CARBOFURAN DEGRADATION BY *BACILLUS* SP. ISOLATED FROM SOILS

INTRODUCTION

Carbofuran (2, 3-dihydro-2, 2-dimethyl-7-benzofuranyl methylcarbamate) is a broad spectrum systemic soil-applied insecticide, nematicide and acaricide that widely used to control pests that harm the agricultural products such as rice, corn, sugarcane, tobacco etc. (Office of Agriculture Regulation, 1994). Until 2000 to 2003, Thailand imported carbofuran in amount of 2,960, 5,270, 3,634 and 1,121 tons, respectively (Office of Agriculture Regulation, 2000-2003). Due to it is widely used to control pests, especially when it uses to control nematode that harms ginger and watermelon by mixing with bottom soil of cultivating hole (Thaveechai *et al*, 1999; Wongsuwan, 1992), it is possible that carbofuran may accumulate and long-term persist in the soils which cause many problems in the environment.

Carbofuran is a potent inhibitor of enzyme cholinesterase activity and is highly toxic to mammals. However, carbosulfan (2, 3-dihydro-2, 2-dimethyl-7-benzofuranyl [(dibutylamino)thio] methylcarbamate) can also be degraded to carbofuran that is more toxic than carbosulfan. And when carbofuran is heavily applied to soil for many years, carbofuran will extremely accumulate in soils. Due to carbofuran is a fairly mobile pesticide, it can be potentially hazardous when runoff from areas of application and results the harmful effects to human when carbofuran contaminates in food as Sakong and Meepansakul (2001) reported that in 4 amphurs, Sakon Nakhon province, was determined 192.35 µg/kg carbofuran in 8.33% of all tomato samples. Besides, carbofuran also accumulates the toxicant in living organisms through food chain, resulting in bio-accumulation.

Carbofuran can be degraded by chemical or physical method and also by microbial activity. The metabolism of microorganisms uses carbofuran as carbon, nitrogen and energy sources. Bacteria which had capability in degrading several pesticides are in group of *Bacillus* sp.

(Mohapatra and Awasthi, 1997; Desaint *et al*, 2000; Karpouzas *et al*, 2000; Bhadbhade *et al*, 2002), *Rhodococcus* sp. (Parekh *et al*, 1994), *Arthrobacter* sp. (Ramanand *et al*, 1988; Bhadbhade *et al*, 2002), *Achromobacter* sp. (Tomasek and Karns, 1989), *Pseudomonas* sp. (Chaudhry and Ali, 1988; Chapalmadugu and Chaudhry, 1993; Mohapatra and Awasthi, 1997), *Rhizobium* sp. (Hashimoto *et al*, 2002), *Sphingomonas* sp. (Feng *et al*, 1997) and *Flavobacterium* sp. (Chaudhry and Ali, 1988). So the carbofuran degradation by soil bacteria isolated from treated soils has been investigated.

OBJECTIVES

1. To isolate and identify carbofuran degrading *Bacillus* sp. by conventional methods.
2. To study the abilities of isolates in degrading carbofuran in minimal medium supplemented with carbofuran at 3, 5 and 7 days by Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC).
3. To study the effect of pH, light and inoculum size on carbofuran degradation in minimal medium .
4. To determine the metabolites produced during the carbofuran degradation process at 7 days.

LITERATURE REVIEWS

Carbofuran

Carbofuran is a broad spectrum pesticide that kills insect, mites and nematode on contact or after ingestion (Cooperative extension offices, 1993). Its IUPAC name is 2, 3-dihydro-2, 2-dimethyl-7-benzofuranyl *N*-methylcarbamate (Bohmont, 2000; Khan, 1980; Milne, 1995). The other names or trade names are Furadan (Bohmont, 2000; Khan, 1980; Matsumura, 1980), Camedan, Camira, NIA 10242 (Matsumura, 1980), ENT 27164 (Matsumura, 1980) and Yaltox (Milne, 1995) etc. Carbofuran is odorless white crystalline solid which molecular weight is 221.25 (Anonymous, 1996). Its chemical formula is $C_{12}H_{15}NO_3$. Figure 1 shows the molecular structure of carbofuran (Bohmont, 2000; Matsumura, 1980)

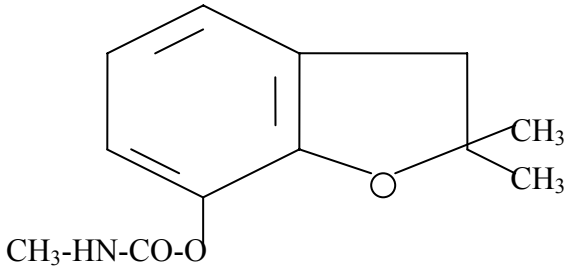


Figure 1 The molecular structure of carbofuran.

The solubility of carbofuran is 320 ppm in water, 15 g/100 g in acetone and less than 1 g/100 g in xylene at 25°C (Anonymous, 2006a). Its melting point is 153-154°C (Anonymous, 2006a). Carbofuran is used against soil and foliar pests of field, fruit, vegetable and forage crops. It is the highly toxic pesticide "Ib" (Matsumura, 1980) to inhalation and ingestion in human but moderately toxic by dermal absorption (Cooperative extension offices, 1993). Carbofuran inhibit enzyme cholinesterase activity. The basis of toxic action is generally associated with the ability to inhibit cholinesterase in the central and peripheral nerve systems where it plays an important role in the transmission of nerve impulses (Biros, 1970). Risks from exposure are especially high for

persons with asthma, diabetes, cardiovascular disease, mechanical obstruction of the gastrointestinal or urogastal tracts. The symptoms when expose to carbofuran are drowsiness, lethargy, fatigue, mental confusion, inability to concentrate, headache, pressure in head, generalized weakness, coma with absence of reflexes, tremors, Cheyne-stakes respiration, dyspnea, convulsions, depression of respiratory centers, lack of control of vomiting, excessive salivation, diarrhea and sweating (Cooperative extension offices, 1993; Krieger, 2001).

Persistence of Pesticides in Soil

The persistence of pesticide in many places is different from each other, depends on many factors. In 1985, Meher *et al*. indicated that half life of carbofuran in soil was ranging from 46 to 117 days, while Siriphontangmun (1991) reported that its half life in soil is from 30 to 60 days. But in 1996, Anonymous showed its half life is 30 to 120 days. No matter what the soils are, carbofuran can persist in soil up to 120 days. Edwards (1973) reported that there are many factors affected the persistence of pesticide in soil as follow

1. Chemical nature of pesticide These include the chemical stability, volatility, solubility, concentration and formulation

2. Type of soil In general, insecticides are retained longer in heavier soils and those with much organic matter, also insecticides are much less toxic to insects in heavier and organic soils. The soil type greatly influences the adsorption of insecticides.

3. Organic matter content The more organic matter is in a soil, the longer an insecticide persists in it.

4. Clay contentA factor that is almost as important as the organic matter is the amount of colloidal material a soil contains. Soils which contain much clay have a much larger internal surface area than sandy soils and so might be expected to retain insecticides longer because there is more area for adsorption.

5. Soil acidity The hydrogen ion concentration may influence the breakdown of insecticides in soil in several ways. Due to that it can affect the stability of clay minerals, the ion exchange capacity, or the rate at which both chemical and bacterial decomposition occurs.

6. Mineral ion content Minerals are important in influencing breakdown of insecticides

in soil. The amounts and kinds of minerals in a soil influence both its type and structure and how long insecticides persist in it as adsorption occurs very rapidly in soils with much iron.

7. TemperatureInsecticides are lost from soil mainly by chemical degradation, bacterial decomposition and volatilization, and all these processes are influenced by temperature, so that at low temperatures these processes slow down and little insecticide is lost. Temperature also influences the adsorption of insecticides in soils because sorption tends to be exothermic, so that increased temperatures decrease adsorption and release insecticides. Furthermore, the solubilities of insecticides usually depend on temperature, so that more insecticide becomes dissolved in the soil moisture as temperature increase.

8. Soil moisture Water can compete for adsorption sites with insecticides because it is a very polar molecule, strongly adsorbed by the soil colloids, and in drier soils there are fewer water molecules to compete with the insecticide molecules for adsorption sites. For most insecticides the adsorption seems to be reversible. Soil humidity can influence the persistence of insecticides in three ways. Firstly, it can influence the adsorption of an insecticide, secondly it can affect the rate at which it diffuses into soil, and finally it can affect the availability of the adsorbed toxicant. The rate of adsorption from the particulate state decreases as humidity increases.

9. Effect of crops Open or fallow soil is exposed to much more wind, sun, and rain than the soil under a growing crop suggest that more remained in the shaded than in the unshaded soil. Another factor is that insecticides are taken up from soil into crops which contributes to their disappearances from soils.

10. Cultivation Insecticides are usually cultivated into the soil as spray drift or run-off from foliage may remain undisturbed on the surface for a long time. The persistence of insecticides differs greatly with the degree of cultivation. When the toxicant is mixed thoroughly into the soil, its persistence will be longer than when not applied to soil, although continued cultivation tends to decrease the persistence.

