การย่อยสลายไอโพรไดโอนโดยแบคทีเรียที่คัดแยกได้จากดิน

นางสาวอรสิริ พลหล้า

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BIODEGRADATION OF IPRODIONE BY A BACTERIUM ISOLATED FROM SOIL

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อรสิริ พลหล้า: การย่อยสลายไอโพรไดโอนโดยแบคทีเรียที่คัดแยกได้จากดิน (BIODEGRADATION OF IPRODIONE BY A BACTERIUM ISOLATED FROM SOIL) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร.อลิสา วังใน, 89 หน้า

ไอโพรไดโอนเป็นสารกำจัดเชื้อรากลุ่มไดคาร์บอกซิไมด์ ที่มีการใช้อย่างแพร่หลายในการควบคุมและยับยั้งโรคที่เกิดจากเชื้อราในสวนผักผลไม้และสนามกอล์ฟ ไอโพรไดโอนสามารถเปลี่ยนรูปไปเป็นสารตัวกลางที่มีความเป็นพิษมากกว่าคือ สาร 3,5-Dichloroaniline (3,5-DCA) งานวิจัยนี้ได้ศึกษาการบำบัดสารไอโพรไดโอนและ 3,5-DCA ด้วยวิธีชีวภาพซึ่งเป็นการศึกษาโดยการอาศัยความสามารถของแบคทีเรียที่คัดแยกได้จากดินในการย่อยสลายสารทั้งสองชนิด แบคทีเรีย *Pseudomonas aeruginosa* สายพันธุ์ I4 ที่ระบุสายพันธุ์ ได้จากการเปรียบเทียบหาลำดับเบสของยีน 16S rDNA สามารถย่อยสลายไอโพรไดโอน (40 ไมโครโมลาร์) และ 3,5-DCA (200 ไมโครโมลาร์) ได้ 73.99 % และ 98.29 % ภายในเวลา 24 ชั่วโมงตามลำดับ การย่อยสลายสารสองชนิดที่มี succinic Acid ที่ความเข้มข้น 5 มิลลิโมลาร์ เป็นสารตั้งต้นร่วมสามารถเพิ่มประสิทธิภาพการย่อยสลายไอโพรไดโอนสูงขึ้นร้อยละ 5.98 นอกจากนั้นสามารถตรวจพบการเหนี่ยวนำให้มีกิจกรรมของเอนไซม์ catechol 1,2 dioxygenase ในระหว่างการย่อยสลายสารไอโพรไดโอนแบคทีเรียสายพันธุ์ *P. aeruginosa* I4 ยังมีความสามารถย่อยสลายสารสารกำจัดเชื้อราในกลุ่มไดคาร์บอกซิไมด์และสารอะนิลีนชนิดอื่นๆได้

สาขาวิชา วิทยาศาสตร์สิ่งแวดล้อม ลายมือชื่อนิสิต.....

ปีการศึกษา 2012 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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Iprodione is a dicarboximide fungicide that is widely used to control fungi and target pests in wineyard and golf courses. Iprodione is naturally transformed to its main intermediate with relatively higher toxicity, 3,5-dichloroaniline (3,5-DCA). Since bioremediation is considered to be an effective method to reduce remaining toxic compounds, the bacterial screening and isolation were carried out to obtain the bacterial isolate able to degrade both chemicals. Through the enrichment technique, *Pseudomonas aeruginosa* strain I4 was isolated. It was identified based on the basic of comparison of 16S rDNA sequence analysis. The strain could degrade 40 μM iprodione and 200 μM 3,5-DCA up to 73.99 % and 98.29 %, respectively, within 24 hours. Among various co-substrates, 5 mM succinic acid could enhance both iprodione and 3,5-DCA degradation. Furthermore, catechol 1,2-dioxygenase (C12DO) of strain *P. aeruginosa* strain I4, respectively representing the *ortho*-cleavage pathway Moreover, the result showed that strain I4 could degraded other dicarboximide fungicide and other chloroaniline.

Field of Study: Environmental Science Student's Signature……

Academic Year: 2012 Advisor's Signature……

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INTRODUCTION

1.1 Statement of problem

Pesticides have important role for crop protection in agricultural activities. However, intensive use of pesticides results in contamination of toxic residues on agricultural food products and in environment. The toxicity and the existence of pesticide and derivatives are of environmental and human health concerns.

Iprodione is a dicarboximide fungicide that is widely used to control several types of fungal strains including *Botrytis*, *Monilinia*, *Sclerotinia*, *Alternaria*, *Fusarium*, *Phoma*, and *Typhula sp.*(US EPA, 1998) for several crops including rice, orchid, grapes, durian, potatoes, mustard greens, green beans, onions, carrots, almonds, lettuce, stone fruits, beans, caneberries, bushberries, canola, cotton, cole crops, crucifer, garlic, peanuts, radish, rutabaga, strawberries and turnip greens. Agricultural applications can be made several different application methods, including ground spray, spray by aircraft, chemigation, in furrow treatment, dip treatment and as a seed treatment to inhibit fugal spore germination and its growth by inhibition of DNA/RNA synthesis and cell division (Steeger and Garber, 2009).

Pollution from pesticide contamination is considered a pervasive problem because of an adverse effect on living organisms. During January – December 2011, 123,000 kg of iprodione was repowered in Thailand (61.09 million bath) (Department of Agriculture, 2011), for agriculture crop protection and golf course maintenance. For these reasons, strict environmental regulations in many countries are applied in order to remove and reduce pesticide and derivative contamination in the environment. To mitigate pesticides and derivative contamination, bioremediation is one of the effective methods to break down and detoxify by the action of microorganism.

There is report on iprodione biodegradation. For example, biodegradation of iprodione by bacteria isolated from soil, *Arthrobacter sp* MA6. is able to degrade the substance and transformation. Substance is transform to chemical product, 3,5-DCA determined by HPLC (Athiel *et al*., 1995).

Iprodione is structurally related to vinclozolin and procymidone. Each of these three pesticides can be catabolized by bacteria and usually accumulated as 3,5-dichloroaniline (3,5-DCA) (Athiel *et al*., 1995). Food Quality Protection Act (FQPA) and U.S. Environmental Protection Agency (EPA) require an estimation of progressive risk from consumption of food and water containing 3,5-DCA derived from iprodione, vinclozolin, and procymidone (US EPA, 1998). Even both iprodione and 3,5-DCA have high toxicity to human, they have been widely used in agricultural areas. Therefore the efficient treatment is required.

1.2 Objectives

1.2.1 To isolate the bacterial strain from soil able to degrade iprodione and 3,5-DCA.

1.2.2 To study the efficiency of biodegradation of iprodione an isolated bacterium.

1.2.3 To optimize biodegradation conditions for iprodione and 3,5-DCA.

1.3 Hypothesis

The isolated bacterium is also able to degrade iprodione and intermediate 3,5-DCA.

1.4 Scope of study

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1.5 Expected result

The isolated bacterium obtained from this study will effectively degrade iprodione and its intermediate under optimal condition. The information may be applied to treat iprodione and/or its intermediate contaminated sites.

CHAPTER II

THEORETICAL BACKGROUND AND LITERATURE REVIEW

2.1 Pesticide

Pesticide is used to control pests. The pests including insects, plant diseases, fungi, weeds, nematodes, snails, slugs, etc. Therefore, insecticides, fungicides and herbicides are all considered types of pesticides. Some pesticides must contact the pest to be killed. Others must be swallowed to be effective. The list of some common categories and their purpose of pesticide is shown in Table 2.1

Pesticides are often grouped into "families" according to their properties, or their act on the pest in the same way. A pesticide product has active ingredients from more than one chemical familes, including:

- Organophosphates pesticides
- Organochlorine insecticides
- Carbamates and Thiocarbamates pesticides
- Pyrethroids pesticides

Pesticides that are related because they address the same type of pests including:

- Fungicides are used to control fungal problems like molds, mildew, and rust.
- Fumigants Produce gas or vapor intended to destroy pests in buildings or soil.
- Herbicides kill or inhibit the growth of unwanted plants, weeds.
- Insecticides are used to control insects.
- Microbial pesticides kill, inhibit, or out compete pests, including insects or other microorganisms.
- Molluscicides are designed to control slugs, snails and other molluscs.
- Nematicides are used to kills nematodes (microscopic, worm-like organisms).
- Rodenticides are used to kills rodents like mice, rats, and gophers.

(US EPA, 2012)

Table 2.1 Types of Pesticides (Canadian Centre for Occupational Health, 2010)

Category	Purpose	Examples
Insecticides	kills or repel insects, ticks and mites	bug sprays, insect repellents, ant and roach baits, garden dusts or sprays commercial farm/orchard sprays, flea shampoos, flea and tick collars, moth balls
Herbicides	kills weeds or unwanted plants	weed killers, weed and feed lawn care products, tree cut/stump treatments
Fungicides	Kills mould, mildew and other fungi.	rose and flower sprays, commercial farm/orchard sprays, treated seeds, paint additives
Rodenticides	kills rodents such as mice and rats	mouse and rat bait stations
Disinfectants	kills bacteria, mould and mildew	bleach, ammonia, kitchen and bathroom cleaners, pool and spa cleaners
Wood preservatives	protects wood from insects and fungi	pressure-treated wood

Fungicides are either chemicals or biological agents that inhibit the growth of fungi or their spores. Modern fungicides do not kill fungi. They simply inhibit fungal growth for a period of days or weeks (Reigart and Roberts, 1999). Fungicides are generally related to one another (Table 2.2). Therefore, fungicides have been intensively used in agricultural activity. Because of the intensive application, fungicide residues have been found on food for human consumption, mostly from post-harvest treatments.

Table 2.2 Chemical groups of fungicides according the Generic names

(Burpee, 2006)

Generic Names	Chemical Group
propamocarb	carbamates
mancozeb, thiram	dithiocarbamates
quintozene, chloroneb, ethazole	aromatic hydrocarbons
iprodione, vinclozolin, procymidon	dicarboxamides
fosetyl aluminum, phosphonate	phosphonates

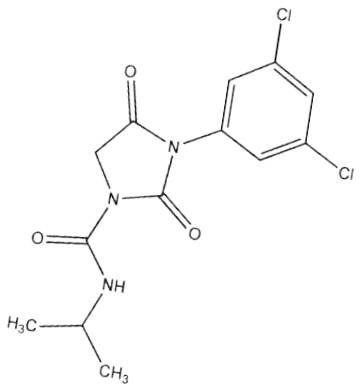
2.2 Iprodione

Iprodione is a broad spectrum contact and/or locally systemic fungicide used to prevent the germination of fungal spores on a variety of field, crops as well as amenity turf. Iprodione is a synthetic compound from the chemical family of dicarboximide. It may also be used as a post-harvest fungicide and for the treatment of seeds.

2.2.1 Chemical and physical properties (US EPA, 1998)

Chemical Name: 3-(3,5-dichlorophenyl)-N-(1-methylethyl)-2,4-dioxo-1- imidazolidinecarboxamide

Molecular Structure:



Molecular Weight: 330.17

Molecular Formula: $C_{13}H_{13}C_{l2}N_3O_3$

Color: white to cream

Odor: none

Melting Point: 136 °C

Solubility at 20 °C (g/l)

water 0.013	acetone 300
acetonitrile 150	ethanol 20
benzene 200	toluene 150

2.2.2 Usage

Iprodione using sites are as followed: (US EPA, 1998)

- Agricultural crops, including almonds, apricots, cherries, nectarines, peaches, pecans, plums, prunes, beans (dried, lima, and snap), blackberries, blueberries, broccoli, bushberries, caneberries, carrots, garlic, grapes, ginseng, gooseberries, huckleberries, lettuce (head and leaf), loganberries, mustard cabbage, Chinese cabbage, dry bulb onions, peanuts, potatoes, raspberries and strawberries.

- Ornamentals, including flowering trees and shrubs, woody shrubs and vines, evergreens, flowering and nonflowering plants, ground covers and shade trees.

- Turfgrass, including sod farms, golf courses and institutional lawn areas of bentgrass, blue grass, Bermuda grass, St. Augustine grass, rye grass, fine fescue or tall fescue.

- Fruit/nut trees, including almonds, apricots, cherries, nectarines, peaches and plums.

- Small fruit/vegetable Garden Crops, including beans (dried, lima, and snap), blackberries, blueberries, broccoli, bushberries, caneberries, carrots, garlic, grapes, ginseng, gooseberries, huckleberries, lettuce (head and leaf), loganberries, mustard cabbage, Chinese cabbage, dry bulb onions, peanuts, potatoes, raspberries and strawberries.

- Ornamentals at residences, including shade trees, evergreens and flowering and non-flowering shrubs.

- Turfgrass, including residential lawn areas.

