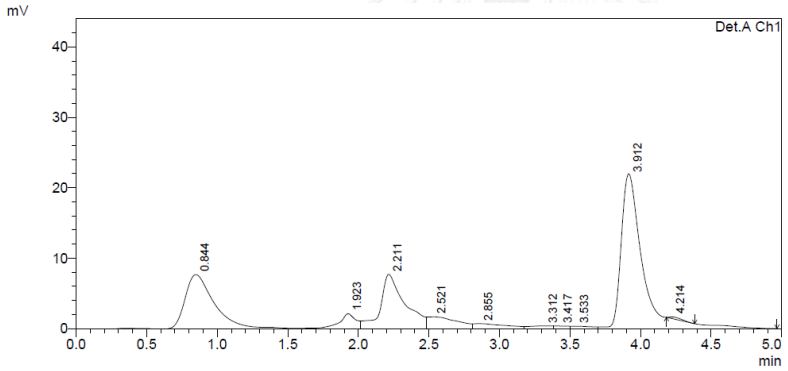
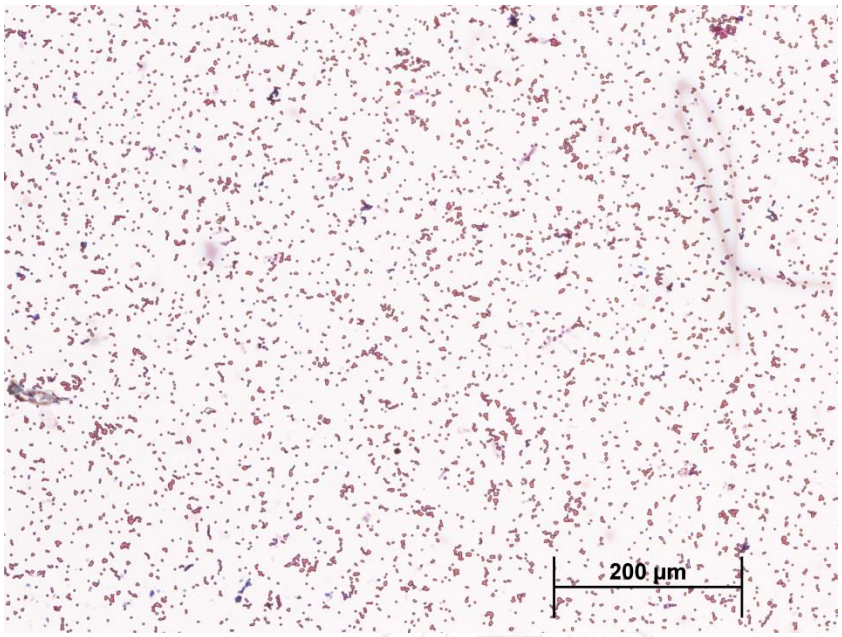


Figure E2. HPLC of 34DCA at 0.1 mM (retention time 3.91 min).

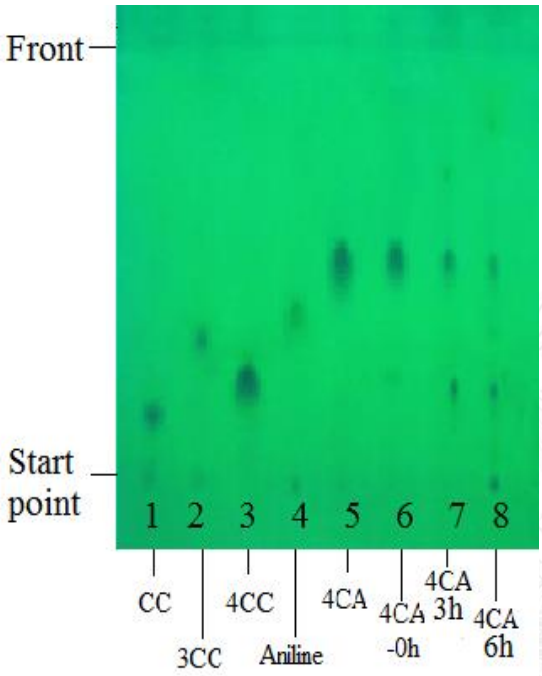
APPENDIX F:
Gram staining of *A. baumannii* GFJ1