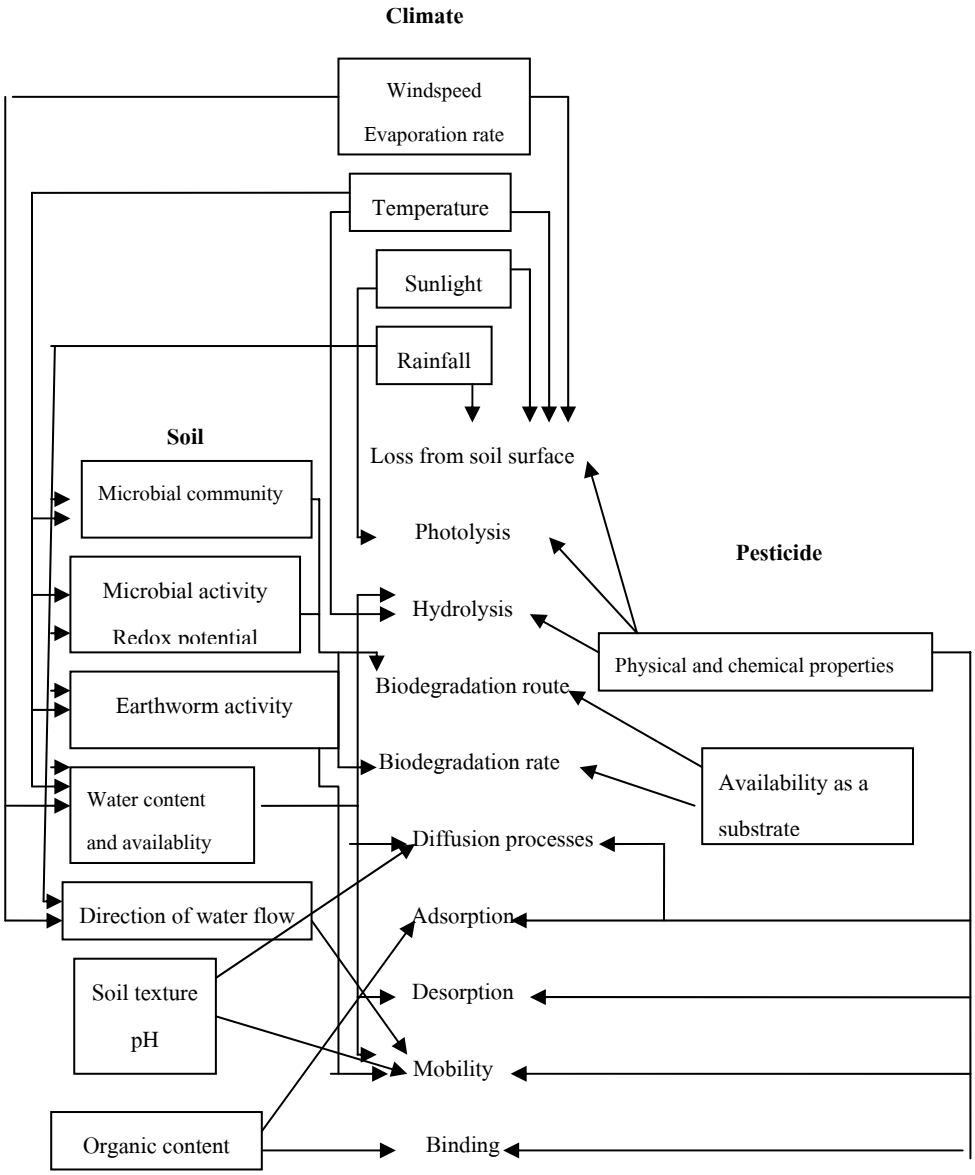


Figure 2 The interactions influencing the fate of the pesticide in soil

Source: Modified from Hutson and Roberts (1994)

Fate of Carbofuran in Soil

Because of its high water solubility and low adsorption coefficient, carbofuran is relatively mobile in soil and in surface runoff. Consequently carbofuran has the potential to contaminate lakes, streams and groundwater. Carbofuran degradation is much quicker in alkaline soils than in acidic soils. While hydrolysis is the major route of degradation in alkaline soils, the slower degradation in acidic and neutral soils is dominated by microbial and chemical mechanisms. Microbial degradation is an important route of carbofuran degradation in neutral soils. Volatilization is not as important as microbial degradation. Photodegradation is generally considered a minor route of carbofuran degradation.

Carbofuran is soluble in water and moderately persistent in soil. Its half-life is 30-120 days. In soil, carbofuran is degraded by chemical hydrolysis and microbial processes. Hydrolysis occurs more rapidly in alkaline soils. Carbofuran breaks down in sunlight. Carbofuran has a high potential for groundwater contamination. Carbofuran is mobile to very mobile in sandy loam, silt clay and silt loam soils; moderately mobile in silt clay loam soils and only slightly mobile in muck soils. (Anonymous, 1996)

Pesticide-Degrading Bacteria and Their Metabolism

There have been previously reported about the soil bacteria that be able to degrade carbofuran such as *Bacillus* sp. (Desaint *et al*., 2000; Karpouzas *et al*., 2000; Mohapatra and Awasthi, 1997), *Pseudomonas* sp. (Chaudhry and Ali, 1988; Chaudhry *et al*., 2002; Desaint *et al*., 2000; Karpouzas *et al*., 2000; Mohapatra and Awasthi, 1997; Parekh *et al*., 1994), *Achromobacter* sp. (Tomasek and Karns, 1989), *Sphingomonas* sp. (Feng *et al*., 1997), *Arthrobacter* sp. (Ramanand *et al*., 1988), *Flavobacterium* sp. (Chaudhry and Ali, 1988; Karpouzas *et al*., 2000)

Typically, pesticide-degrading bacteria hydrolysed or oxidized of the parent pesticide molecules, but frequently do not necessarily result in detoxification (in fact, metabolites are occasionally more toxic) and frequently do not yield metabolites which are inherently biodegradable. In most cases, the bacteria produced a hydrolase enzyme that cleaved the *N*-methylcarbamate linkage of the pesticide resulting in the complete loss of pesticide activity (Shelton and Karns, 1998). Figure 3 shows some microbial reaction that effect *N*-methylcarbamate.

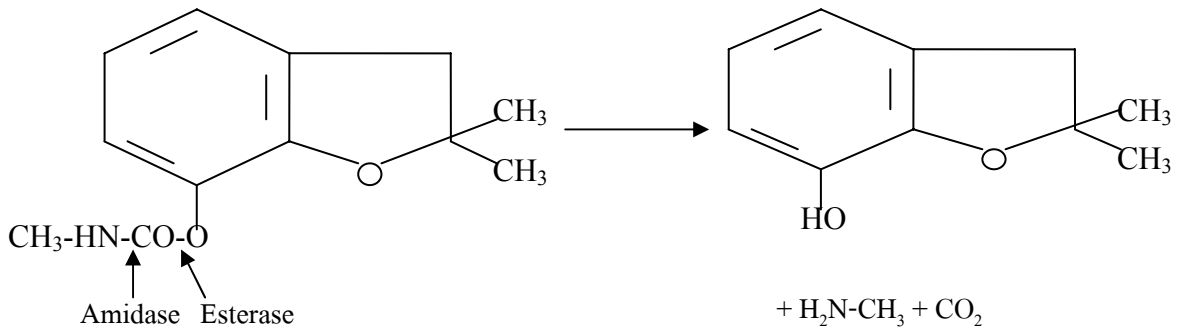


Figure 3 Some microbial reaction that effect *N*-methylcarbamate.
Source: Shelton and Karns (1998)

There are frequently several different biochemical mechanisms for the degradation of organic molecules. The methylamine produced as a result of this hydrolysis is used by many of

the organisms as a source the nitrogen for growth but some of the organisms use as a carbon source. A few of the organisms mineralize the phenolic metabolites as carbon and energy sources. In 1988, Chaudry and Ali reported about one culture appeared to attack carbofuran by oxidizing the phenolic portion of the molecule without hydrolyzing the carbamate linkage. Chaudhry et al., 2002 reported that an inducible oxidative enzyme, hydroxylase, meadiated the conversion of carbofuran to 4-hydroxycarbofuran and a constitutively synthesized enzyme hydrolase transformed carbofuran to 7-phenol.

Microorganisms in soil are known to degrade the carbamate pesticides via hydrolysis and oxidation. The rate and patterns of degradation were different in each soils (Parekh *et al*., 1994). Microbial metabolites of carbofuran are 7-phenol (2, 3-dihydro-2, 2-dimethyl-7-hydroxybenzofuran), (Chaudhry and Ali, 1988; Chaudhry *et al*., 2002; Parekh *et al*., 1994; Ramanand *et al*., 1988; Topp *et al*., 1993), 3-hydroxycarbofuran (2, 3-dihydro-2, 2-dimethyl-3-hydroxybenzofuran-7-yl methylcarbamate) (Ramanand *et al*., 1988), 4-hydroxycarbofuran (2, 3-dihydro-2, 2-dimethyl-4-hydroxybenzofuran-7-yl methylcarbamate) (Chaudhry *et al*., 2002), 3-hydroxycarbofuran 7-phenol (2, 3-dihydro-2, 2-dimethyl-3, 7-dihydroxybenzofuran), 3-ketocarbofuran (2, 3-dihydro-2, 2-dimethyl-3-keto-benzofuran-7-yl methylcarbamate), 3-ketocarbofuran 7-phenol (2, 3-dihydro-2, 2-dimethyl-3-keto-7-hydroxybenzofuran), 5-hydroxycarbofuran (2, 3-dihydro-2, 2-dimethyl-5-hydroxybenzofuran-7-yl methylcarbamate), CO_2 (Chaudhry and Ali, 1988; Ramanand *et al*., 1988; Topp *et al*., 1993), methylamine (Chaudhry and Ali, 1988; Topp *et al*., 1993) and 2-hydroxy-3-(3-methylpropan-2-ol) phenol (Kim *et al*., 2004). All the possible metabolites (except CO_2) were shown on figure 4.