Target Pests:

Types of fungi that iprodione is used to prevent, treat, and control included (but is not limited to) the following:

- Dollar spot (*Lanzia* spp. and *Moellerodiscus* spp.), Brown patch *(Rhizoctonia solani*), Leaf spot and Melting out (*Drechslera* spp.) , Fusarium blight (*Fusarium* spp.), Gray snow mold (*Typhula* spp.) and Pink snow mold (*Fusarium nivale*), Corticum red thread (*Laetisaria fuciformis*) on turfgrass

- Aerial web blight (*Rhizoctonia* sp.), Alternaria leaf blight (*Alternaria zinniae*), Botrytis blight (*Botrytis* sp.), Ink spot (*Drechslera iridis*), Ray blight (*Ascochyta chrysanthami*), Tulip fire (*Botrytis tulipae*), and Fusarium corn rot (*Fusarium oxysporum*) on ornamentals

- Sclerotinia blight (*Sclerotinia minor*) on peanuts

- Sheath blight (*Rhizoctonia solani*), Brown spot (*Bipolaris oryzai*), Sheath spot (*Rhizoctonia oryzae*) and Narrow brown leaf spot (*Cercospora oryzae*) on rice

- Brown rot blossom blight (*Monilinia* spp.), Fruit brown rot (*Monilinia* spp.), Shot hole (*Stigmina carpophila*), Scab (*Ventura carpophila*), and Cherry leaf spot (*Blumeriella jaapii*) on stone fruit

- Bunch rot (*Botrytis cinerea*) on grapes

- Gray mold (*Botrytis cinerea*), White mold (*Sclerotinia sclerotiorum*) on beans, Black leg (*Leptosphaeria maculans*) on broccoli, Alternaria blight (*Alternaria dauci*) and Black crown rot (*Alternaria radicina*) on carrots, White rot (*Sclerotium cepivorum*) on garlic, Lettuce drop (*Sclerotinia* spp.) and Brown rot (*Rhizoctonia solani*) on lettuce, and Early blight (*Alaternaria solani*) and White mold (*Sclerotinia sclerotiorum*) on potatoes. (US EPA, 1998)

2.2.3 Human health assessment (US EPA, 1998)

2.2.3.1 Toxicity

In studies using laboratory animals, iprodione generally has been shown to be of low acute toxicity. It is slightly toxic by the eye, dermal and oral routes and has been placed in Toxicity Category III (the second lowest of four categories) for these effects. In acute inhalation and as a dermal sensitizer, iprodione is practically non-toxic (Category IV). Iprodione was not mutagenic in several

studies. However, iprodione has been classified as a likely human carcinogen, based on evidence of tumors in both sexes of mouse (liver) and in the male rat.

2.2.3.2 Dietary exposure

People may be exposed to residues of iprodione through the diet and drinking water.

2.2.3.3 Occupational and residential exposure

Handlers of iprodione may be exposed to iprodione during and after normal use of liquid, wettable powder, dry flowable, and granular formulations. For dermal exposure, no short- and intermediate-term dermal risk for iprodione. For inhalation exposure, the current used of iprodione does not indicate a concern for long-term exposure or risk. Based on the use patterns and potential exposures, nineteen exposure scenarios for handlers were identified and assessed for iprodione

2.2.3.4 Human risk assessment

Aggregate cancer risk from iprodione (from dietary, residential and water exposure) with risk mitigation measures in place is 1.8×10^{-6}, which is within the range that the US EPA currently considers acceptable. With personal protective equipment (PPE) in place, risk to handlers of iprodione are considered acceptable. The Agency has also determined that a restricted-entry interval (REI) of 24-hours reduces the post-application risks posed by iprodione to workers.

The cumulative carcinogenic risk estimate for consumption of food and wine containing residues of 3,5-DCA as a result of use of iprodione, vinclozolin, and procymidone is 9.5×10^{-7}.

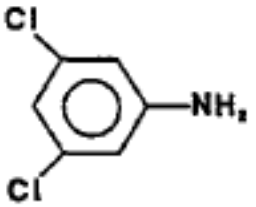
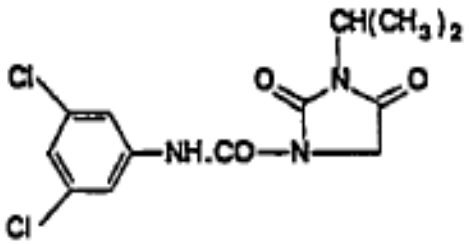
2.2.4 Environmental fate

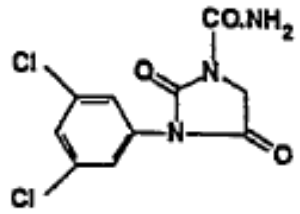
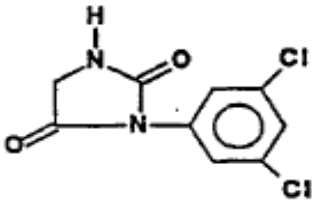
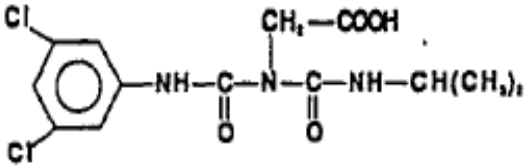
The major routes of dissipation of iprodione are hydrolysis in neutral and alkaline environments (half-life pH 7 = 4.7 days; pH 9 = 27 minutes) and microbial degradation under both aerobic and anaerobic conditions. The overall result of these mechanisms of dissipation appears to indicate that iprodione has low to intermediate persistence in the environment. The results obtained in the field confirm the expected low persistence of iprodione ($t_{1/2}$ = 3-7 days). Despite the fact that iprodione is mobile to highly mobile in some soils, it is unlikely that it will leach to ground water because of its rapid degradation in the environment. For aquatic systems, there was no strong evidence of effective mechanisms of iprodione degradation other than hydrolysis.

However, the problem of iprodione biodegradation is the accumulation of toxic intermediate. The major degradation intermediates observed in laboratory and field studies are summarized in Table 2.3. The information in Table 2.3 shows the degradation fate and maximum percentage of the parent compound at which each of the degradation intermediate appeared in the studies. The only degradation that the Health Effects Division has reported to be of toxicological concern is 3,5-DCA and it has been found in several of the laboratory studies. As the

result, the assessment includes consideration for the exposure of both iprodione and 3,5-DCA (Steeger and Garber, 2009).

Table 2.3 Iprodione degradation intermediate observed in environment fate studies (Steeger and Garber, 2009)

Chemical Name	Chemical Structure	Study in Which Found (Maximum % of Parent)
3,5-dichloroaniline		Soil Photolysis (28%) Aerobic Soil (9%) Aerobic Soil (3.9%) Aerobic Aquatic (10%) Anaerobic Aquatic (3.6%)
3-(1-methylethyl)-N-(3,5dichlorophenyl)-2,4-dioxo1-imidazolidinecarboxamide		Hydrolysis (pH 7) (45.6%) Hydrolysis (pH 9) (93%) Soil Photolysis (7.7%) Aerobic Soil (29%) Aerobic Aquatic (65%) Anaerobic Aquatic (60%) Terrestrial Field (--) Aquatic Field (--)

Chemical Name	Chemical Structure	Study in Which Found (Maximum % of Parent)
3-(3,5-dichlorophenyl)2,4-dioxo-1-imidazolidinecarboxamide		Aerobic Aquatic (15%) Terrestrial Field (--)
N-(3,5-dichlorophenyl)-2(1-methylethyl)-1ureylenecarboxamide		Aquatic Field (--)
3-(3,5-dichlorophenyl)2,4-dioxoimidazolidine		Soil Photolysis (14%) Aerobic Soil (9.5%)
[(dichloro-3,5-phenyl)-1isopropylcarbamoyl-3]-2acetic acid		Hydrolysis (pH 5) (12%) Hydrolysis (pH 7) (10.1%)

2.3 Biodegradation

Biodegradation is a process in which the enzymes produced by microorganisms breakdown or assimilate the organic substances. Such living organisms use these organic substances as a carbon and/or energy source.

Biodegradation of pesticides can occur in soil. Pesticides reaching to the soil are acted upon by several physical, chemical, and biological forces. However, physical and chemical forces are acting upon/degrading the pesticides to some extent. Microorganisms play major role in the degradation of pesticides. Many soil microorganisms have the ability to act upon pesticides and convert them into simpler, non-toxic compounds. This degradation process of pesticides and conversion into non-toxic compounds by microorganisms is known as biodegradation.

The chemical reactions leading to biodegradation of pesticides fall into several broad categories, which are discussed in brief in the following paragraphs.

a) Detoxification: Conversion of the pesticide molecule to a non-toxic compound. Detoxification is not synonymous with degradation. Since a single chance in the side chain of a complex molecule may render the chemical non-toxic.

b) Degradation: The breaking down / transformation of a complex substrate into simpler products leading finally to mineralization. Degradation is often considered to be synonymous with mineralization, e.g. Thirum (fungicide) is degraded by a strain of *Pseudomonas* and the degradation products are dimethlamine, proteins, sulpholipaids, etc.

c) Conjugation (complex formation or addition reaction): The reaction in which an organism make the substrate complex or combines the pesticide with cell metabolites. Conjugation or the formation of additional product is accomplished by those organisms catalyzing the reaction of addition of an amino acid, organic acid or methyl group of the substrate.

d) Changing the spectrum of toxicity: Some fungicides/pesticides are designed to control one particular group of organisms / pests, but they are metabolized to yield products inhibitory to entirely dissimilar groups of organisms, for e.g. the fungicide PCNB fungicide was converted in soil to chlorinated benzoic acids that killed weed.

Biodegradation of pesticides / herbicides is greatly influenced by the soil factors like moisture, temperature, pH and organic matter content, in addition to microbial population and pesticide solubility. Optimum temperature, moisture and organic matter in soil provide congenial environment for the break down or retention of any pesticide added in the soil. Most of the organic pesticides degraded within a short period (3-6 months) under tropical conditions. Metabolic activities of bacteria, fungi and actinomycetes have the significant role in the degradation of pesticides. (http://agriinfo.in/?page=topic&superid=5&topicid=174)

2.4 Biodegradation of iprodione

Iprodione-degrading bacteria

Arthrobacter sp. was able to transform iprodione in a medium containing iprodione as its sole source of carbon and nitrogen. There was an evidence of a biological transformation by the appearance of at least three metabolites: the two newly identified metabolites 1 and 2 and 3,5-DCA. They were not detected in the sterile controls. The metabolite 2 concentration increased as the metabolite 1 concentration decreased, suggesting a possible transformation of metabolite 1 into metabolite 2 (Figure 2.1) (Athiel *et al*., 1995).

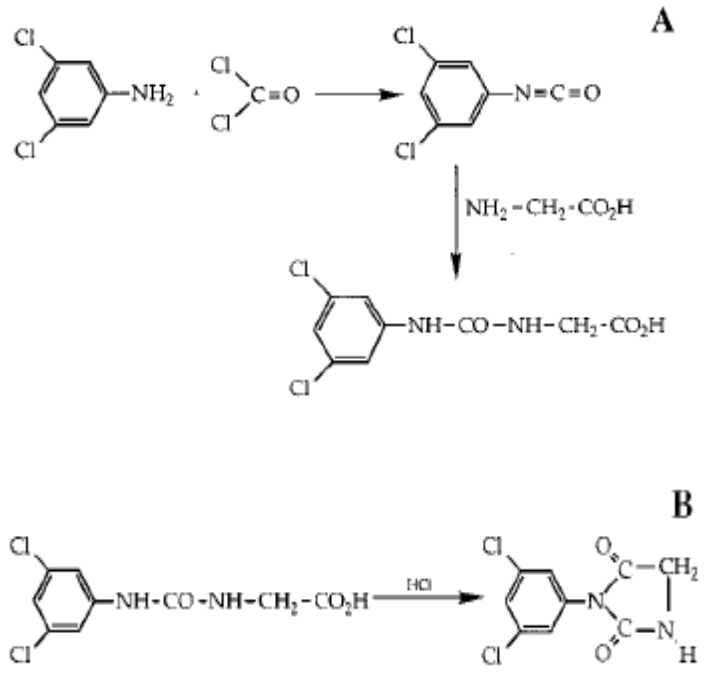


Figure 2.1 Chemical synthesis of metabolites 2 (A) and 1 (B) (Athiel *et al*., 1995)

Walker (1987) reported a two-stage degradation of iprodione; the final metabolite was 3,5-D, but the intermediate metabolite was not identified. In contrast, only the iprodione isomer and hydantoic acid were identified in the noninoculated medium. This is in agreement with results showing that iprodione was physicochemically converted into a major cyclic form and an open form (Belafdal *et al*., 1989). A scheme of the biological degradation of iprodione is proposed in (Figure 2.2) First, the urea function of iprodione is broken, producing a 3,5-dichlorophenyl dicarboximide (metabolite 1). Metabolite 1 is then converted, by chemical hydrolysis, into (3,5-dichlorophenylurea)acetic acid (metabolite 2). These metabolites had never been described in studies on iprodione degradation in soil. (Athiel *et al*., 1995)