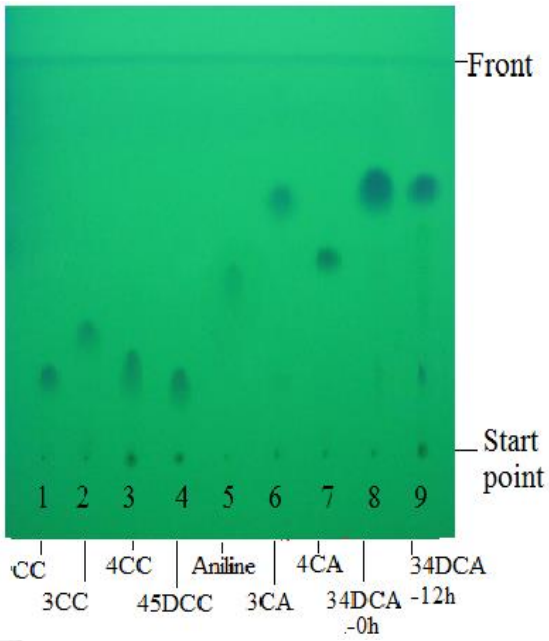
APPENDIX G:

The TLC analysis of intermediates of aerobic 4CA (A) and 34DCA (B) degradation by *A. baumannii* GFJ1 in solvent 2 (benzene : acid acetic = 9 : 1)

A

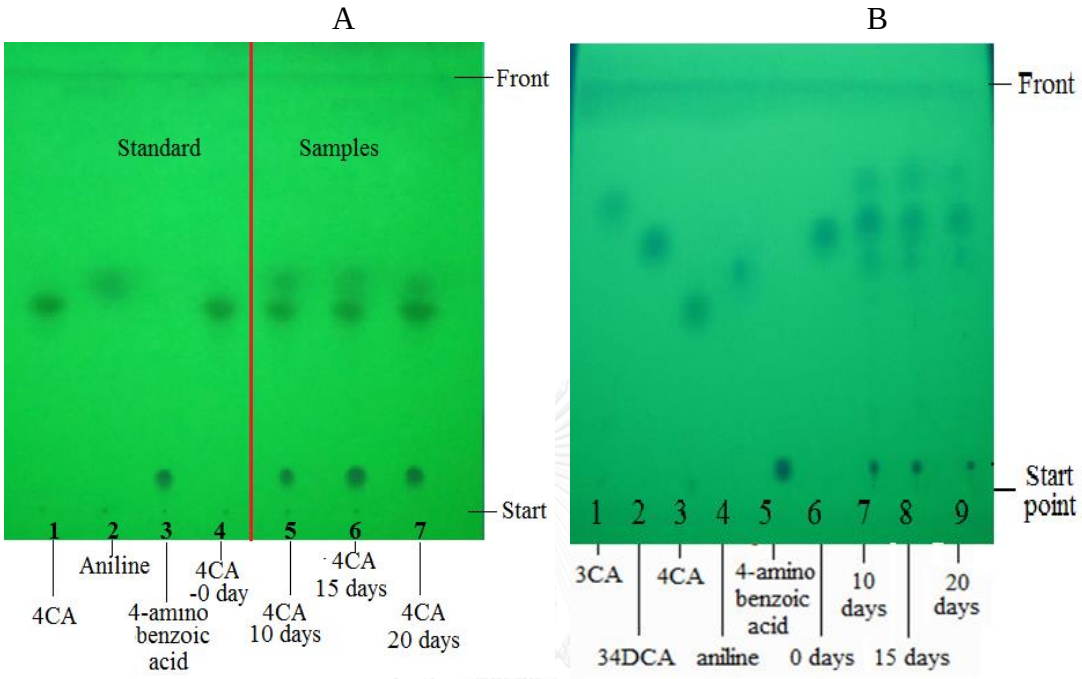


B



APPENDIX H:

The TLC analysis of intermediates of anaerobic 4CA (A) 34DCA (B) degradation by *A. baumannii* GFJ1 in solvent 1 (Hexane : benzene : acetone = 10 : 20 : 1).



APPENDIX I:

16s rRNA sequence

AGATTAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGGGGGAAG
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CTGCCTATTAGTGGGGGACAACATCTCGAAAGGGATGCTAATACCGCATA
CGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGATGA
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TGATGGCc

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Mr. Ha Danh Duc was born on February 8th, 1977 in Nghe An province, Viet Nam. He received Bachelor's Degree in College of Education, Faculty of Biology, Vinh University in 2000. He pursued his Master degree study in the Biotechnology, Can Tho University in 2004 and finished in December 2007. He pursued her PhD. degree study in the Environmental Management, Chulalongkorn University, Bangkok, Thailand in April 2011.

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