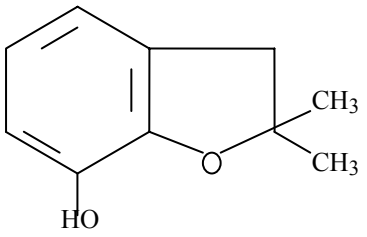
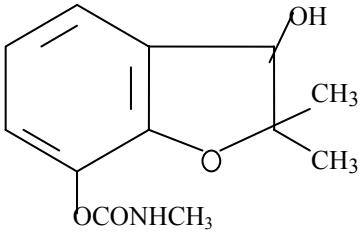
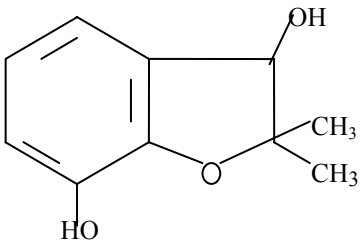
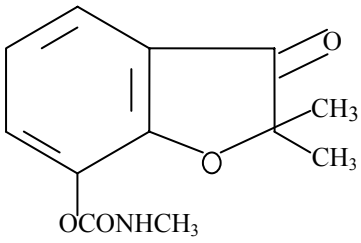
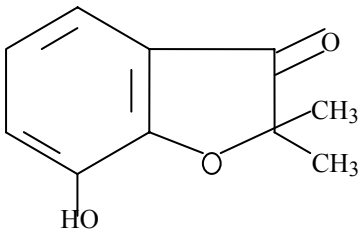
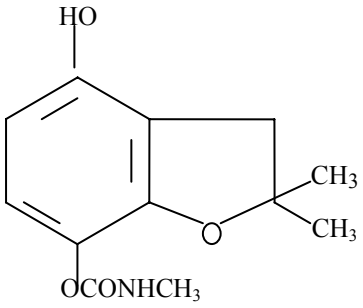
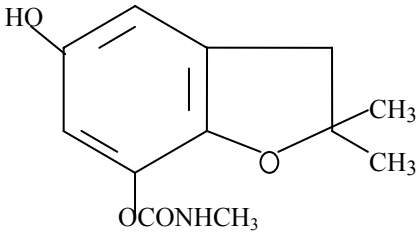
Structure of metabolite	Name of metabolite
	7-phenol (2, 3-dihydro-2, 2-dimethyl-7-hydroxybenzofuran)
	3-hydroxycarbofuran (2, 3-dihydro-2, 2-dimethyl-3-hydroxybenzofuran-7-yl methylcarbamate)
	3-hydroxycarbofuran 7-phenol (2, 3-dihydro-2, 2-dimethyl-3, 7-dihydroxybenzofuran)
	3-ketocarbofuran (2, 3-dihydro-2, 2-dimethyl-3-keto-benzofuran-7-yl methylcarbamate)
	3-ketocarbofuran 7-phenol (2, 3-dihydro-2, 2-dimethyl-3-keto-7-hydroxybenzofuran)

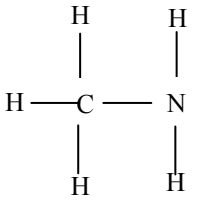
Figure 4 The structure of metabolites produced during the carbofuran degradation process.



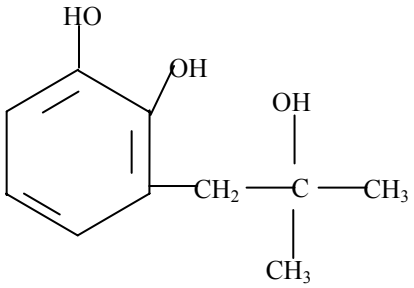
4-hydroxycarbofuran (2, 3-dihydro-2, 2-dimethyl-4-hydroxybenzofuran-7-yl methylcarbamate)



5-hydroxycarbofuran (2, 3-dihydro-2, 2-dimethyl-5-hydroxybenzofuran-7-yl methylcarbamate)



methylamine



2-hydroxy-3-(3-methylpropan-2-ol) phenol

Figure 4 (continued) The structure of metabolites produced during the carbofuran degradation process.

Genetic studies of *N*-methylcarbamate-degrading bacteria suggest a major role of plasmids in the spread of a unique genetic character throughout the microbial community

(Shelton and Karns, 1998). The *mcd* gene (methylcarbamate degradation) of a 14 kbp *Eco*RI DNA fragment encoding the carbofuran hydrolase in *Achromobacter* sp. strain WM111 was shown to be carried on a large plasmid (Tomasek and Karns, 1989). *Sphingomonas* sp. strain CF06 that is capable of using carbofuran as a sole carbon source and nitrogen contains 5 plasmids (Loss of the plasmids induced by growth at 42 °C resulted in the inability to grow on carbofuran as a sole carbon source). Of the 5 plasmids, 4 are rich in insertion sequence elements and contain large regions of overlap (Feng *et al*., 1997). Methylotrophic bacterium strain ER2 contained 2 plasmids (120 and130 kb) 120-kb plasmid was very similar to pDL11 plasmid of *Achromobacter* sp. strain WM111. This *mcd* gene was cloned and used as a probe to determine the origin and cellular location of the genes encoding *N*-methylcarbamate hydrolase in several other carbofuran-degrading bacteria.

MATERIALS AND METHODS

Materials

1. Two soil samples from Bangsapan, Chaikasem district, Prachuap Khiri Khan province.
2. High Performance Liquid Chromatography (HPLC): Shimadzu model 554
3. Chromatographic column stainless steel packed with C_{18} bounded silica gel
4. TLC plate silica gel F_{254} plate (Merck, Germany)
5. Shaker
6. pH meter
7. Methanol (HPLC grade)
8. Water (HPLC grade)
9. Dichloromethane (PR grade)
10. Ethyl acetate (PR grade)
11. Acetone (PR grade)
12. Hexane (PR grade)
13. Chloroform (PR grade)
14. Furadan 3%G
15. Standard Carbofuran 99% purity

Methods

1. Preparation of Soils and Properties Measurement

Soils from 2 agricultural field locations in Bangsapan, Chaikasem district, Prachuap Khiri Khan province, were randomly sampled for 1 kg. The samples were air-dried, sieved through 2 mm. mesh size and determined for pH, Electrical Conductivity (EC), water holding

capacity, total nitrogen, available phosphorus and exchangeable potassium content. All properties were carried on as described by Attanandana, and Chancharoensook, 1999.

2. Enrichment of Carbofuran Degrading Bacteria

An enrichment cultures were prepared in triplicate by adding normal recommendation rate (1X), 0.05 g and double recommendation rate (2X), 0.10 g of Furadan 3%G to 40 g soils at 15-day intervals. The treated soils were incubated for 3 months in closed and dark condition at room temperature together with untreated soils.

3. Enumeration of Total Number of Culturable Soil Microorganisms and Carbofuran Degrading Microorganisms from Carbofuran Treated Soils.

After 3 months incubation, soil samples treated as mention above were enumerated for total number of culturable soil bacteria on Plate Count Agar (PCA) and the population of carbofuran degrading microorganisms on minimal medium containing filtered-steriled carbofuran (MMC) (Appendix A) by spread plate method pararelled with untreated control.

4. Isolation and Characterization of Microorganisms from Carbofuran Treated Soils.

After 3 months enrichment, population of soil bacteria were determined on Plate Count Agar and minimal medium containing 20 ppm carbofuran and incubated at room temperature until the colonies were appeared. The results were checked and compared with untreated soils. The carbofuran degrading bacteria were isolated by method modified from Ramanand *et al*. (1988). Twenty ml minimal medium supplemented with 20 ppm steriled standard carbofuran in Erlenmeyer flask 100 ml were inoculated with 0.5 ml suspension of treated soils. When the media turned turbid, it might take about 4-5 days, the colonies were isolated by plating on minimal agar containing carbofuran and picking the different colonies as much as possible and subsequently streaked for the isolation of single colonies. Those isolates were characterized for morphological,

biochemical and physiological characteristics, described by Gordon (1989) and Sneath in Bergey's Manual of Systematic Bacteriology (1986). The working and preserved stock cultures were maintained on nutrient agar (NA). The selected isolates were used for further studies.

5. Biodegradation of Carbofuran in Minimal Medium

The isolates were cultured for carbofuran degradation by method modified from Chaudhry and Ali, 1988 and Ramanand *et al*., 1988, the isolates were grown on NA for 18-24 hours and then transferred to 50 ml minimal medium containing 1% glucose and 20 ppm carbofuran in Erlenmeyer flask 250 ml. When inoculum density reached to 10^6 cells/ml, cultured cells were harvested and washed with minimal medium without carbofuran for 3 times and then inoculated to 50 ml minimal medium containing 20 ppm as final concentration of carbofuran in Erlenmeyer flask 250 ml. The cultures were shaked at 150 rpm, room temperature with aluminum foil wrapping to avoid light exposure. The inoculated medium was taken to extract and analyze the carbofuran residues at every 3, 5 and 7 days, compared with control.

6. Extraction of Carbofuran Residues from Minimal Medium

Fifty milliliters cultured minimal medium were centrifuged at 3000 rpm for 30 minutes to separate cells from the supernatant. The supernatant were extracted with 15 ml dichloromethane 3 minutes for 3 times (Anonymous, 2000). The lower solvent fraction was collected and fluxed by N_2 stream until dryness. The residues were redissolved in 1 ml methanol and taken to analyze with TLC and HPLC method.

7. Residue Analysis with TLC and HPLC

Carbofuran residues were spotted on TLC aluminum plate siliga gel 60 F_{254} (Merck, Germany) in solvent systems i.e., ethyl acetate, acetone and mixture of hexane: ethyl acetate: chloroform (70:20:5) (Getz, 1980). The spotted plate was detected as black or dark purple band

under UV light at 254 nm and compared to R_f value of standard carbofuran. Limit of detection of this method was also determined.

For HPLC method, as described by Taumcharearn and Sriplakij, 1995, the carbofuran residues were analyzed using methanol: water (1:1) as mobile phase. The condition of analytical method was as follow: Stainless steel chromatographic column packed with C_{18} bounded silica gel, guard column; RP-18, 1 ml/min flow rate, 280 nm detector wavelength and 40 $^{\circ}$C column temperature. Limit of detection under this condition was also determined.

8. Effect of pH, Light and Inoculum Size on Degradation of Carbofuran

Some effective isolates were selected to study the effect of pH, light and inoculum size on degradation of carbofuran in minimal medium containing 20 ppm carbofuran. The isolates were cultured and extracted as described above but vary pH of medium as 5 and 8, with or without aluminum foil wrapping and inoculum size as 10^4 and 10^8 compared with control, the residues were extracted and analyzed by HPLC.

9. Determination the Metabolites Occurred During Carbofuran Degradation Process

The most effective isolate in degrading carbofuran was selected to study the metabolites produced during the degradation process by GC-MS methods. The isolates were cultured, and extracted as mentioned above. The samples were sent to analyze by GC-MS (Aqilent 6890) contributed by the Laboratory Center for Food and Agricultural Products (LCFA). The condition of GC-MS was HP-5-MS column, He as carrier gas, 1 ml/min flow rate.