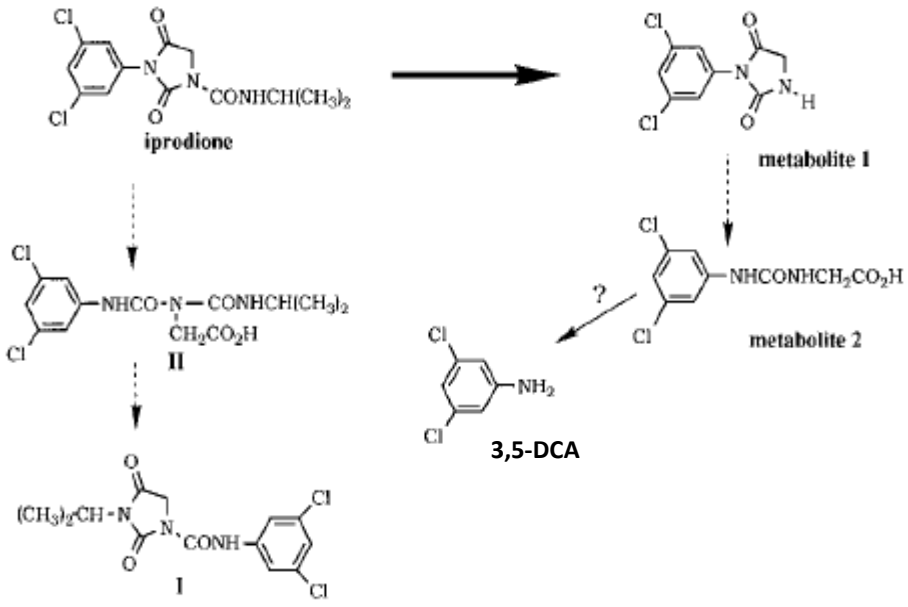


Figure 2.2 Proposed pathways for iprodione transformation. Dashed arrows, chemical pathway; solid arrow, pathway proposed for strain MA6. (Athiel *et al*., 1995)

2.5 3,5-dichloroaniline (3,5-DCA)

3,5-DCA is a common metabolite in a class of fungicides, and dicarboximides (vinclozolin, chlozolinate, iprodione, procymidone) which are commonly used in agriculture. (Vanni et al., 2000)

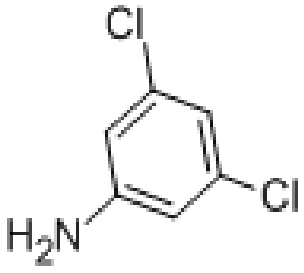
Chemical and physical properties (Syracuse Research Corporation, 2009)

Chemical Name: 3,5-Dichloroaniline

Molecular Weight: 162.02

Molecular Formula: $C_6H_5C_{l2}N$

Molecular Structure:



Color : white crystals

Water solubility: 784 mg/l

Occupational exposure to 3,5-DCA may occur through inhalation and dermal contact with this compound at workplaces where 3,5-DCA is produced or used.

The general population may be exposed to 3,5-DCA via drinking water and pesticide, dye, and pharmaceutical products containing 3,5-DCA.

2.6 Bacteria related to this study

The bacteria reported to be involved in the degradation of iprodione and 3,5-DCA are as followed:

2.6.1 *Arthrobacter*

The MA6 strain was tentatively identified as an *Arthrobacter* sp. according to its morphological and biochemical characteristics. A bacterial strain able to transform iprodione was isolated from a fast iprodione-degrading soil by enrichment procedures. Transformation pathway was through 0.02 M 3,5-dichloroaniline production as measured by a rapid colorimetric method (Athiel *et al*., 1995).

2.6.2 *Pseudomonas*

Three bacterial strains were isolated from soils adapted to iprodione and identified as *Pseudomonas fluorescens*, *Pseudomonas* sp. and *Pseudomonas paucimobilis*. The first, two strains transformed iprodione to *N*-(3,5-dichlorophenyl)-2,4-dioxoimidazolidine (II) and under restrictive conditions to 3,5-dichlorophenylurea acetic acid (III); the latter subsequently degraded II to III and III to 3,5-dichloroaniline (3,5-DCA). they constructed bacterial combinations consisting of *Pseudomonas paucimobilis* and one of the iprodione

degraders and showed that these combinations transformed iprodione into 3,5-DCA. It was known that 3,5-DCA was the major metabolite found in treated soils, suggesting that such a bacterial combination might be responsible for degrading iprodione into 3,5-DCA in adapted soils. Plasmids could only be isolated in *Pseudomonas fluorescens* but they did not investigate if one of these was involved in the ability to degrade 25 μMole/l of iprodione (Mercadier *et al*., 1997).

2.6.3 *Rhodococcus*

The biodegradation of vinclozolin and/or 3,5-dichloroaniline-degrading bacteria were isolated from pesticide-polluted agriculture soil. Among the isolated bacteria, a *Rhodococcus* sp. was identified from a 16S rDNA sequence analysis and named *Rhodococcus* sp. T1-1. The degradation ratios for vinclozolin or 3,5-dichloroaniline in a minimal medium containing vinclozolin (200 μg/ml) or 3,5-dichloroaniline (120 μg/ml) were 90% and 84.1%, respectively. Moreover, *Rhodococcus* sp. T1-1 also showed an effective capability to biodegrade dichloroaniline isomers on enrichment cultures in which they were contained. Therefore, these results suggest that *Rhodococcus* sp. T1-1 can bioremediate vinclozolin as well as 3,5-dichloroaniline.(Lee J et al., 2008)

CHAPTER III

MATERIALS AND METHODS

3.1 Equipments

All equipments used in this study as follow.

3.1.1 Autoclave NLS-3020 (Sanyo Electric Co.,Ltd, Japan)

3.1.2 Autoclave HV-110 (Hirayama, Japan)

3.1.3 Balance(Mettler Toledo, Switzerland)

3.1.4 Centrifuge (Beckman coulter, USA)

3.1.5 French press Cell Disrupter (Thermo Electron Corporation, USA)

3.1.6 Hyperclone column; 5u BDS C18 130A 250 x 4.6 mm 5µ (Phenomenex, USA

3.1.7 High Performance Liquid Chromatography (HPLC) LC20 AD (Shimazu, Japan)

3.1.8 Hotplate stirrer (IKA, Germany)

3.1.9 Incubator shaker ZHWY-100B (Zhicheng, China)

3.1.10 Incubator oven (Memmert, Germany)

3.1.11 pH meter (Mettler Toledo, USA)

3.1.12 Spectrophotometer (Thermo scientific, USA)

3.2 Chemicals

Analytical grade chemicals

3.2.1 Aniline (Merck, Germany)

3.2.2 Agar (Scharlau Microbiology , Spain)

3.2.3 Bovine serum albumin (BSA) (Sigma-Aldrish, USA)

3.2.4 $CaCl_2.2H_2O$ (Merck, Germany)

3.2.5 CH_3COONa (BDH, England)

3.2.6 $CuSO_4.5H_2O$ (Scharlau Microbiology, Germany)

3.2.7 $C_{12}H_{25}O_4SNa$ (SDS) (Sigma-Aldrish, USA)

3.2.8 $CoCl_2.6H_2O$ (Merck, Germany)

3.2.9 Absolute ethanol 99.9% purity (Merck, Germany)

3.2.10 $FeSO_4.\ 7H_2O$ (BDH, England)

3.2.11 Glycerol (Univar, Australia)

3.2.12 H_3BO_4 (Merck, Germany)

3.2.13 KH_2PO_4 (Carlo Erba Reagenti , Italy)

3.2.14 $MgSO_4.7H_2O$ (Carlo Erba Reagenti, Italy)

3.2.15 $MnSO_4.H_2O$ (Merck, Germany)

3.2.16 MoO_3 (Merck, Germany)

3.2.17 NaCl (BDH, England)

3.2.18 $NaNO_3$ (Carlo Erba Reagenti, Italy)

3.2.19 NaOH (Merck, Germany)

3.2.20 Na_2HPO_4 (Fluka, Germany)

3.2.21 Succinic acid (Merck, Germany)

3.2.22 Tris (USB, USA)

3.2.23 Tryptone (Himedia, India)

3.2.24 Yeast extract (Scharlau Microbiology , Spain)

3.2.25 $ZnSO_4.7H_2O$ (Merck, Germany)

3.2.26 3,5-Dichloroaniline 98% purity (Merck, Germany)

HPLC grade chemicals

3.2.27 Acetonitrile 99% purity (Burdick & Jackson, USA)

3.2.28 Catechol 99.5% purity (Sigma-Aldrish, USA)

3.2.29 2-chloroaniline 99% purity (Chem Service, Inc., USA)

3.2.30 3-chloroaniline 99% purity (Chem Service, Inc., USA)

3.2.31 3,4-dichloroaniline 99% purity (Chem Service, Inc. , USA)

3.2.32 4-chloroaniline 99% purity (Chem Service, Inc., USA)

3.2.33 4-chlorocatechol 97% purity (Aldrish, Germany)

Chemical of fungicides

3.2.34 Iprodione (commercial formulation) (Bayer Cropscience, Thailand)

3.2.35 Iprodione 97% purity (Sigma-Aldrish, USA)

3.2.36 Procymidone 99.9% purity (Sigma-Aldrish , USA)

3.2.37 Vinclozolin 99.5% purity (Sigma-Aldrish, USA)

3.3 Screening, isolation and identification of iprodione-degrading bacteria

3.3.1 Screening and isolation of iprodione-degrading bacteria

Soil samples were collected from agricultural area (mango groove, guava groove, rose apple groove), Thailand, with history of fungicide use, and were used for isolation of iprodione-degrading bacteria by following enrichment procedure. Five grams of soil sample were added to 100 ml of the mineral medium supplemented with iprodione (a commercial formulation) at the concentration equivalent to 200 μM. There samples were incubated at 30 °C for 72 hours on a rotary shaker (200 rpm). Then one millilitre of soil suspension was transferred into broth containing MM medium supplemented with 200 μM iprodione and incubated for an additional 16 hours under the same conditions. The enriched culture (50 μl) was spread on MM agar plates containing 200 μM iprodione, incubated 30 °C for 3 days. Bacterial single colonies formed on the plate were streaked on a MM agar plate with 200 μM iprodione.

3.3.2 Identification of iprodione-degrading bacteria.

3.3.2.1 Morphology

Colony morphology was used to study shape, size of bacteria and to classify bacteria into gram reaction. Bacteria were determined by observing strained cell and visualized through microscopy.

3.3.2.2 16S ribosomal DNA gene sequencing

Bacterial isolate was identified by 16S ribosomal DNA sequence analysis. Briefly, cell chromosomal DNA was extracted by standard method (Sambrook *et al*., 1989). The 16S rDNA gene fragment was amplified from the genomic DNA of each bacteria by polyamerase chain reaction (PCR) amplification was conducted using 63f and 1387R PCR primer (Marchesi *et al*., 1998). A reaction mixture (total volume of 25 µl) contained 1X *taq* buffer with $(NH_4)_2SO_4$ 0.2 mM dNTP, 2 mM $MgCl_2$, 0.4 mM of each primer, DNA sample 1 µl (500 g DNA), *taq* polyamerase 5 units/µl (Fermentas, USA). The reaction mixture was subjected to pre-denaturation at 94 °C for 3 min, 30 cycles consisting of denaturation at 95 °C for 1 min, 55 °C and 1 min for annealing, chain extention at 72 °C for 1.3 min with an additional extension time of 72 °C for 5 min. The PCR product was sequenced and its nucleotide sequence compared with NCBI database (www.ncbi.nlm.nih.gov).

3.4 Biodegradation of iprodione and 3,5-DCA by iprodione-degrading bacterium

3.4.1 Iprodione utilization

The ability of iprodione degrading-bacteria to utilize iprodione as a sole carbon source was determined by measurement of growth in MM containing 200 µM iprodione.

Iprodione-degrading bacteria were grown in MM medium agar plate containing 200 µM iprodione for 3 days. Then a single colony of the bacterial isolate was transfered

into a test tube containing LB medium 5 ml and 200 μM iprodione. The starter was incubated at 30 °C on the rotary shaker for 16 hours before using as a inoculum (2 % v/v) in MM (100 ml) with 200 μM iprodione. Cell were grown at 30 °C on the rotary shaker at 200 rpm in Erlenmeyer flask containing MM (100 ml). At time interval, the culture was sampling (1 ml) to determined 1) cell growth by measuring cell optical density with a spectrophotometer at 600 nm. and 2) iprodione utilization using HPLC analysis.

3.4.2 Iprodione utilization *via* resting cell technique

For resting cell study, overnight grown cells were harvested by centrifugation at 10,000 rpm for 20 min, at 4 °C, washed with 0.85 % NaCl, and resuspended in 10 ml of MM. Iprodione or 3,5-DCA solution were added to the final concentration of 40 μM and 100 μM, respectively. The test was conducted with and without a co-substrate (5 mM sodium acetate, 5 mM succinic acid, 5 mM ammonium sulphate and 0.2 % yeast extract). Samples were collected at time interval. The residual chemical was determined using HPLC and cell protein concentration was estimated.

3.4.3 Enzymatic assays for catechol 1,2-dioxynase (C12DO), chlorocatechol 1,2-dioxynase (CC12DO), catechol 2,3-dioxygenase (C23DO) and chlorocatechol 2,3-dioxygenase (CC23DO) activities

3.4.3.1 Preparation of bacteria for enzyme assay

Bacteria were grown in 5 conditions as follow:

(1) MM supplemented with 5 mM succinic acid containing 40 μM iprodione

(2) MM supplemented with 5 mM succinic acid MM medium containing 100 μM 3,5-DCA

(3) MM supplemented with 5 mM succinic acid containing 100 μM catechol

(4) MM supplemented with 5 mM succinic acid containing 100 μM 4-chlorocatechol

Cells were incubated on rotary shaker at 200 rpm, 30 °C for 16 hour

3.4.3.2 Preparation of cell-free extract for enzyme assays

Cells were harvested by centrifugation at 10,000 rpm for 20 min, at 4 °C, washed twice with 0.85% NaCl and suspended with 50 mM Tris-HCl buffer pH 8.0 Cells were disrupted using French pressure cell (Thermo Electron Corporation, USA) twice, at 12,000 psi. Cell debris was removed by centrifugation at 12,000 rpm for 20 minutes at 4 °C. The clear supernatant was used as a crude extract for enzyme assays. The protein

was determined by the method of modified Lowry method, using bovine serum albumin (BSA) as a standard. One unit of enzyme activity was defined as the amount of enzyme required to catalyze formation of 1 µmole of product per minute under the specified assay conditions. Specific activities were expressed as units per milligram of protein.

3.4.3.3 Enzymatic assays

The activity of bacterial enzymes were measured by spectrophotometer monitoring the change in absorbance of each product (Table 3.1). Assays were performed to measure the activity of enzyme against substrate. The reaction mixture (final volume 1 ml) contains enzyme, substrate and 50 mM Tris-HCl buffer pH 8.0.

Table 3.1 Information of enzyme assay measured spectrophotometrically

Enzyme	Substrate	Product	Absorbance (nm)
catechol 1,2-dioxynase (C12DO)	Catechol	Muconic acid	260
chlorocatechol 2,3-dioxygenase (C23DO)	4-Chlorocatechol	3-Chloromuconic acid	260
catechol 2,3-dioxygenase (C23DO)	Catechol	2-Hydroxymuconic semialdehyde	375
chlorocatechol 2,3-dioxygenase (CC23DO)	4-Chlorocatechol	5-Chloro-2-hydroxymuconic semialdehyde	379

3.5 Optimization of iprodione degradation

Based on the results of screening experiment, kinetic studies were carried out using the resting cell technique. For process optimum conditions, the following parameters: co-substrate supplements (type and concentration), pH and temperature were tested.

3.5.1 Effect of co-substrate

Either sodium acetate (5 mM) or succinic acid (5 mM) was used as a co-substrate carbon source, while ammonium sulphate (5 mM) and 2% yeast extract was used as a nitrogen source. In the presence of iprodione (40 μM) in MM medium supplemented with each co-substrate. Culture was incubated in shaker at 200 rpm, at 30 °C. Then bacterial culture was collected for resting cell technique and samples was collected at time interval. The residual chemical was determined using HPLC.

3.5.2 Effect of iprodione concentrations

Bacterial ability to degrade iprodione was tested with various concentrations of iprodione between 10 - 200 μM. Culture was incubated in shaker at 200 rpm, at 30 °C. Culture was collected for resting cell technique, samples were collected at time interval, The residual was determined using HPLC. The substrate degradation rates were then calculated.

3.5.3 Effect of pH

Biodegradation test was conducted as previously described, but at various pHs. The medium was adjusted to pH 5.0, 6.0, 7.0, and 8.0. After inoculation, all flasks were incubated in shaker at 200 rpm, at 30 ˚C. Culture was collected for resting cell technique, samples were collected at time interval. The residual was determined using HPLC.

3.5.4 Effect of temperature

Bacteria culture effluent was adjusted at varying at different incubation temperatures; 30 ˚C, 37 ˚C, 40 ˚C and 45 ˚C. After inoculation, all flasks were incubated in shaker at 200 rpm. Bacterial culture was collected for resting cell technique, sample was collected at time interval, The residual was determined using HPLC.

3.6 Kinetic degradation

Biodegradation of chemicals is one of the major processes, which determines the fate of organic compounds in environments. Thus, biodegradation logically plays a key role in the environmental risk assessment of substances (Dimitrov *et al*., 2007).

The degradation kinetics of iprodione using a stability-indicating HPLC method is demonstrated. The degradation rate kinetics of iprodione were determined by plotting the concentration of iprodione remaining versus a time for different concentration of

iprodione. The kinetic parameters were analyzed in order to investigate substrate. The kinetics parameter such as apparent order degradation rate constant (K_s^{app}), and k_{max} (specific degradation rate; $mM.h^{-1}.mg\ prot^{-1}$) were obtained. The kinetic parameters, k_{max} and K_s^{app} values, of each substrate were derived by a linear regression fitting of data points according a non-growth Michaelis–Menten model developed by (Schmidt *et al*., 1985).

3.7 Range of iprodione and 3,5-DCA structural related compounds

Several types of chloroaniline at 200 μM 2-chloroaniline, 3-chloroaniline, 4-chloroaniline 2,3-dichloroaniline, 2,4-dichloroaniline,3,4-dichloroaniline and 3,5-dichloroaniline were used to examine the substrate range biodegradation. Bacterial culture was grown on 100 ml of MM with 5 μM succinic acid, cells were harvested by centrifugation at 10,000 rpm, washed with 0.85 % NaCl, and resuspended in 10 ml of MM. Samples were collected at time interval. The residual was determined using HPLC.

Then the last was conducted with dichlorophenyl dicarboximide fungicides i.e. procymidone and vinclozolin. At 40 μM bacterial culture was grown on 100 ml of MM with 5 mM succinic acid, cells were harvested by centrifugation at 10,000 rpm, washed with 0.85 % NaCl, and resuspended in 10 ml of MM. Samples were collected at time interval. The residual chemical was determined using HPLC.

3.8 Analytical method

3.8.1 Determination of protein concentration

Cell suspension (400) µl was centrifuged at 10,000 rpm, for 10 min. The supernatant was removed. Cell pellet and washed twice with 0.85 % (w/v) NaCl, heated at 100°C for 10 min in a heating block, and then the supernatant was used to determine protein concentration.

Reagent preparation

Complex-forming reagent was prepared immediately before use by mixing the following stock solutions.

Solution A : 2% sodium carbonated in 0.1 M sodium hydroxide

containing 0.5% sodium dodecyl sulfate (SDS)

Solution B : 0.5% copper sulfate in potassium sodium tartate

Solution C : phenol solution (Folin-Clocahen' s reagent)

Method

The test sample was adjusted sample to 0.4 ml using sterile deionized water. Then, two milliliter of the fresh mixed solution A and B (A:B, 50:1) was added and rapidly mixed and incubated at 30 °C for 10 min. Solution C (0.2 ml) was then,

mixed immediately, incubated at 30 °C for 30 min and finally measure absorbance at 750 nm by a spectrophotometer. The standard curve was done using bovine serum albumin (BSA).

3.8.2 HPLC analysis

Iprodione was determined using High Performance Liquid Chrotography (HPLC). Samples were collected at time interval, harvested to remove cells by centrifugation at 10,000 rpm 10 min. Cell-free supernatant was mix 1:1 (v/v) with acetonitrile, filtered with 0.45 µM syringe filter into a 1.5 ml glass of vial. The pump was operated at 1 ml/min, with a UV detection wavelength set at 240 nm. The mobile phase consisted of acetonitrile /water 70:30 (v/v), flow rate was 1 ml/min, injection volume was 20 µl, the analytical column was hyperclone column (5u BDS C18 130A 250 x 4.6 mm). Under these conditions, iprodione was eluted and detected in 5.27 min. In this study, peak area was used as a detector response, and it calculated by comparison of the peak area in the chromatogram of extracts with those in the chromatogram of standard solutions.

CHAPTER IV

RESULTS

4.1 Screening, isolation and identification of iprodione-degrading bacteria

4.1.1 Screening and isolation of iprodione-degrading bacteria

By using an enrichment technique with 200 μM iprodione (a commercial formula) as sole carbon and energy sources, bacterial strains were isolated from soils sample from agricultural area. Enrichment technique was used to increase quantity of bacteria with capability of iprodione degradation. Screened on mineral medium (MM) (Appendix A) agar plate with of 200 μM iprodione, fifteen colonies of potential iprodione-degrading bacteria were obtained. After the second round of growth of iprodione containing MM agar plate, only seven types of colony could grow with reasonable growth rate in liquid MM supplemented with 200 μM iprodione (Figure 4.1). Further test result in only one type of bacterial isolated, which showed positive result on iprodione degradation. These seven bacteria isolates were cultured in MM with 200 μM iprodione were for 3 days. The growth of bacteria at 30 °C was observed by measuring the optical density at 600 nm using spectrophotometer during 3 days of incubation. Cell suspension was collected to analyze iprodione utilization using High Performance liquid Chromatography (HPLC) (Table 4.1). Seven isolated strains include I1, I2, I4, I6, I7, I8 and I12.

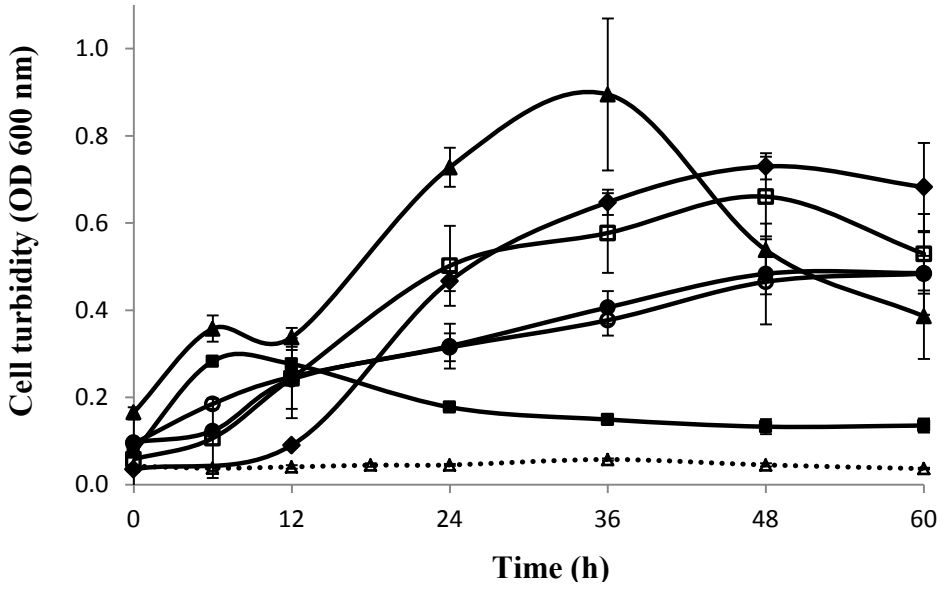


Figure 4.1 Growth of seven bacterial isolate: I1(o), I2 (●), I4 (□), I6 (■), I7 (♦), I12 (▲) in MM, in the presence of 200 µM of iprodione (a commercial formula) and abiotic control (Δ). The data are means from three independent experiments with vertical bars representing standard errors of the means.

Table 4.1 Iprodione degradation and growth of the bacterial isolates

Bacterial strains	Growth		Degradation	
	Growth rate (h^{-1})	Final OD (600 nm)	Specific deg. rate (0-12h) (mM.h-1.mg cell prot-1)	Total degradation (3 days)
I1	0.008 ± 0.099	0.205 ± 0.035	0.013 ± 0.01	29.71 ± 1.34
I2	0.013 ± 0.001	0.489 ± 0.069	0.039 ± 0.00	45.36 ± 9.29
I4	0.004 ± 0.56	0.530 ± 0.016	0.145 ± 0.02	92.54 ±2.84
I6	0.010 ± 0.005	0.464 ± 0.020	0.022 ± 0.01	26.15 ± 7.23
I7	0.005 ± 0.45	0.624 ± 0.083	0.031 ± 0.01	20.81 ± 8.54
I8	0.017 ± 0.58	0.134 ± 0.134	0.019 ± 0.02	22.22 ± 4.62
I12	0.014 ± 0.59	0.561 ± 0.26	0	0

4.1.2 Identification of iprodione-degrading bacteria

4.1.2.1 The morphological characteristic of bacteria

The morphological characteristic was done by observing colony appearance, color, and Gram's straining. Furthermore, the characteristic of colony on MM agar plate containing 0.2 mM iprodione has been shown in (Table 4.2). One of bacteria strain capable of iprodione degradation, which was isolated from soil sample of mango groove were identified for their morphological characteristic by bacteria Gram's straining and chemical solution straining. Similar to many of the *P.*

aeruginosa colony morphology variants previously described in the literature, the result showed that the isolate is a gram-negative bacterium as shown in Figure 4.2.

Table 4.2 Characteristic of bacterial colonies for screening iprodione-degrading bacteria on MM agar plate containing 200 μM iprodione.