RESULTS AND DISCUSSIONS

Properties of Soil Samples

Two soil samples from Bangsapan, Chaikasem district, Prachuap Khiri Khan province, were checked for pH, Electrical Conductivity (EC), maximum water holding capacity, total nitrogen, available phosphorus and exchangeable potassium content. The soil properties were shown in Table 1.

Table 1 The properties of 2 soil samples collected from Bangsapan, Chaikasem district, Prachaup Khiri Khan province.

Properties	Soil samples	
	A	B
Texture	Sandy loam	Sandy loam
pH	5.7	4.9
Electrical Conductivity (EC) (dS/m)	$5.5x10^{-4}$	$8.0 x10^{-4}$
Water holding capacity (%)	54.3	50.5
Total nitrogen (ppm)	150	135
Available phosphorus (ppm)	66.0	57.6
Exchangeable potassium (ppm)	201.4	181.7

Both soil A and B were sandy loam. Soil B was more acidic than soil A but soil A showed slightly higher acidic in nitrogen, phosphorus and potassium content than those in soil B.

Area of Bangsapan, Chaikasem district, Prachaup Khiri Khan province consisted of 13 soil profiles in various series indicating that soil types are medium fertile and suitable for vegetative production (Land Development Department, 2006). Land use information showed that agriculture production area in Bangsapan were abundant and had potential for cultivation.

However, some areas must be conserved for being the natural forest area and a small spring source.

Number of Culturable Microorganisms from Carbofuran Treated Soils

Soil samples were treated with Furadan 3%G at normal rate recommendation (1X) and double recommendation rate (2X) concentration. The population of culturable bacteria were enumerated on Plate Count Agar (PCA) whereas culturable carbofuran degrading microorganisms were enumerated on minimal medium containing filtered-steriled carbofuran (MMC) after 3 months of carbofuran treated into soils. Control treatments were carried out by means of no Furadan application.

Table 2 Effect of carbofuran concentration at 1X and 2X to the number of culturable microorganisms of soil A and B compared with different culture.

Soil	carbofuran treated condition	Number of colonies on PCA (cfu/g)	Number of colonies on MMC (cfu/g)
A	control	$1.1X10^{7}$	$1.6X10^{5}$
	1X	$5.3X10^{6}$	$3.6X10^{5}$
	2X	$3.3X10^{6}$	$1.8X10^{5}$
B	control	$1.5X10^{8}$	$1.8X10^{5}$
	1X	$6.1X10^{6}$	$1.5X10^{5}$
	2X	$4.4X10^{6}$	$1.2X10^{5}$

The population of bacteria on soil A on PCA at untreated condition was more than those at concentration 1X and 2X, it meant that carbofuran affected in reducing the number of bacteria in soils and this effect was correlated to the concentration of carbofuran too. The higher concentration, the more number of bacteria reduced, as shown in number of colonies at 1X was more than number of colonies at 2X.

As well as the number of bacteria of soil B on PCA, carbofuran reduced the number of bacteria at concentration 1X and 2X, when compared with untreated control, at $1.4x10^8$cfu/g and $1.5x10^8$cfu/g, respectively. It meant that, when compared to the number of bacteria of soil A in the same concentration, carbofuran affected in reducing the number of bacteria in soil B more than soil A. This decreasing effect was correlated to the concentration of carbofuran too; the number of bacteria at concentration 1X was more than number of bacteria at 2X at $1.7x10^6$cfu/g.

Bacteria that survived in the condition of carbofuran treatment in PCA were tolerated to carbofuran and adapted themselves to live with carbofuran, whether they utilized carbofuran as carbon or nitrogen source or not. But bacteria that survived in carbofuran and were able to grow on MMC were bacteria that capable in degrading carbofuran because MMC contained K_2HPO_4, $MgSO_4.7H_2O$, $CaSO_4$ and $FeSO_4.7H_2O$ as basal medium and carbofuran as sole carbon and nitrogen source. Thus only bacteria which able to utilize carbofuran as carbon and nitrogen source could grow on this medium.

Number of culturable carbofuran degrading bacteria of soil A on MMC at untreated condition was less than those at concentration 1X at $2.0x10^5$cfu/g and 2X at $2.0x10^4$cfu/g, it meant that carbofuran affected in inducing the number of carbofuran degrading bacteria in soils but in higher concentration, when 2X was compared with 1X, the number of carbofuran degrading bacteria were decreased at $1.8x10^5$ cfu/g. It could possibly mean that carbofuran could induce the number of bacteria when treated with low concentration but carbofuran became more toxic when used with higher concentration, the number of bacteria was reduced.

In the other hands, the number of culturable carbofuran degrading bacteria of soil B on MMC, carbofuran reduced the number of culturable carbofuran degrading bacteria at concentration 1X and 2X, when compared with untreated control, at $3.0x10^4$cfu/g and $6.0x10^4$cfu/g, respectively. And when compared the number of carbofuran degrading bacteria between 1X and 2X, the number of bacteria in 1X was more than those in 2X at $3x10^4$cfu/g. It could possibly mean that more carbofuran brought more toxic to the number of bacteria in soil B.

There were many reports about the effect of carbofuran on number of bacteria in soil such as in 2003, Das *et al*. reported that when carbofuran was conducted in clay loam alluvial soil at rate of 1.0 kg. a.i./ha, the population of bacteria in the rhizosphere soil was stimulated. In the other hands, Das and Mukherjee (1998) reported that the effect of carbofuran when applied in alluvial soil increased the total bacteria in soil. In control condition, the population of bacteria in soil was $1.1x10^7$cfu/g, while in carbofuran treatment condition, the number of bacteria was $1.3x10^7$cfu/g. As well as in 2000, Das and Mukherjee reported that when applied in sandy clay soil at 1.0 k g. a.i/ha, carbofuran generally induced the population of bacteria. In control condition, the population of bacteria in soil was $6.19x10^6$cfu/g, while in carbofuran treatment condition, the number of bacteria was $8.27x10^6$cfu/g.

From the mentioned reports, it was found that the effect of carbofuran was completely different when compared with the effect of carbofuran in this study. It might cause from the different incubation condition of different country.

Carbofuran affected in reducing total number of culturable soil microorganisms It was found that the higher concentration, the more number of bacteria reduced. Carbofuran affected in inducing the number of carbofuran degrading bacteria but in higher concentration carbofuran became more toxic caused the number of bacteria was reduced.

Isolation and Characterization of Carbofuran Degrading Microorganisms.

After 3 months, carbofuran degrading microorganisms were isolated from carbofuran treated soils on minimal agar supplemented with 20 µg/ml steriled standard carbofuran and streaked for single colonies. 66 isolates were obtained with 33 isolates from soil A and 33 isolates from soil B.

All 66 isolates were characterized by morphological, biochemical and physiological properties. In this study, 4 isolates which were gram positive cocci with positive results in

catalase test had not been studied. Sixty two isolates were gram positive, endospore forming and rod shape cells. All of these isolates gave positive results in catalase test and grouped as *Bacillus* sp. They were identified in species level by biochemical and physiological properties, described by Gordon (1989) and Sneath in Bergey's Manual of Systematic Bacteriology (1986). Sixty two isolates were grouped in *Bacillus* group IA, IB, II and III, respectively as following.

Table 3 Grouping of carbofuran degrading *Bacillus* sp. by morphological properties.

List of isolates	*Bacillus*			
	group IA	group IB	group II	group III
P001, P002, P006, P007, P008, P009, P010, P011, P012, P021, P022, P023, P024, P026, P027, P028, P029, P030, P031, P033, K002, K003, K004, K005, K006, K009, K011, K014, K015, K018, K024, K026, K031, K032, K033	√			
P013, P014, P015, P016, P017, P018, P019, P020, K008, K010, K013, K016, K019, K021, K025, K027, K029		√		
P003, P004, P005, K001, K007, K012, K017, K020, K023			√	
K022				√

Data from Table 3 showed that 35 isolates were belonged to group IA, 17 isolates in group IB, 9 isolates in group II and only 1 isolate in group III. Isolates of group IA showed $>1\mu m$ of their cell width as well as the presence of unstained globules and cylindrical or ellipsoidal endospore with non swollen sporangium. Whereas isolates of group IB showed $< 1\mu m$ of their cell width and the existence of cylindrical or ellipsoidal endospore with non swollen sporangium along with the absence of unstained globules. However, all 10 isolates with $<1\mu m$ of their cell

width and the presence of ellipsoidal endospore with swollen sporangium caused isolate P003, P004, P005, K001, K007, K012, K017, K020 and K023 belonged to group II. Consequently, isolate K022 with $<$1μm of their width and the existence of spherical endospore with swollen sporangium was belonged to group III.

Besides, the data also showed that the number of *Bacillus* group IA isolates was more than *Bacillus* group IB, II and III isolates. This might cause by group IA was the predominant in soil and their ability to grow faster than other groups. Thus, their fast grower colonies were isolated and collected more than the slow grower colonies. In the other hands, it also noticed that there were no gram negative cultures isolated from this study. It might cause by the condition of incubation before isolation, soils were dry off and affected with carbofuran, gram negative cultures might not able to tolerate and grow in this dry and toxic condition. When soil cultures were isolated and characterized, only gram positive cultures were obtained.

All 35 isolates belonged in group IA was classified into 2 groups as *B. megaterium* and *B. thuringiensis* according to their different biochemical and physiological characteristics. List of isolates belonged in each group were shown in Table 4.

Table 4 List of *Bacillus* isolates that classified in group IA identified as *B. megaterium* and *B. thuringiensis*.

Bacterial cultures	List of isolates
Bacillus megaterium	18 isolates P001, P002, P007, P008, P009, P011, P021, P022, P028, P029, K002, K003, K004, K005, K009, K014, K018, K024
Bacillus thuringiensis	17 isolates P006, P010, P012, P023, P024, P026, P027, P030, P031, P033, K006, K011, K015, K026, K031, K032, K033

Eighteen isolates of *B. megaterium* were isolated from soil A 10 isolates and soil B 8 isolates. Whereas, 17 isolates of *B. thuringiensis* were isolated from soil A 10 isolates and soil B 7 isolates. Morphological, physiological and biochemical properties that characterized these 2 species were shown as following. (Figure 5 and 6 and Table 9 and 10)

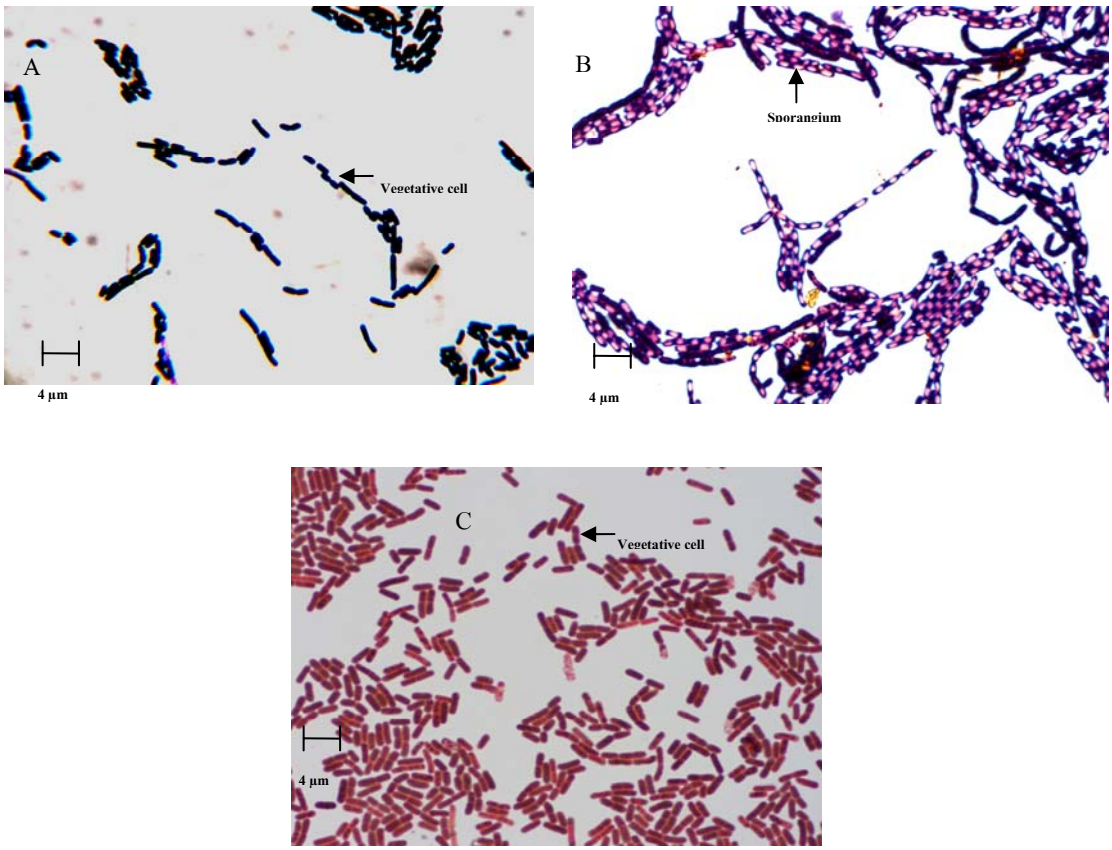


Figure 5 *B. megaterium* isolate

A. Isolate P007 grown on NA not more than 24 hours showed vegetative cells and cell arrangement.

B. Isolate P007 showed its sporangium after 24 hours.

C. Isolate P009 grown on glucose agar not more than 24 hours showed its poly-β-hydroxybutylate granules in protoplasm.

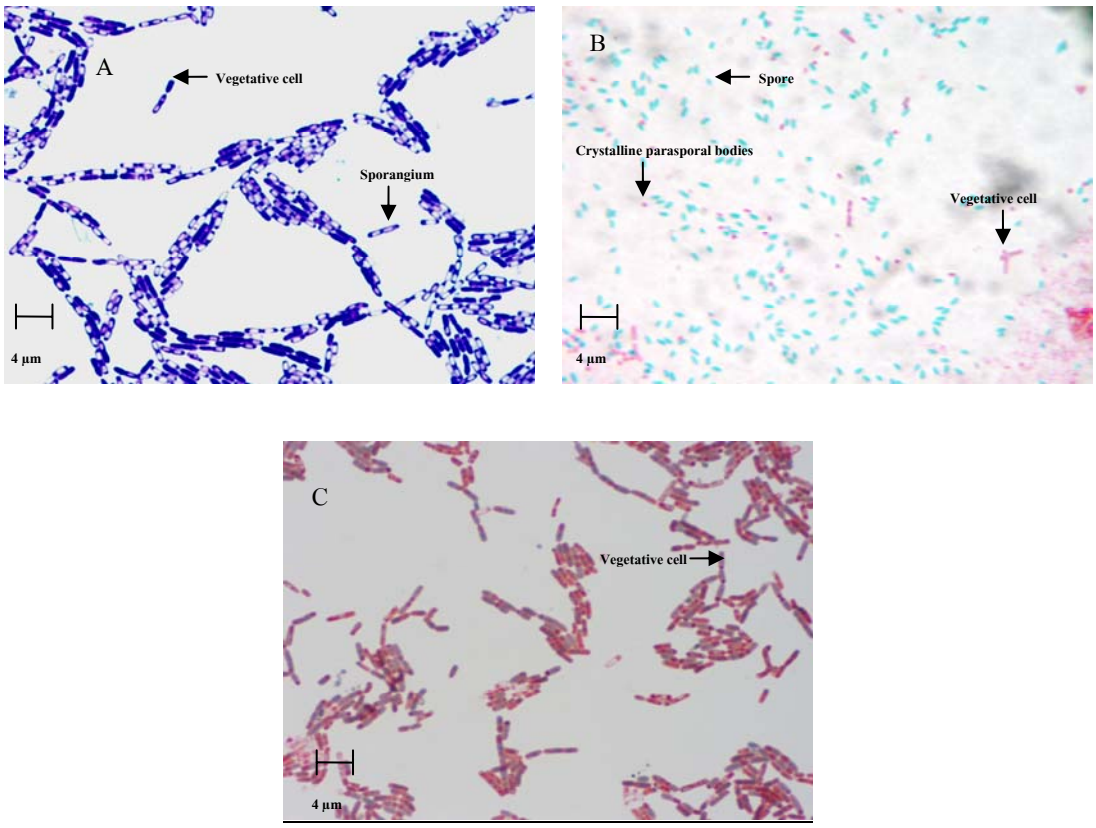


Figure 6 *B. thuringiensis* isolate

A. Isolate P023 grown on NA not more than 24 hours showed vegetative cells, sporangium and cell arrangement.

B. Isolate P010 showed vegetative cells, spores and crystalline parasporal bodies.

C. Isolate K011 grown on glucose agar not more than 24 hours showed its poly-β-hydroxybutylate granules in protoplasm.

Table 5 Biochemical and physiological characteristics of isolate P001, P002, P007, P008, P009, P011, P021, P028, P029, K002, K003, K004, K005, K009, K014, K018 and K024 compared with *B. megaterium*.

Properties	Subgroup [a]					*B. megaterium* [b]
	1	2	3	4	5	
Cell width (μm)	>1.0	≥1.0	>1.0	≥1.0	>1.0	1.2-1.5
Cell length (μm)	4.0-5.0	3.5-5.0	3.5	3.0-4.0	3.0-4.0	2.0-5.0
Unstained globules in protoplasm	+	+	+	+	+	+
Spore shape	cylindrical	cylindrical/ ellipsoidal	cylindrical	cylindrical/ ellipsoidal	cylindrical	ellipsoidal /round
Swollen sporangium	-	-	-	-	-	-
Spore position	central/ paracentral	central/ paracentral	central/ paracentral	central/ paracentral	central/ paracentral	central/ paracentral
Crystalline parasporal bodies	-	-	-	-	-	-
Anaerobic growth	-	-	-	-	-	-
VP	-	-	-	-	-	-
pH of VP broth	4.41-5.18	4.75-5.37	4.99	4.70-6.18	5.48/6.17	4.5-6.8
Reduction of NO_3 to NO_2	-	-	+	+	+	b
Acid production from						
D-glucose	+	+	+	+	+	+
L-arabinose	+	+	+	-	+ (exp K005)	a
D-xylose	-	+	+	+	+	a
D-mannitol	+	+	+	+	+	+
Hydrolysis of						
Casein	+	+	-	+	+	+
Gelatin	+	+	+	+	+	+
Starch	+	+	-	+	+	+
Lecithinase reaction	-	-	-	-	-	-
Utilization of						
Citrate	+	+	+	+	+	+
Propionate	-	-	-	-	-	ND
Growth in						
5% NaCl	+	+	+	-	+	ND
7% NaCl	+	+	+	-	+	d
10% NaCl	- (except P001)	+ (except P008)	-	-	+	ND
Deamination of phenylalanine	+	+ (except K014)	+	-	-	d
Hydrolysis of						
Urea	+	+	+	+	+	d
Esculin	+	+	+	+	+	+

Remark a) Subgroup 1: isolate P001, P002, P007
Subgroup 2: isolate P008, P009, P021, P029, K014, K018, K024
Subgroup 3: isolate P011
Subgroup 4: isolate P022, P028, K002, K003, K004
Subgroup 5: K005, K009

b) Data cited from described by Gordon (1989) and Sneath in Bergey's Manual of Systematic Bacteriology (1986).

c) Symbol code: + = 85 to 100% of the strains positive; a = 50 to 84% of the strains positive; b = 15 to 49% of the strains positive; d = 11 to 89% of the strains positive; ND = no data; - = 0 to 14% of the strains negative.

Table 6 Biochemical and physiological characteristics of isolate P006, P010, P012, P023, P024, P026, P027, P030, P031, P033, K006, K011, K015, K026, K031, K032 and K033 compared with *B. thuringiensis*.