Agricultural area source	**Isolations**	**Colonies characteristics**			
		Color	**Form**	**Surface**	**Edge**
Guava groove	I1	Clear	Circular	Smooth	Entire
	I2	White	Punctiform	Smooth	Entire
Mango	I4	Green	Irregular	Raised	Entire
groove	I6	Yelllow	Circular	Smooth	Entire
	I7	Green	Circular	Raised	Entire
Rose apple	I8	White	Circular	Smooth	Entire
groove	I12	White	Circular	Raised	Entire

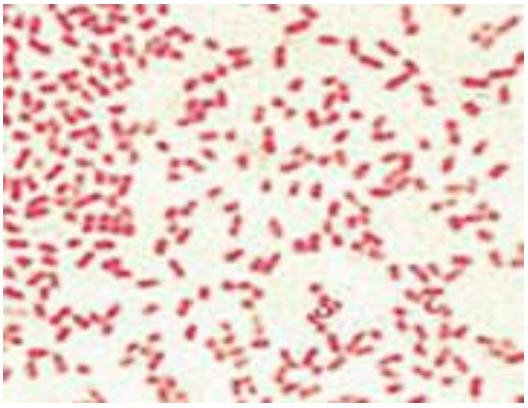


Figure 4.2 Gram's straining and the morphology of strain I4

4.1.2.1 Determination of 16S ribosomal DNA gene for bacteria identification

16S rDNA gene sequence analysis was used to identify iprodione-degrading bacteria strain I4. The specific primers, 63f and 1387r (Marchesi et al., 1998) were used to amplify the target sequence. These primers were found to be more useful for identification of bacterial species. After PCR amplification, PCR products were analyzed by 0.8% agarose gel electrophoresis. The length of the amplified 16S rDNA fragment product was approximately 1.3 kbps. Subsequently, the Nucleotide Blast program was used to compare and analyze the 16S rDNA sequence against NCBI database (www.ncbi.nlm.nih.gov). The alignment result of 16S rDNA sequences are shown in Appendix C and summarized in Table 4.3.

Table 4.3 Identification of iprodione-degrading bacteria using 16S rDNA gene sequence comparison

Culture	Sequence identity	Accession number	Source of bacteria strain	Note	References	Bacteria
I4	99%	CP402496.1	Soil	Effect biocontrol agent *Pseudomonas aeruginosa* M18	(Wu *et al*., 2011)	*Pseudomonas aeruginosa* M18
				Biodegradation of aniline in natural environments.	(Konopka *et al*., 1989)	*Pseudomonas aeruginosa* K
				Iprodione degradation	(Lo H *et al*., 1990)	*Pseudomonas sp. and Pseudomonas paucimobilis*

4.2 Degradation of iprodione by iprodione-degrading bacteria

4.2.1 Iprodione degradation

P. aeruginosa I4 utilized the fungicide iprodione as a sole source of carbon and energy for its growth. The growth of *P. aeruginosa* I4 in MM containing 200 μM iprodione (a commercial formula) shown in Figure 4.3. Within 3 days of growth the total iprodione degradation of *P. aeruginosa* I4 was at 82.96 % ± 1.28, while the decrease of iprodione in abiotic control was only 1.47 % ± 6.43. *P. aeruginosa* I4 exhibited the highest percentage of total degradation. Loss of iprodione in abiotic control might be caused by the effect of photo-oxidation. Iprodione degradation was then determined as the specific iprodione degradation rate expressing the disappearance rate of iprodione in three days on cell protein basis (calculation is shown in Appendix B). The specific degradation rate of *P. aeruginosa* I4 was 28.83 ± 0.246 (uM/h/mg protein) and growth rate was 0.012 ± 0.001 (h^{-1}).

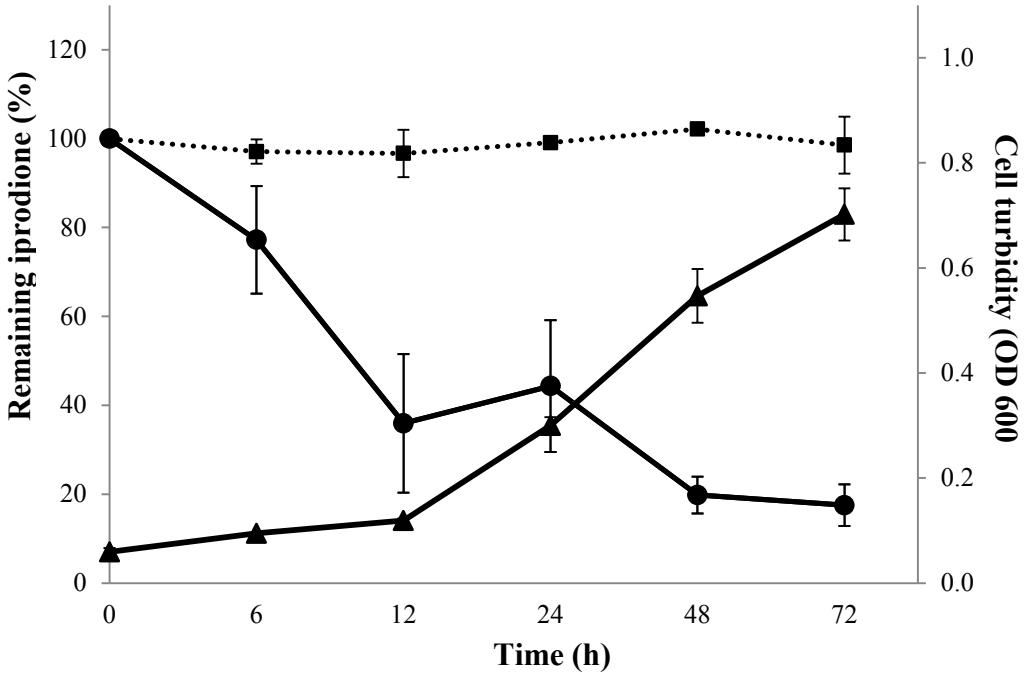


Figure 4.3 Utilization of iprodione (●) during growth (▲) by *P. aeruginosa* I4 in MM containing 200 µM iprodione. And abiotic control (■). The data are means from three independent experiment with vertical bars representing standard errors of the means.

4.2.2 Effect of a co-substrate on iprodione degradation

Type and concentration of additional carbon and/or nitrogen source was varied to promote cell growth and cell degradation efficiency. This experiment purposed to selected the efficiency of co-substrate during the entire incubation, though iprodione was degraded via resting cell technique. First, iprodione (used commercial formula) degradation without additional supplement , The total

degradation of iprodione co-substrate The following co-substrates: 5 mM sodium acetate, 5 mM succinic acid, 5 mM ammonium sulphate and 0.2 % yeast extract was supplemented in MM medium and the total degradation of iprodione was investigated in comparison to that without co-substrate.

The degradation test with succinic acid supplementation showed 68.01 % ± 4.96 higher total degradation than those sodium acetate, ammonium sulphate and yeast extract, The supplementation conditions that supplemented with 5 mM and degradation of *P. aeruginosa* I4 (Figure 4.4) showed the total degradation 68.01 ± 4.96 and specific degradation rate, 0.02 ± 0.001 uM.h-1.mg.prot-1 within 24 hour (Table 4.4)

Table 4.4 Biodegradation ability of the isolated bacterium in co-substrate *via* resting cell technique.

Bacteria	**Co-substrate condition**	**% Total degradation 24 hour**	**Specific deg. rate (0-12h) ($\mu M.h^{-1}$.mg cell $prot^{-1}$)**
P. aeruginosa I4	5 mM sodium acetate	7.68± 0.72	0
	5 mM succinic acid	68.01 ± 4.96	0.02 ± 0.001
	5 mM ammonium sulphate	10.65 ± 8.55	0.004 ± 0.002
	0.2 % yeast extract	30.02 ± 0.42	0.005 ± 0.000

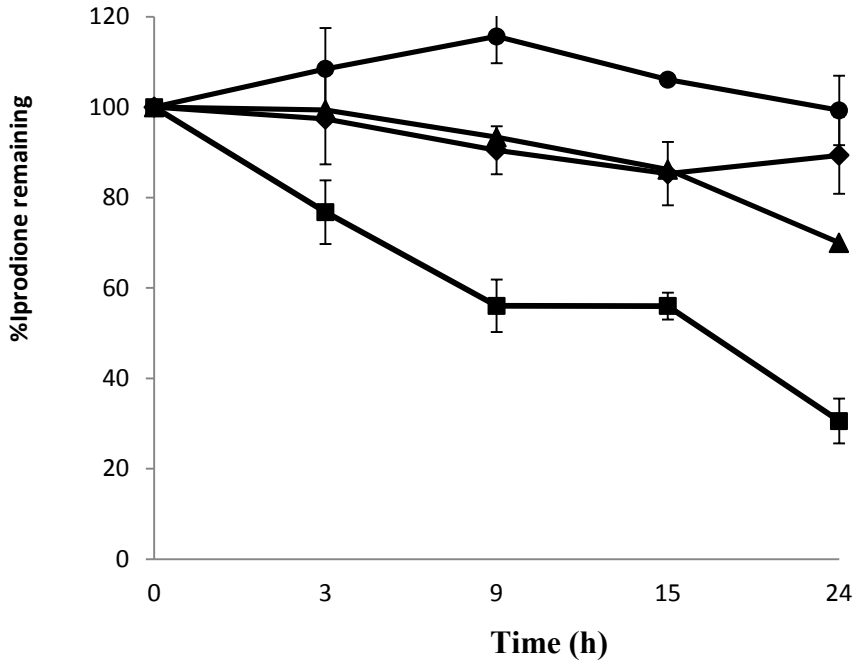


Figure 4.4 Biodegradation of iprodione (40 µM) at various co-substrate; 5mM succinic acid (■), 5mM sodium acetate (●), 5mM ammonium sulfate (♦), 0.2% yeast extract (▲).

4.3 Iprodione degradation kinetic

Ability of *P. aeruginosa* I4 on degradation of iprodione (used iprodione purity 97 %) was further tested at various concentrations as follow: 10, 20, 30, 40, 80, 100 and 200 µM in MM medium supplemented with additional 5 mM succinic acid, using a resting cell technique. During the incubation cell suspension was interval taken, centrifuged and the supernatant was analyzed for the degradation

of iprodione using High Performance Liquid Chromatography (HPLC). Total iprodione degradation at various concentrations as follow: 10, 20, 30, 40, 80, 100 and 200 μM were 48.20 ± 1.87, 64.30 ± 2.84 , 65.56 ± 3.47, 73.99 ± 5.03, 48.68 ± 6.39, 34.87 ± 4.88 and 15.64 ± 11.01 % (Figure 4.4).

The highest total removal achieved at 40 μM (Table 4.5). Iprodione biotranformation kinetic was determined as the specific iprodione degradation rate expressing the disappearance rate of iprodione in twenty-four hour on cell protein basis. Abiotic control (uninoculated cell) was performed as a control. The specific iprodione degradation rate was calculated from the rate of iprodione degradation devided by cell protein concentration. Biodegradation kinetic of iprodione by *P. aeruginosa* I4 followed the saturation kinetic of a Michaelis-Menten-like relationship (Figure 4.5). The specific degradation rate of *P. aeruginosa* I4 tested at various concentrations as follow: 10, 20, 30, 40, 80, 100 and 200 μM were 0.02 ± 0.02, 0.024 ± 0.01, 0.030 ± 0.00, 0.037 ± 0.00, 0.032 ± 0.01, 0.012 ± 0.00 and 0.011 ± 0.00 $\mu M.h^{-1}.mg\ cell\ prot^{-1}$.

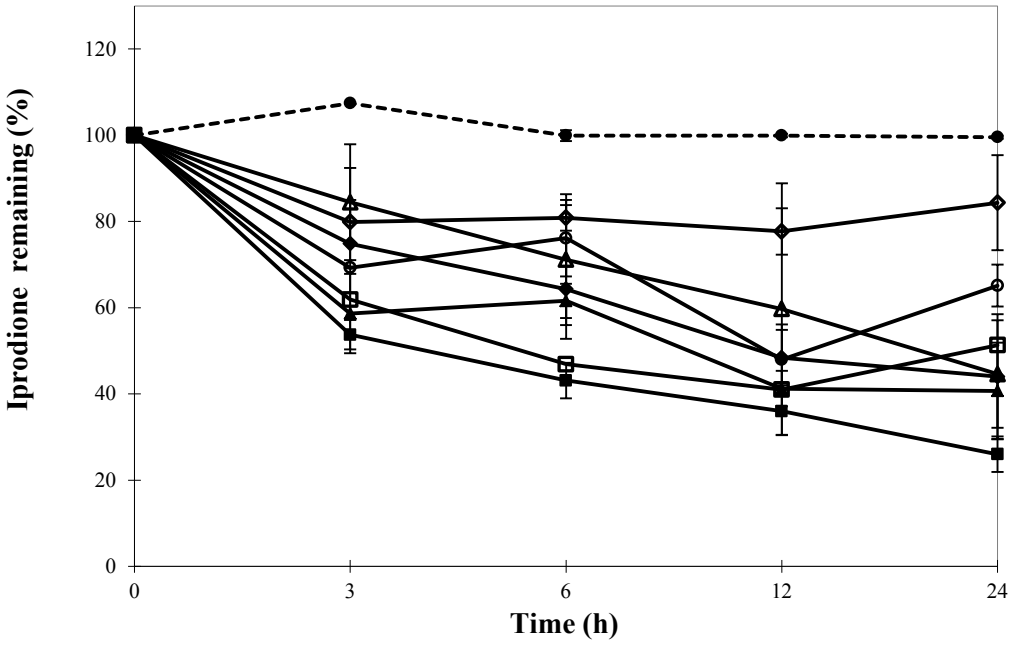


Figure 4.5 Biodegradation of iprodione by *P. aeruginosa* I4 at various concentrations: 10 µM (Δ), 20 µM (♦), 30 µM (▲), 40 µM (■), 80 µM (□), 100 µM (o), 200 µM (◊) grown in mineral medium and abiotic control iprodione (●) in MM containing iprodione. The data are means from three independent experiment with vertical bars representing standard errors of the means.

Table 4.5 Iprodione degradation rate and total removal within 24 hours by *P. aeruginosa* I4

Concentration (µM)	**Degradation rate (µM/h)**	**% Total degradation**	**Specific deg. rate (0-12h) (µM.h^{-1}.mg cell prot^{-1})**
10	0.48 ± 0.25	48.20 ± 1.87	0.020 ± 0.02
20	1.13 ± 0.38	64.30 ± 2.84	0.024 ± 0.01
30	1.97 ± 0.20	65.56 ± 3.47	0.030 ± 0.00
40	3.85 ± 0.34	73.99 ± 5.03	0.037 ± 0.00
80	5.18 ± 1.16	48.68 ± 6.39	0.032 ± 0.01
100	5.12 ± 0.03	34.87 ± 4.88	0.012 ± 0.00
200	5.80 ± 1.80	15.64 ± 11.01	0.011 ± 0.00

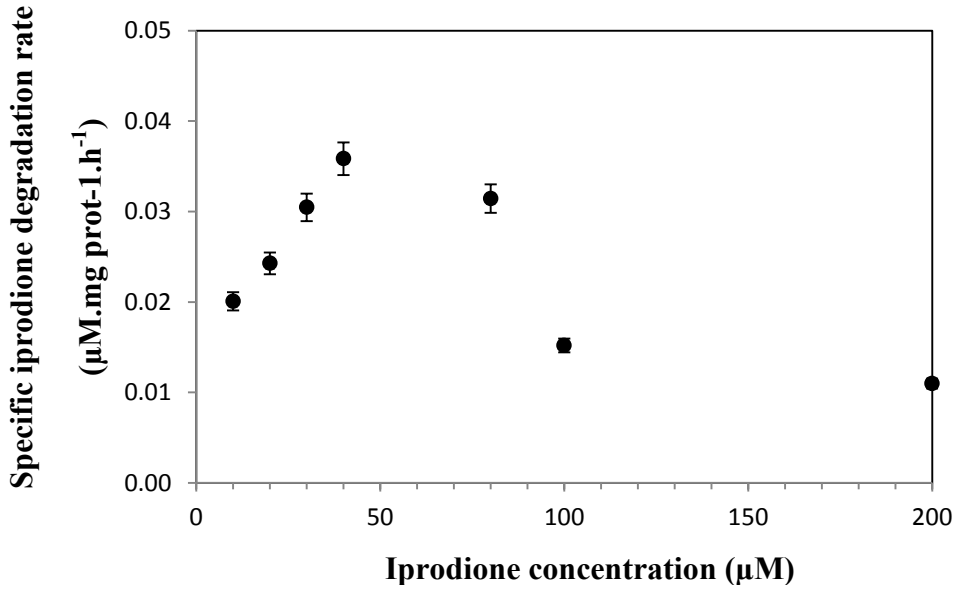


Figure 4.6 Relationship between the specific biodegradation rate and the concentrations of iprodione (●) by *P. aeruginosa* I4. The data are means from three independent experiment with vertical bars representing standard errors of the means.

From previous studies, Walker (1987) reported a two-stage degradation of iprodione in which, the final metabolite, 3,5-DCA was yielded. It is a degradation product of iprodione . In the present study, investigation of biodegradation by *P. aeruginosa* I4, it has unique character that is able to completely degrade either iprodione and its intermediate (3,5-DCA). Although it was stated that 3,5-DCA is hardly degraded by previous reports. This experiment tested at various concentrations as follow: 50, 80, 100, 200, 250, 300 and 400 µM (Table 4.6). Biodegradation kinetic of 3,5-DCA by *P. aeruginosa* I4 followed the saturation

kinetic of a Michaelis-Menten-like relationship, showed between the specific biodegradation rate and the concentrations (Figure 4.7).

Table 4.6 3,5-DCA degradation rate and total removal within 24 hours by *P. aeruginosa* I4

Concentration (µM)	**Degradation rate (µM/h)**	**% Total degradation**	**Specific deg. rate (0-12h) (µM.h^{-1}.mg cell prot^{-1})**
50	5.39 ± 0.00	87.90 ± 1.87	0.029 ± 0.00
80	9.75 ± 0.00	93.16 ± 9.38	0.049 ± 0.00
150	19.85 ± 0.00	93.78 ± 1.04	0.096 ± 0.00
200	28.61 ± 0.00	96.36 ± 0.72	0.147 ± 0.01
250	37.09 ± 0.00	95.88 ± 1.25	0.215 ± 0.01
300	40.08 ± 0.00	98.29 ± 10.21	0.257 ± 0.03
400	32.09 ± 0.46	95.48 ± 8.49	0.173 ± 0.00

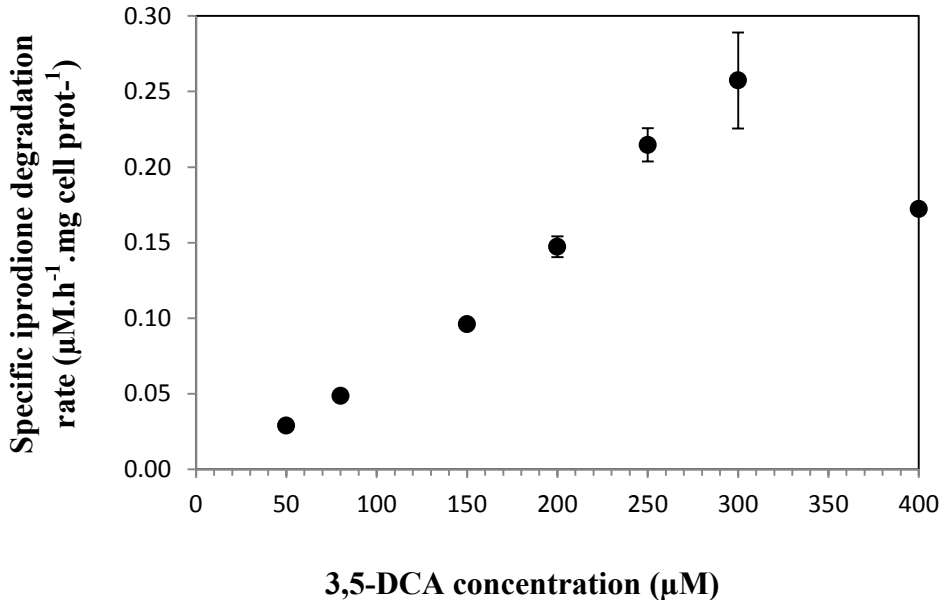


Figure 4.7 Relationship between the specific biodegradation rate and the concentrations of 3,5-DCA (●) by *P. aeruginosa* I4. The data are means from three independent experiment with vertical bars representing standard errors of the means.

Table 4.7 Apparent kinetic of the biodegradation of iprodione and 3,5-DCA by *P. aeruginosa* I4

Substrate	K_s^{app} **(mM)**	K_{max} **(mM.h^{-1}.mg prot^{-1})**	K_{max}/K_s^{app}
Iprodione	0.038 ± 0.01	3.70E-05 ± 0.00	0.001
3,5-DCA	1.691 ± 0.49	0.240 ± 0.00	0.142

4.4 Determination of enzymes involving iprodione-degradation

The modified *ortho*-cleavage pathway reveals the enzymes that have been evolved to handle chlorinated substrates which are different from *ortho-cleavage* pathway (Schlomann M., 1994). Chlorocatechol 1,2-dioxygenase is an important enzyme that can be detected in modified *ortho*-cleavage pathway, while catechol 2,3-dioxygenase can be detected *meta*-cleavage pathway (Harwood and Parales, 1996.) In this study, the activities of catechol 1,2-dioxygenase (*ortho*-cleavage pathway), chlorocatechol 1,2-dioxygenase (a modified *ortho*-cleavage pathway), catechol 2,3-dioxygenase and chlorocatechol 2,3-dioxygenase (*meta*-cleavage pathway) were examined from iprodione-grown cells in five different growth conditions.

In the enzymatic study of *P. aeruginosa* I4, the activity of the enzyme of *ortho*-cleavage pathway was highest when compared that of other pathways. The maximum activity is 3.384.9 ± 0.04 μmole/min.mg protein^{-1} for the catechol 1,2-dioxygenase which was grown on MM containing 5 μM succinic acid and 100 μM catechol for overnight. The specific activity of these enzymes were determined by measuring the apparent product using a spectrophotometer. The result is as shown in Table 4.8.

Table 4.8 Specific activities of enzymes (cell free extract) involving iprodione degradation pathway of *P. aeruginosa* I4.

Cell grown in medium type	***ortho*-cleavage pathway (µmole/min.mg protein)**	**Modified *ortho*-cleavage pathway (µmole/min.mg protein)**	***meta*-cleavage pathway (µmole/min.mg protein)**	
	C12DO	**CC12DO**	**C23DO**	**CC23DO**
MM + S[b]	540.01 ± 0.08	56.90 ± 0.11	4.80 ± 0.06	6.50 ± 0.09
MM + S[b] MM + S+ IP[a]	950.30 ± 0.04	80.46 ± 0.07	23.75 ± 0.00	3.21 ± 0.02
MM + S + 3,5-DCA[c]	3002.5 ± 0.04	265.30 ± 0.03	19.58 ± 0.01	17.68 ± 0.02
MM + S + catechol[d]	3384.9 ± 0.04	286.62 ± 0.03	28.20 ± 0.0	4.77 ± 0.01
MM + S + 4-chlorocatechol[e]	2582.2 ± 0.02	129.5 ± 0.03	0.00 ± 0.00	6.47 ± 0.02

a MM + S+ 40 µM IP: mineral medium containing 5 mM succinic acid and 40 µM ipordione

b MM + S: mineral medium containing 5 mM succinic acid

c MM + S + 100 µM 3,5-DCA: mineral medium containing 5 µM succinic acid and 100 µM 3,5-DCA

d MM + S + 100 µM catechol: mineral medium containing 100 µM succinic acid and 100 µM catechol

e MM + S + 100 µM 4-chlorocatechol: mineral medium containing 100 µM succinic acid and 100 µM 4-chlorocatechol

4.5 Optimization iprodione degradation

The optimization of growth conditions was investigated in order to increase the total degradation. Various temperatures were determined as follows: 30 °C, 37 °C, 40 °C, 45 °C and various pH as followed: 5.0, 6.0, 7.0, 8.0. The optimum conditions increase the total iprodione degradation by 65.77 % ± 9.24 (Table 4.9). The optimal degradation conditions of iprodione by *P. aeruginosa* I4 were 40 µM iprodione, at 37 °C and pH 7.0. The result obtained was reasonable because *P. aeruginosa*' s growth is 37°C, and it is not able to grow at temperatures as high as 42°C. (Todar, 2009) In this study, the optimum temperature is 37°C, which yielded degradation rate of 1.56 ±0.03 (h^{-1}), total degradation of 65.77 ± 9.24%, specific degradation rate of 0.014 ± 0.00 ($\mu M.h^{-1}$.mg cell $prot^{-1}$), while the optimum pH 7.0, which increase degradation rate to 3.56 ± 0.34 (h^{-1}), total degradation to 63.63 ± 7.82%, specific degradation rate to 0.018 ± 0.00 ($\mu M.h^{-1}$.mg cell $prot^{-1}$)

Table 4.9 Optimization of iprodione degradation

Optimum conditions		Degradation rate (h^{-1})	% Total degradation	Specific deg. rate (0-12h) ($\mu M.h^{-1}.mg$ cell $prot^{-1}$)
At 37 °C	pH 5.0	0	0	0
	pH 6.0	1.42 ± 1.13	65.35 ± 5.63	0.005 ± 0.01
	pH 7.0	3.56 ± 0.34	63.63 ± 7.82	0.018 ± 0.00
	pH8.0	0	0	0
pH 7.0	30 °C	1.46 ± 2.98	54.95 ± 15.83	0.011 ± 0.01
	37 °C	1.56 ±0.03	65.77 ± 9.24	0.014 ± 0.00
	40 °C	0	0	0
	45 °C	0	0	0

4.6 Range of iprodione and 3,5-DCA structural related compounds

Ability of *P. aeruginosa* I4 on degradation of iprodione and others substrates including 2-chloroaniline, 2,3-dichloroaniline, 2,4-dichloroaniline, 2,3,4-tri chloroaniline, 2,4,6-tri chloroaniline, 3,4-dichloroaniline and 3,5-dichloroaniline was examined. The bacteria strain was cultured in MM supplemented with 5 mM succinic acid and the substrate tested at 200 μM. Cell were grown for 3 days, cell suspension was collected to analyze all substrates utilization via resting cell technique, and detected High Performance liquid Chromatography (HPLC) (Table 4.10).

Furthermore, the ability of cells to degrade chemical in groups of dicarboximides fungicides as follow: 40 μM vinclozolin, 40 μM procymidone were determined *via* a resting cell technique (Table 4.11).