Properties	Subgroup [a]		*B. thuringiensis* [b]
	1	2	
Cell width (μm)	>1.0	>1.0	1.0-1.2
Cell length (μm)	2.5	2.5-4.0	3.0-5.0
Unstained globules in protoplasm	+	+	+
Spore shape	ellipsoidal	cylindrical/ellipsoidal	ellipsoidal
Swollen sporangium	-	-	-
Spore position	central/paracentral	central/paracentral	central/paracentral
Crystalline parasporal bodies	+	+	a
Anaerobic growth	+	+	+
VP	-	+	d
pH of VP broth	5.08	4.54-4.97	4.3-5.6
Reduction of NO_3 to NO_2	-	+	+
Lecithinase reaction	+	+	+
Acid production from			
D-glucose	+	+	+
L- arabinose	-	-	-
D- xylose	-	-	-
D- mannitol	-	-	-
Hydrolysis of			
Casein	+	+	+
Gelatin	+	+ (except K032, K033)	+
Starch	-	+	+
Growth in			
5% NaCl	+	+	+
7% NaCl	+	+	+
10% NaCl	-	+ (except P033, K033)	ND
Utilization of			
Citrate	+	+ (except P012, K006)	+
Propionate	+	-	ND

Remark a) Subgroup 1: isolate P006
Subgroup 2: isolate P010, P012, P023, P024, P026, P027, P030, P031, P033, K006, K011, K015, K026, K031, K032, K033

b) Data cited from described by Gordon (1989), Sneath in Bergey's Manual of Systematic Bacteriology (1986) and Barjac in The Aerobic Endospore-forming Bacteria: Classification and Identification (1981).

c) Symbol code: + = 85 to 100% of the strains positive; d = 11 to 89% of the strains positive; ND = no data; - = 0 to 14% of the strains negative.

Results from Figure 5-6 as well as Table 5 and 6 showed that it was difficult to distinguish *B. megaterium* and *B. thuringiensis* by cell width and cell arrangement under light microscope. Thus, biochemical properties, particularly anaerobic growth, lecithinase reaction and crystalline parasporal bodies existence could differentiate these two bacteria. As *B. megaterium* showed negative results in anaerobic growth, lecithinase reaction and crystalline parasporal bodies existence whereas the *B. thuringiensis* gave positive results.

Results from Figure 5 and Table 5, when compared with *B. megaterium* as reference, indicated that isolate P001, P002, P007, P008, P009, P011, P021, P022, P028, P029, K002, K003, K004, K005, K009, K014, K018 and K024 were mostly identified as *B. megaterium*. Negative results in hydrolysis of casein and starch of isolate P011 caused the difficulty to identify this bacteria. Further study to clarify should be done.

Results from Figure 6 and Table 6, when compared with *B. thuringiensis* as reference, showed that isolate P006, P010, P012, P023, P024, P026, P027, P030, P031, P033, K006, K011, K015, K026, K031, K032 and K033 were identified as *B. thuringiensis*. Negative results in nitrate reduction and hydrolysis of starch of isolate P006, the negative results in hydrolysis of gelatin of isolate K032 and K033 and utilization of citrate of isolate P012 and K006 showed variation among this species. However, in case of insect pathogen, characteristics in nitrate reduction, hydrolysis of starch and utilization of citrate were varied (Barjac, 1981).

Bacillus spp. that belonged to *Bacillus* group IB, 15 of 17 isolates were identified as *B amyloliquefaciens*, others were unidentified according to their different biochemical characteristics as shown in Table 7.

Table 7 Isolates of *Bacillus sp.* belonged in group IB.

List of isolates	Identified as
15 isolates P013, P014, P015, P016, P018, P017, P019, P020, K008, K013, K016, K019, K021, K025, K027	*Bacillus amyloliquefaciens*
2 isolates K010, K029	Unidentified

As shown in Table 7, 15 isolates were identified as *B. amyloliquefaciens*. Eight isolates originated from soil A whereas 7 isolates were from soil B. Two isolates from soil B closed to *B. badius* and *B. firmus*.

Most characteristics between *B. amyloliquefaciens* and *B. subtilis* were almost identical. However, the key physiological characteristic to differentiate *B. amyloliquefaciens* from *B. subtilis* is the result in acid production from lactose, *B. amyloliquefaciens* gave positive result while the latter gave the opposite result (Roberts *et al*, 1996). Morphological, physiological and biochemical characteristics of 15 isolates identified as *B. amyloliquefaciens* had shown in Figure 7 and Table 8.

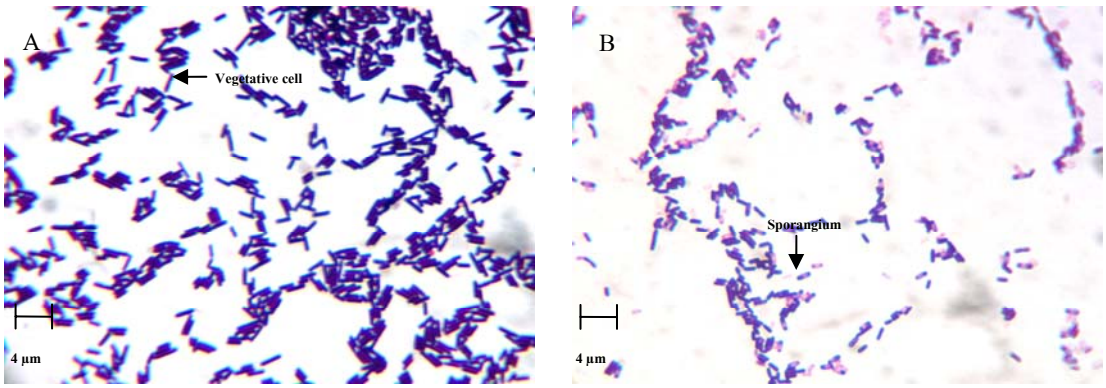


Figure 7 *B. amyloliquefaciens* isolate

A. Isolate P013 grown on NA not more than 24 hours showed vegetative cells and cell arrangement.

B. Isolate P020 showed its sporangium after 24 hours.

Table 8 Biochemical and physiological characteristics of isolate P013, P014, P015, P016, P017, P018, P019, P020, K008, K013, K016, K019, K021, K025 and K027 compared with *B. amyloliquefaciens*.

Properties	a)	*B. amyloliquefaciens*[b)]
Cell width (μm)	<1.0	0.7-0.8
Cell length (μm)	2.0-3.5	2.0-3.0
Unstained globules in protoplasm	-	-
Spore shape	cylindrical	ellipsoidal/cylindrical
Swollen sporangium	-	-
Spore position	central/paracentral	central/paracentral
Crystalline parasporal bodies	-	-
Anaerobic growth	-	-
VP	+	+
pH of VP broth	5.32-6.23	ND
Reduction of NO_3 to NO_2	+	+
Acid production from		
D-glucose	+	+
L-arabinose	+	+
D-xylose	+	+
D-mannitol	+	+
Lactose	+	+
Hydrolysis of		
Casein	+	+
Gelatin	+	ND
Starch	+	+
Lecithinase reaction	-	-
Utilization of		
Citrate	+	+
Propionate	-	ND
Growth in		
5% NaCl	+	+
7% NaCl	+	+
10% NaCl	+	ND

Remark a) Subgroup 1: isolate P013, P014, P015, P016, P017, P018, P019, P020, K008, K013, K016, K019, K021, K025, K027

b) Data cited from Gordon (1989), Sneath in Bergey's Manual of Systematic Bacteriology (1986) and Roberts *et al* (1996).

c) Symbol code: + = 85 to 100% of the strains positive; - = 0 to 14% of the strains negative; ND = No Data.

In the other hands, in *Bacillus* group IB, there was 2 isolate, K010 and K029, did not identified as any species. The characteristics of isolate K010 and K029 were shown in Figure 8 and 9 and Table 9.

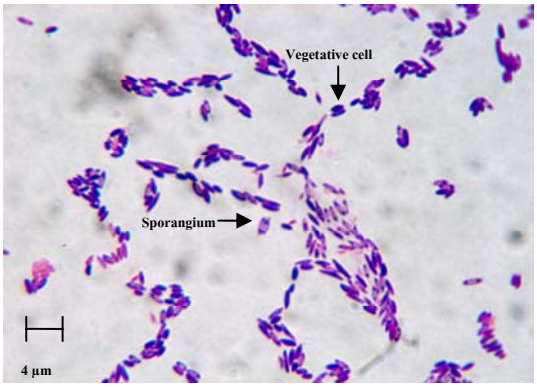


Figure 8 Isolate K010, representing of *Bacillus* group IB, grown on NA not more than 24 hours showed vegetative cells, sporangium and cell arrangement.

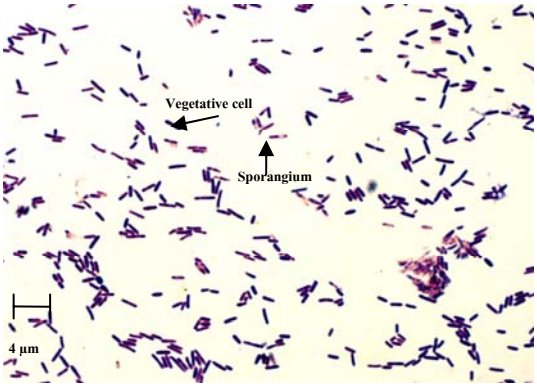


Figure 9 Isolate K029, representing of *Bacillus* group IB, grown on NA not more than 24 hours showed vegetative cells, sporangium and cell arrangement.

Table 9 Biochemical and physiological characteristics of unidentified isolate K010 and K029.