Table 4.10 Biodegradation of chloroaniline of *P. aeruginosa* I4

Substrates (200 µM)	**Degradation rate (µM/h)**	**% Total degradation**	**Specific deg. rate (0-12h) (µM.h^{-1}.mg cell prot^{-1})**
aniline	0	0	0
2-chloroaniline	5.09 ± 4.98	83.23 ± 3.56	18.20 ± 0.01
3-chloroaniline	0	0	0
4-chloroaniline	0	0	0
2,3-dichloroaniline	4.34 ± 1.28	40.16 ± 9.59	17.35 ± 0.00
2,4-dichloroaniline	4.14 ± 2.83	55.29 ± 15.57	15.48 ± 0.00
3,4-dichloroaniline	8.38 ± 6.82	82.90 ± 4.31	62.04 ± 0.01
3,5-dichloroaniline	8.38 ± 6.82	82.90 ± 4.31	0.062 ± 0.01
2,3,4-trichloroaniline	0	0	0
2,4,5-trichloroaniline	0	0	0

Table 4.11 Biodegradation of of vinclozolin and procymidone by *P. aeruginosa* I4

Substrates (40 µM)	**Degradation rate (µM/h)**	**% Total degradation**	**Specific deg. rate (0-12h) (µM.h^{-1}.mg cell prot^{-1})**
procymidone	0.94 ± 0.17	52.42 ± 3.67	0.005 ± 0.00
vinclozolin	2.55 ± 0.24	85.04 ± 13.46	0.014 ± 0.00
iprodione	3.85 ± 0.34	73.99 ± 5.03	0.037 ± 0.00

CHAPTER V

DISCUSSIONS

5.1 Screening, isolation and identification of iprodione-degrading bacteria

The iprodione-degrading bacteria were isolated from agriculture soil with a history of fungicide exposure (propineb, cabendazim). The properties of soil of which could be briefly described as loam soil, confirmed by its information at Damnoen Saduak soil series: Dn (Department of Agriculture, 2011). Multiple genera of iprodione-degrading bacteria, including *Pseudomonas* and *Bacillus* have been isolated in previous studies, while an enhanced degradation of iprodione and vinclozolin had been observed in France and the United Kingdom and in New Zealand (Slade and Fullerton, 1992). Although field studies and laboratory data confirmed that the microbial degradation play a major role for fungicides mitigation in environment (Martin *et al*., 1990), much less information is available on the microbial degradation of fungicide iprodione. Walker (1987) demonstrated that 3,5-DCA was a degradation product of iprodione and vinclozolin and appeared concomitantly with the disappearance of these fungicide (Walker, 1987).

The bacterial strain was isolated from soils adapted to iprodione and identified as *P. aeruginosa* I4. It could degrade both iprodione and 3,5-DCA and was further tested at various concentrations of both chemicals using a resting cell technique. They

could use iprodione as a growth substrate and an inducer for the degradation pathway. This study is different from previous studies because so far only one report succeeded in isolating pure strain involved in iprodione and 3,5-DCA biodegradation (Head et al., 1988). Most studies reported bacterial strians able to degrade iprodione only.

The isolated bacterium *P. aeruginosa* I4 is a gram negative bacteria which was isolated in this study as an iprodione-metabolizing strain. This confirms the fact that *Pseudomonas* is diverse in environment and plays an important role on environmental cleanup process. Examples of other *Pseudomonas sp.* Which have been reported as iprodione-degrading bacteria include soil-isolated *Pseudomonas fluorescens*, *Pseudomonas* sp. and *Pseudomonas paucimobilis*. (Mercadier *et al*., 1997). In addition to *Pseudomonas* sp., the ability of an iprodione-resistant strain of *Zygosaccharomyces rouxii* to degrade iprodione at a concentration of 1 mg/L was reported (Zadra *et al*., 2006). The yeast *Z. rouxii* was also noted for its ability to grow at high osmolarity (Zadra *et al*., 2006), but its biodegradation of iprodione produced 3,5-DCA.

5.2 Biodegradation of iprodione by iprodione-degrading bacteria

5.2.1 Iprodione biodegradation kinetics

Batch experiments were conducted to examine the effect of concentration of iprodione on biodegradation efficiency of *P. aeruginosa* I4. Iprodione was detected at

a retention time of 5.2 min at a wavelength of 240 nm of HPLC analysis tested with seven different substrate concentrations.

The biodegradation process can be quantified *via* different test measurements such as biological oxygen demand (BOD), CO_2 production, primary and ultimate half-lives, kinetic constants, formation of reactive intermediates and stable products, etc (Dimitrov *et al*., 2007). Conceptually this implies expressing as the relationship between degradation rate and substrate concentration. The kinetics of iprodione degradation by *P.* I4 analyzed by a, nonlinear regression of the data fitted the Michaelis-Menten model (Schmidt *et al*., 1985) and resulted in a half-saturation constant of 38.41 ± 13.61 μM and K_{max} of 0.037 $\mu M.h^{-1}.mg\ prot^{-1}$. The profile also suggests that substrate inhibition was occurred.

Example of the substrate inhibited phenomena using pure culture isolates which provide a good overview of the range of degradation kinetics behavior are as follow. A pure bacterial culture of *Stenotrophomonas maltophilia*, capable of using aniline as a sole carbon source, was isolated(Zissi *et al*., 1999). Kinetic experiments were conducted to develop a mathematical model that describes accurately the growth and utilization rates of the microorganism on the aniline and an alternate carbon source (glucose) individually and on their mixture. The proposed model was found capable of describing accurately cellular growth as well as aniline and glucose biodegradation.(Zissi et al., 1999).

Degradation of the fungicide thiophanate-methyl (TM) by *Enterobacter* sp. TDS-1 and *Bacillus* sp.TDS-2 isolated from sandy soil. The final rate of TM degradation was reduced by 21% and 27% for strains TDS-1 and TDS-2. This additional carbon source changed the TM degradation kinetics. This effect was linked to substrate competition and/or a strong decrease of medium pH. Isolates degraded TM (100 mg kg^{-1}) in soil with rate constants of 0.186 and 0.210 day^{-1}, following first-order rate kinetics, and the time in which the initial TM concentration was reduced by 50% (DT50) in soils inoculated with strains TDS-1 and TDS-2 were 6.3 and 5.1 days, respectively. Analysis of TM degradation products in soil showed that the tested strains may have the potential to transform carbendazim to 2-aminobenzimidazol, and may be useful for a bioremediation of carbendazim -polluted soil (Zissi *et al*., 1999).

5.2.2 Determination of enzymes involving in iprodione degradation

To further study iprodione degradation pathway in *P. aeruginosa* I4, the test was conducted to see if it could grow on 3,5-DCA, which is an intermediate in the proposed iprodione degradation pathway. In addition, Iprodione degradation pathway was investigated through enzyme determination from *P. aeruginosa* I4 grown in iprodione, 3,5-DCA in comparison to those of controls. The following enzyme activities: catechol 1,2-dioxygenase, chlorocatechol 2,3-dioxygenase, catechol 2,3-

dioxygenase and chlorocatechol 2,3-dioxygenase were determined from cell-free extract of *P. aeruginosa* I4 grown on various substrates.

In the enzymatic study of *P. aeruginosa* I4, the activities of the enzyme of *ortho*-cleavage pathway was higher than that of the modified *ortho*-cleavage pathway and the *meta*-cleavage pathway.

In previous study, *Pseudomonas aeruginosa* 3mT exhibited the ability to degrade high concentrations of 3-chlorobenzoate (3-CBA) and 4-chlorobenzoate (4-CBA). Fairly high titres of chlorocatechol 1,2-dioxygenase activity were obtained in extracts of cells grown on 3-CBA and 4-CBA. 3-CBA, and more than 99% of 4-CBA were degraded through the formation of the respective chlorocatechol, *via* a modified *ortho*-pathway.

5.3 Effect of additional co-substrate of iprodione degradation

The biodegradation conditions of iprodione and 3,5-DCA by P. *aeruginosa* I4 were optimized in order to increase the degradation rate and/or improve cell growth. The conditions supplementation with 5 mM succinic acid supported the cell growth and cell activity.

5.4 Range of iprodione and 3,5-DCA structural related compounds

Dichloroanilines (DCAs) are widely used as industrial chemicals in commercial syntheses. 3,4-DCA and 3,5-DCA are particularly noxious, harmful to human, followed by that of 2,3-DCA, 2,4-DCA and 2,5-DCA (Claver *et al*., 2006). In this study, the ability of P. I4 to degrade substrate range of chloroanilines and another fungicide with structural related to that of iprodione (i.e. procymidone and vinclozolin) was examined.

Bacteria strain *P. aeruginosa* I4 could degrade 2-CA, 2,3-DCA, 2,4-DCA, 2, 3,4-DCA, 3,5-DCA, procymidone and vinclozoline, in MM supplemented with succinic acid, but not to some extents other isomers (3-CA, 4-CA, 2,3,4-TCA, 2,3,5-TCA).

CHAPTER VI

CONCLUSIONS

In conclusion, P. I4 was isolated from agricultural soil by enrichment method as an iprodione-degrading bacterium and identified by using 16s rDNA gene sequence alignment.

P. I4 has ability to completely degrade both iprodione and 3,5-DCA ; therefore, there is no toxic product left as a threat to the environment. The degradation pathway of this isolated strain was suggested by 1) the transient accumulation of 3,5-DCA, a known degradation product of iprodione, which appeared concomitantly with the disappearance of these fungicides (Walker, 1987); 2) the determination of enzyme involving in iprodione degradation. The presence of high activities of catechol 1,2-dioxigenase in the cell-free extract of *P. aeruginosa* I4 grown on iprodione confirmed that iprodione was degraded *via ortho*-cleavage pathway in P. I4.

In addition to iprodione and 3,5-DCA, P. I4 was able to degrade DCA isomers and another dicarboximide fungicide as follow: 2-CA, 2,3-DCA, 2,4-DCA, 3,4-DCA, vinclozolin and procymidone.

Complete degradation of the fungicide iprodione by *P. aeruginosa* I4 could be potentially useful in the bioremediation of soils. However, the modification of iprodione fate and toxicity, which may be caused by soil and 3,5-DCA contaminated interactions and other abiotic factors in environment should be investigated further. In addition, the knowledge about the biochemical pathways and respective genes involved in biodegradation should be embraced.

CHAPTER VII

SUGGESTIONS AND FUTURE WORKS

The research results described that the isolated *P. aeruginosa* I4 strain possesses ability for iprodione degradation.

This study was based on a laboratory scale investigation. Further studies should be to determine the optimum environmental conditions favorable for their application in bioremediation.

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APPENDICES

APPENDIX A

Culture Medium

A1 The mineral medium (MM)

The mineral medium used for screening, isolation, cultivation and degradation test was comprised of mineral solution and trace elements as follow: (Dejonghe *et al*., 2003).

I. Mineral solution (l^{-1})

Na_2HPO_4	1.4196 g
KH_2PO_4	1.3609 g
$MgSO_4.7H_2O$	0.0985 g
$CaCl_2.2H_2O$	0.0059 g

II. Trace element (100 ml^{-1})

H3BO4	0.116 g
$FeSO_4.7H_2O$	0.278 g
$ZnSO_4.7H_2O$	0.115 g
$MnSO_4.H_2O$	0.169 g

$CuSO_4.5H_2O$	0.038 g
$CoCl_2.6H_2O$	0.024 g
MoO_3	0.01 g

The trace element solution was prepared as stock solution by dissolving compounds in 100 ml distilled water, autoclaved at 121 °C for 15 minutes, The solution was prepared separately from the mineral solution and was used at 0.1% (v/v) in the mineral solution.

A2 Luria bertani medium (LB)

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

LB medium was dissolved in 1 liter of distilled water, adjusted pH to 7 and autoclaved at 121 °C for 15 minutes.

APPENDIX C

Sequence result

The following sequence data is the sequence of PCR product of 16S rDNA gene sequence of isolated strain I4 with forward primer (63f) and reverse primer (1387r) as C1 and C2. Full-length sequence is partially reliable C3 . For nucleotide blast, each sequence from f-and r-primer are done separately (C4, C5). However, both search results are identified as *P. aeruginosa.*(> 99%) and similar to *P. aeruginosa* PAO1 type strain 100 % identities (C6)

C1 63f

>110810-50_I24_I4-f-63f.ab1 876

NNNNAANNGGNNCTTCGGGATGCCGGCGA*GCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGAT*
AACGTTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGA
GCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTC
ACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCA
GCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGCTAGTTAATACCTGGTT
GTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCG
*GAATTACTGGGCGTAAAGCGCGCGT*AGGTGGTTCAGCAAGTTGGAGGTGAAATCCCCGGGCTCAACCTGGGAACTGCC
TCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCATAGATATAGGA
AGGAACACCTGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTATCCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCT
AACGCGATAAGTCGACCGCCTGGGGAGTACGGTCGCAATGTTAAAACTCAAATGAATTGTACGGGGGCCCGCACTA
GCGGATGG

Reliable signal

C2 1387r

>110810-50_J24_I4-r-1387r.ab1 987

GNNCNNNCGNNNATTCTGATTCACGATTAC**TAGCGATTCCGACTTCACGCAGTCGAGTTGCAGACTGCGATCCG**
GACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTTTGTACCGACCATTGTAGCACG
TGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGT
CTCCTTAGAGTGCCCACCCGAGGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAA
CATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGCACCAATCCATCTCT
GGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGC
TTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTATCGCG
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CTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCACCCTCTACCGTACTCTAGCTCAGTAGTTTTGGAGGCA
GTTCCCAGGTTGAGCCCGGGGATTTCACCTCCAACTTGCTGAACCACCTACGCACGCTTTACGCCCAGGAAATCCGA
TTAACGCTTGCACCCTTCGTATTACCGCGGCTGCTGGCACGAAGATAAGCCTGGTGCTTATTCTTTTTGGTAACATCA
CNCCACAGGTATTAACTAGCTGTCCCTCCTCCCAAACTTTAAAGTGATGTGGAATCCGAGACCTGATTACTTCTCCCG
AGGGTGGCG

Reliable signal

Rev>110810-50_J24_I4-r-1387r

GCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGG
TGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGG
ATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC
CCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACC
GGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTAC
AAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGAC
TGCGTGAAGTCGGAATCGCTA

C3 Assembled sequence

GCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCA
TACGTCCTACGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTG
GGGTAAAGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAG
AAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGCTAGTTAATACCTGGTTGTTTTGACGTTACCAAC
AGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCG
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GAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGT
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CGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGA
GCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGG
ATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTT
AAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCG
GTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCT
ACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGAT
CGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTA

C 4 63f-blast

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Pseudomonas sp. CG2(2007) 16S ribosomal RNA gene, partial sequence	889	889	100%	0	10%	EU100399.1
Pseudomonas mendocina strain B6-1 16S ribosomal RNA gene, partial sequence	889	889	100%	0	100%	EF208965.1
Pseudomonas sp. CG2(2006) 16S ribosomal RNA gene, partial sequence	889	889	100%	0	100%	EF119841.1
Pseudomonas sp. HR 26 16S ribosomal RNA gene, partial sequence	889	889	100%	0	100%	AY032726.1
Pseudomonas resinovorans strain mol11 16S ribosomal RNA gene, partial sequence	883	883	100%	0	99%	HM031472.1
Pseudomonas mendocina strain PCP37 16S ribosomal RNA gene, partial sequence	878	878	100%	0	99%	HM439420.1
Pseudomonas sp. DRJS2 16S ribosomal RNA gene, partial sequence	878	878	100%	0	99%	FJ032311.1
Pseudomonas sp. LC563 16S ribosomal RNA gene, partial sequence	872	872	100%	0	99%	JQ014614.1
Pseudomonas sp. LC254 16S ribosomal RNA gene, partial sequence	872	872	100%	0	99%	JQ014476.1
Pseudomonas sp. HI-G1 16S ribosomal RNA gene, partial sequence	872	872	100%	0	99%	DQ205300.1
Pseudomonas sp. MI-o1 16S ribosomal RNA gene, partial sequence	872	872	100%	0	99%	DQ180954.1
Pseudomonas sp. INBio2893C 16S ribosomal RNA gene, partial sequence	867	867	100%	0	99%	HM771055.1
Pseudomonas sp. 1.4.4 16S ribosomal RNA gene, partial sequence	861	861	98%	0	99%	HM192783.1
Pseudomonas sp. HRB-9 16S ribosomal RNA gene, partial sequence	861	861	100%	0	99%	DQ973489.1
Pseudomonas resinovorans strain LAM 9 16S ribosomal RNA gene, partial sequence	861	861	100%	0	99%	EU019983.1
Pseudomonas sp. WW9 16S ribosomal RNA gene, partial sequence	861	861	100%	0	99%	EF433551.1
Pseudomonas sp. J11 gene for 16S rRNA, partial sequence	859	859	100%	0	99%	AB097174.1

C5 1387r-blast

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Pseudomonas sp. BRO5A1 16S ribosomal RNA gene, partial sequence	887	887	100%	0	100%	KC715841.1
Pseudomonas sp. ARLB49 16S ribosomal RNA gene, partial sequence	887	887	100%	0	100%	KC715839.1
Pseudomonas sp. ARLB45B 16S ribosomal RNA gene, partial sequence	887	887	100%	0	100%	KC715837.1
Pseudomonas aeruginosa B136-33, complete genome	887	3544	100%	0	100%	CP004061.1
Pseudomonas aeruginosa strain Z992 16S ribosomal RNA gene, partial sequence	887	887	100%	0	100%	KC212091.1
Pseudomonas aeruginosa strain Z91 16S ribosomal RNA gene, partial sequence	887	887	100%	0	100%	KC212090.1
Pseudomonas aeruginosa strain Z981 16S ribosomal RNA gene, partial sequence	887	887	100%	0	100%	KC212089.1
Pseudomonas aeruginosa strain 34SS 16S ribosomal RNA gene, partial sequence	887	887	100%	0	100%	KC433742.1
Pseudomonas aeruginosa strain 34S 16S ribosomal RNA gene, partial sequence	887	887	100%	0	100%	KC433741.1
Pseudomonas aeruginosa strain P3 16S ribosomal RNA gene, partial sequence	887	887	100%	0	100%	KC417305.1
Pseudomonas aeruginosa strain P2 16S ribosomal RNA gene, partial sequence	887	887	100%	0	100%	KC417304.1
Pseudomonas aeruginosa strain P1 16S ribosomal RNA gene, partial sequence	887	887	100%	0	100%	KC417303.1
Pseudomonas aeruginosa strain R21 16S ribosomal RNA gene, partial sequence	887	887	100%	0	100%	KC188783.1
Pseudomonas aeruginosa PAO1 strain PAO1 16S ribosomal RNA, complete sequence	887	887	100%	0	100%	NR_074828.1
Uncultured bacterium clone UUF112 16S ribosomal RNA gene, partial sequence	887	887	100%	0	100%	KC245267.1

C 6

Pseudomonas aeruginosa PAO1 strain PAO1 16S ribosomal RNA, complete sequence

Sequence ID: ref|NR_074828.1| **Length:** 1536 **Number of Matches:** 1

Range 1: 861 to 1340 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
887 bits(480)	0.0	480/480(100%)	0/480(0%)	Plus/Minus

```
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Sbjct  1340  TAGCGATTCCGACTTCACGCAGTCGAGTTGCAGACTGCGATCCGGACTACGATCGGTTTT  1281

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Sbjct  1280  ATGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTTTGTACCGACCATTGTAGCACGTGT  1221

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Query  301   TGTGTCTGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCAGCATGTCAAGGCC  360
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Sbjct  980   AGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCC  921

Query  421   CCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTATCGC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  920   CCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTATCGC  861
```

APPENDIX D

Calculation of iprodione residual

Determination iprodione remaining and degradation

Remaining percentage of iprodione was calculated from HPLC peak height when compared to that of standard curved. The following equations were used to calculate the percentage of iprodione degradation. For example;

A. Remaining percentage of iprodione after 24 hr of incubation was

$$100 \times \frac{\text{Peak height reported at 24 hr}}{\text{Peak height reported at 0 hr}}$$

B. Degradation percentage of iprodione after 24 hr of incubation was

100- Remaining percentage of iprodione

APPENDIX E

Retention times of iprodione and 3,5-DCA

HPLC peak of 200 µM iprodione and 3,5-DCA, the compound was analyzed by UV detector at 240 nm, the mobile phase was acetonitrile: water (70:30 v/v). Retention time of iprodione and 3,5-DCA were 5.3 and 4.5 min.

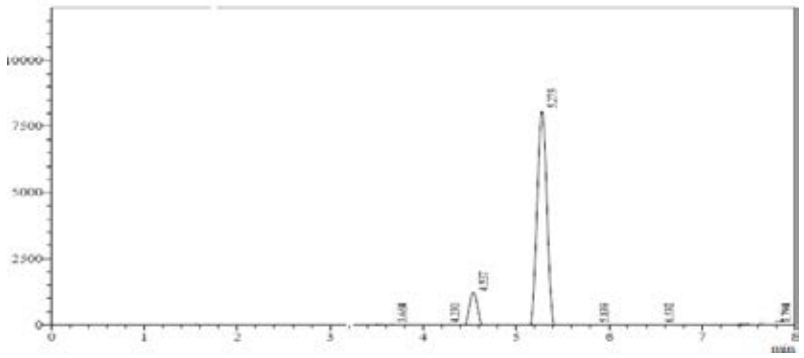


Figure E1 HPLC chromatogram of 200 µM iprodione

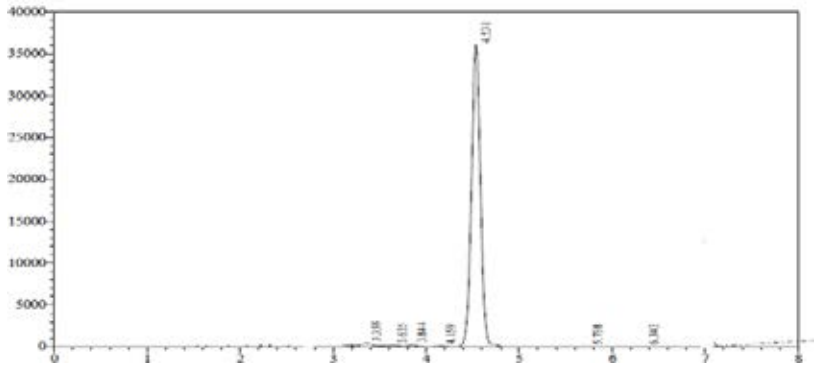
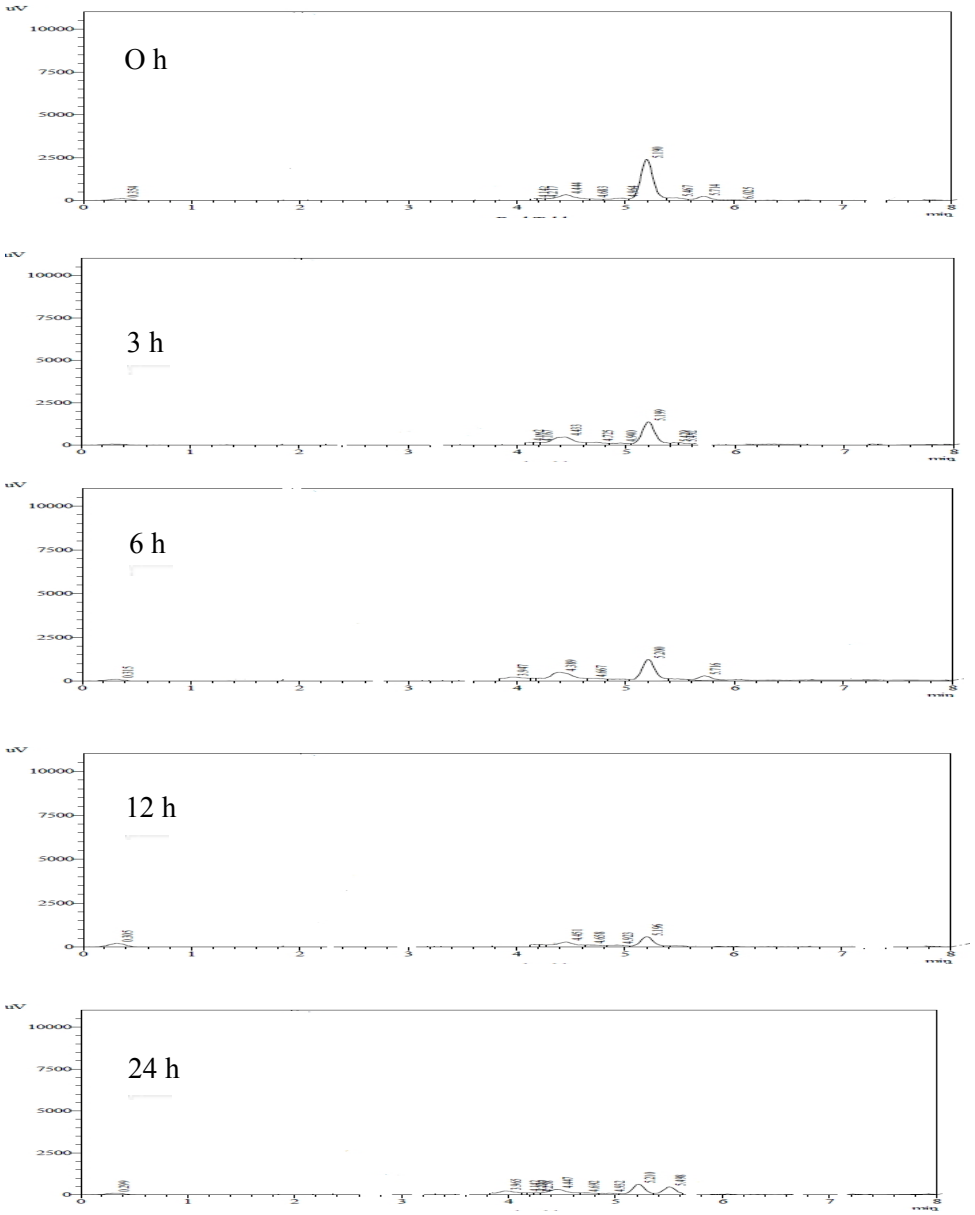


Figure E2 HPLC chromatogram of 200 µM 3,5-DCA

APPENDIX F

HPLC peak of iprodione degradation

HPLC peak of 40 μM iprodione degradation test of *P. aeruginosa* I4, retention time of iprodione is 5.2 min determined under the specific HPLC condition.



BIOGRAPHY

Miss Aornsiri Polla born in Loei province, Thailand. She graduated with the Bachelor Degree of Science in Environment from Suandusit Rajabhat university. She study for Master degree of Environment Science in from Chulalongkorn University until 2012.