Properties	K010	K029
Cell width (μm)	<1.0	<1.0
Cell length (μm)	3.0	2.5
Unstained globules in protoplasm	-	-
Spore shape	cylindrical	cylindrical
Swollen sporangium	-	-
Spore position	subterminal	subterminal
Crystalline parasporal bodies	-	-
Anaerobic growth	-	-
VP	-	-
pH of VP broth	7.70	4.97
Reduction of NO_3 to NO_2	+	-
Acid production from		
D-glucose	+	+
L-arabinose	-	-
D-xylose	-	-
D-mannitol	+	-
Lactose	-	-
Hydrolysis of		
Casein	+	+
Gelatin	+	+
Starch	-	+
Lecithinase reaction	-	-
Utilization of		
Citrate	+	-
Propionate	-	-
Growth in		
5% NaCl	-	+
7% NaCl	-	+
10% NaCl	-	-
pH 5.7	ND	+

Remark a) Symbol code: + = 85 to 100% of the strains positive; d = 11-89% of the strains positive; - = 0 to 14% of the strains negative; ND = No Data.

From Figure 8 and 9 and Table 9, isolate K010 and K029 were still unidentified by this method. Further study to classify this strain should be done.

However, there were 9 isolates belonged to *Bacillus* group II, comprised with the ellipsoidal spores that distended sporangium. Six isolates were closed to *B. brevis*, 1 isolate was closed to *B. circulans* and 2 isolates were unidentified, according to their different biochemical characteristics. List of isolates closed to each group was shown as Table 10.

Table 10 List of *Bacillus* isolates belonged in group II.

List of isolates	Identified as	Closed to
6 isolates P003, P004, P005, K007, K012, K023	-	*Bacillus brevis*
1 isolate K020	-	*Bacillus circulans*
2 isolates K001, K017	Unidentified	

There were 6 isolates closed to *B. brevis*, 3 isolates isolated from soil A and 3 isolates from soil B. However there was only one isolate from soil B closed to *B. circulans*. In the other hands, there were 2 unidentified isolates which isolated from soil B.

Key test to differentiate *B. brevis* from others *Bacillus* sp. were the negative results in VP test pH 8.0-8.6 in VP broth after 7 days, anaerobic growth and acid production from arabinose, xylose and lactose as well as the positive result in acid production from glucose and mannitol in some strains. Phenotypic characteristics of these 6 isolates indicated that they are closely related to *B. brevis* as shown on Figure 10 and Table 11.

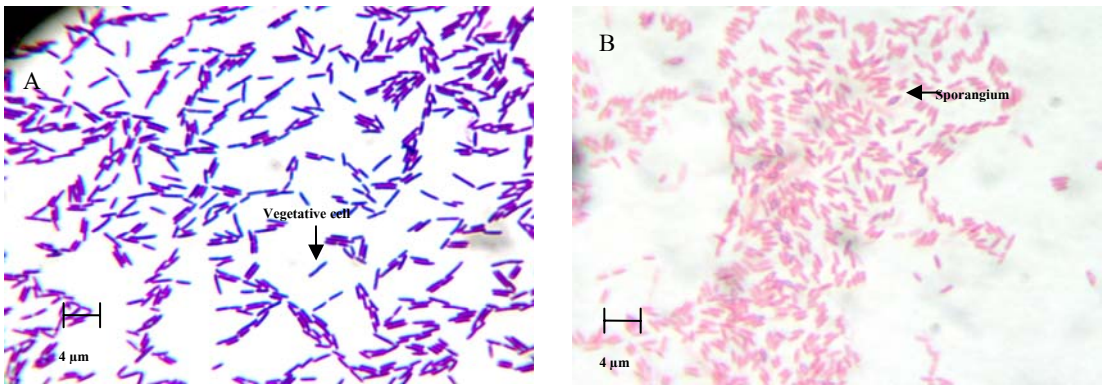


Figure 10 *Bacillus* sp. closely to *B. brevis.*

A. Isolate P005 grown on NA not more than 24 hours showed vegetative cells and cell arrangement.

B. Isolate K012 showed its sporangium after 24 hours.

Table 11 Biochemical and physiological characteristics of isolate P003, P004, P005, K007, K012 and K023 compared with *B. brevis*.

Properties	Subgroup [a]			*B. brevis* [b]
	1	2	3	
Cell width (μm)	<1.0	≤1.0	<1.0	0.6-0.9
Cell length (μm)	2.0-3.0	2.5	3.0	1.5-4.0
Unstained globules in protoplasm	-	-	-	-
Spore shape	ellipsoidal	ellipsoidal	ellipsoidal	cylindrical/ellipsoidal
Swollen sporangium	+	+	+	+
Spore position	central/ paracentral	central	paracentral	central/paracentral/ terminal /subterminal
Crystalline parasporal bodies	-	-	-	-
Anaerobic growth	-	-	-	-
VP	-	-	-	-
pH of VP broth	8.12-8.15	8.17	8.25	8.0-8.6
Reduction of NO_3 to NO_2	+	+	+	a
Acid production from				
D-glucose	+	-	+	d
L-arabinose	-	-	-	-
D-xylose	-	-	+	-
D-mannitol	+	-	+	d
Lactose	-	-	-	-
Hydrolysis of				
Casein	+	+	-	d
Gelatin	+	+	-	d
Starch	-	-	+	d
Lecithinase reaction	-	-	-	-
Utilization of				
Citrate	+	+	+	b
Propionate	-	-	-	ND
Growth in				
5% NaCl	-	-	-	-
7% NaCl	-	-	-	-
10% NaCl	-	-	-	-

Remark a) Subgroup 1: isolate P003, P004, P005 and K012
Subgroup 2: isolate K007
Subgroup 3: isolate K023

b) Data cited from Gordon (1989) and Sneath in Bergey's Manual of Systematic Bacteriology (1986).

c) Symbol code: + = 85 to 100% of the strains positive; a = 50 to 84% of the strains positive; b = 15 to 49% of the strains positive; d = 11-89% of the strains positive; - = 0 to 14% of the strains negative; ND = No Data.

Results from above showed that isolate P003, P004, P005, K007 and K012 were almost similar to *B. brevis*. However, these isolates were not identified as *B. brevis* but still identified as *Bacillus* sp. that closed to *B. brevis*. Further study to classify this strain should be done.

Three isolates of *Bacillus* group II, isolate K001, K017 and K020. Their taxonomic positions were still unclear. Only isolate K020 was closed to *B. circulans* while others were unidentified. Their characteristics were showed on Figure 11 and Table 12.

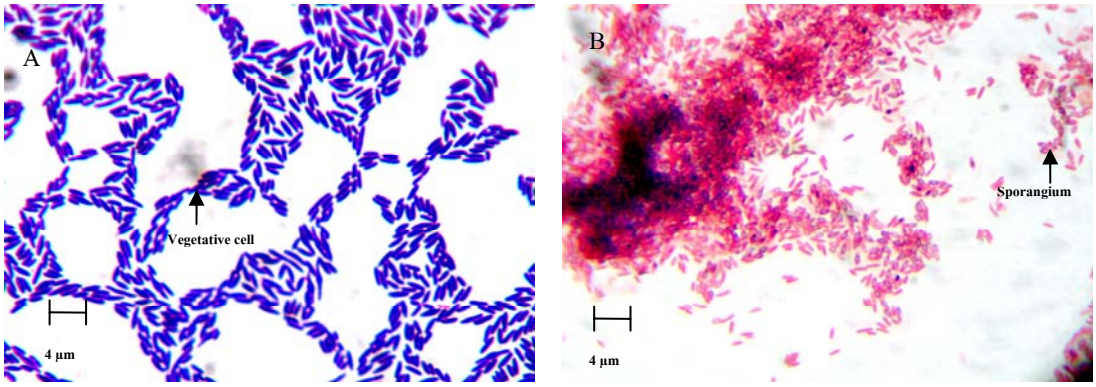


Figure 11 Unidentified *Bacillus* sp. (closed to *B. circulans*)

A. Isolate K001 grown on NA not more than 24 hours showed vegetative cells and cell arrangement.

B. Isolate K020 showed its sporangium after 24 hours.

Table 12 Biochemical and physiological characteristics of unidentified isolate K001, K017 and K020 compared with *B. circulans*.

Properties	K001	K017	K020	*B. circulans* [a)]
Cell width (μm)	<1.0	<1.0	<1.0	0.5-0.7
Cell length (μm)	3.0	2.5	3.0	2.0-5.0
Unstained globules in protoplasm	-	-	-	-
Spore shape	ellipsoidal	cylindrical	ellipsoidal	cylindrical/ellipsoidal
Swollen sporangium	+	+	+	+
Spore position	central /paracentral	central	central	central/paracentral /terminal /subterminal
Crystalline parasporal bodies	-	-	-	-
Anaerobic growth	-	-	-	a
VP	-	-	-	-
pH of VP broth	5.29	4.89	4.58	4.5-6.6
Reduction of NO_3 to NO_2	+	+	-	b
Acid production from				
D-glucose	+	+	+	+
L-arabinose	+	+	+	+
D-xylose	+	-	+	+
D-mannitol	-	-	+	+
Lactose	+	+	+	+
Hydrolysis of				
Casein	+	+	-	b
Gelatin	+	-	+	b
Starch	-	+	-	+
Lecithinase reaction	-	-	-	-
Utilization of				
Citrate	+	-	+	b
Propionate	-	-	+	ND
Growth in				
5% NaCl	-	-	+	a
7% NaCl	-	-	+	a
10% NaCl	-	-	-	-

Remark a) Data cited from Gordon (1989) and Sneath in Bergey's Manual of Systematic Bacteriology (1986).

b) Symbol code: + = 85 to 100% of the strains positive; a = 50 to 84% of the strains positive; b = 15 to 49% of the strains positive; - = 0 to 14% of the strains negative; ND = No Data.

However, 1 isolate of *Bacillus* group III, isolate K022, comprised with the spherical spores that distended sporangium, this isolate was identified as *B. sphaericus*. Key tests to differentiate *B. sphaericus* from other species were the negative results in anaerobic growth and acid production from glucose, arabinose, xylose, mannitol and lactose. The morphological, physiological and biochemical characteristics of isolate K022 was shown as Figure 12 and Table 13.

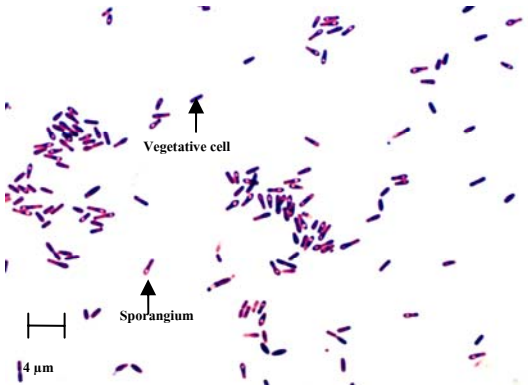


Figure 12 Isolate K022, representing of *Bacillus* group III identified as *B. sphaericus*, grown on NA not more than 24 hours showed vegetative cells, sporangium and cell arrangement.

Table 13 Biochemical and physiological characteristics of isolate K022 compared with *B. sphaericus*.

Properties	K022	*B. sphaericus* [a)]
Cell width (μm)	1.0	0.6-1.0
Cell length (μm)	3.0	1.5-5.0
Unstained globules in protoplasm	-	-
Spore shape	Spherical	Spherical
Swollen sporangium	+	+
Spore position	Terminal	Terminal/ subterminal
Crystalline parasporal bodies	-	-
Anaerobic growth	-	-
VP	-	-
pH of VP broth	7.87	7.4-8.6
Reduction of NO_3 to NO_2	-	-
Acid production from		
D-glucose	-	-
L-arabinose	-	-
D-xylose	-	-
D-mannitol	-	-
Lactose	-	-
Hydrolysis of		
Casein	-	b
Gelatin	+	b
Starch	-	-
Lecithinase reaction	-	-
Utilization of		
Citrate	+	b
Propionate	-	ND
Growth in		
5% NaCl	-	b
7% NaCl	-	b
10% NaCl	-	-

Remark a) Data cited from Gordon (1989) and Sneath in Bergey's Manual of Systematic Bacteriology (1986).

b) Symbol code: + = 85 to 100% of the strains positive; b = 15 to 49% of the strains positive; - = 0 to 14% of the strains negative; ND = No Data.

Sixty two cultures characterized as *Bacillus* sp. were isolated from soil #A 31 isolates and soil # B 31 isolates. According to the morphological, biochemical and physiological properties, 62 isolates were grouped into group IA 35 isolates, group IB 17 isolates, group II 9 isolates and group III 1 isolate. In group IA, 35 isolates were identified as *B. megaterium* 17 isolates and *B. thuringiensis* 17 isolates. Only isolate P011 was not clearly identified but closed to *B. megaterium*. Fifteen of 17 isolates from group IB were identified as *B. amyloliquefaciens* and 2 isolates, K010 and K029, were still unidentified. However in group II, 6 isolates were *Bacillus* sp. that closely to *B. brevis*, 1 isolate closed to *B. circulans* and 2 isolates were still unidentified. In group III, isolate K022 was clearly identified as *B. sphaericus*.

All 66 isolates, after grouped and classified in species level, were determined the carbofuran degradation abilities. The cultures were grown, harvested and analyzed compared with uncultured control.

Carbofuran Residue Determination by TLC

From 66 isolates, 5 isolates were randomly sampled for primary screening method of carbofuran degrading ability by TLC. When silica gel 60 F_{254} plate was used as stationary phase, band of carbofuran was not clear in ethyl acetate system and not able to be detected when acetone was used as mobile phase. Carbofuran was detected at R_f value 0.18 under UV light at 254 nm using mixture of hexane: ethyl acetate: chloroform (70:20:5) as mobile phase. The results indicated that there were carbofuran degradation of all 5 isolates as detected at the same R_f value of standard carbofuran and the minimum detected concentration by visualization was 20 ppm (data not shown).

Carbofuran Residue Determination by HPLC

Standard carbofuran was injected to HPLC to obtain the retention time and limit of detection. Chromatogram of carbofuran was shown in Figure 13 under the condition using methanol: water (1:1) with flow rate 1 ml/min as mobile phase, Chromatographic column stainless steel packed with C_{18} bounded silica gel as stationary phase, detector wavelength was 280 nm and column temperature at 40 °C.

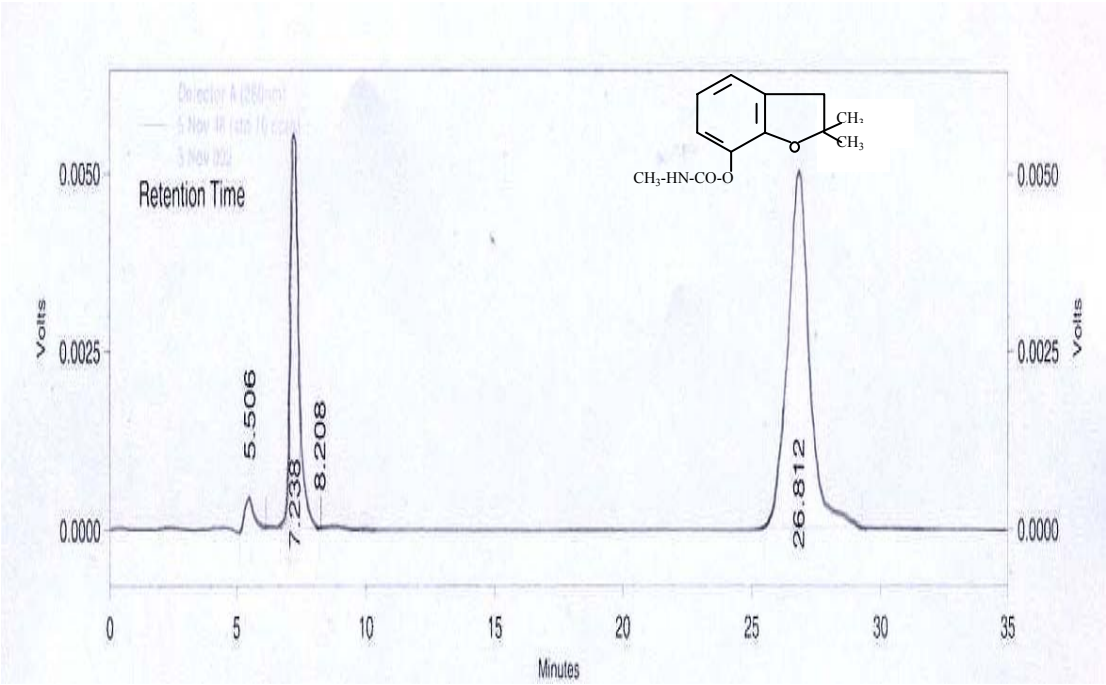


Figure 13 Chromatogram of standard carbofuran which had retention time at 26.812 minutes.

As shown in Figure 13, solvent peak and carbofuran peak were appeared at 7.238 and 26.812 minutes, respectively. The carbofuran concentrations at 0, 5, 10 and 20 ppm were determined to set the standard curve between area and concentration. The standard curve of carbofuran was shown as Figure 14 with the limit of detection of this method was 0.2 ppm.

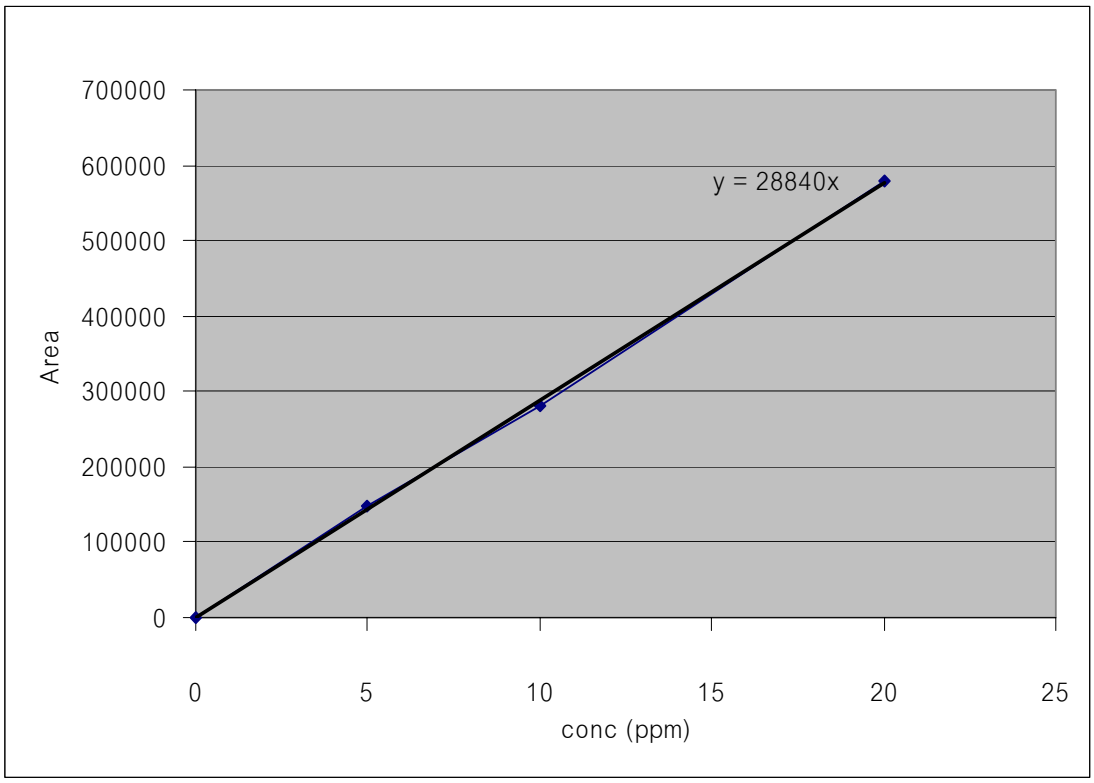


Figure 14 Standard curve of carbofuran compared between peak area and concentration.

Recovery of the extraction method was studied from minimal medium spiked with carbofuran. Extraction by dichloromethane gave 102% recovery of residue with the deviation value of 6%. The carbofuran residues in control treatment spiked with carbofuran in all experiments were 107, 100 and 97 % at day 3, 5 and 7, respectively. The decrement at day 7 might caused by chemical hydrolysis. The degradations of all 62 isolated bacteria were shown in Figure 15-21.

Degradation Abilities of 66 Isolates

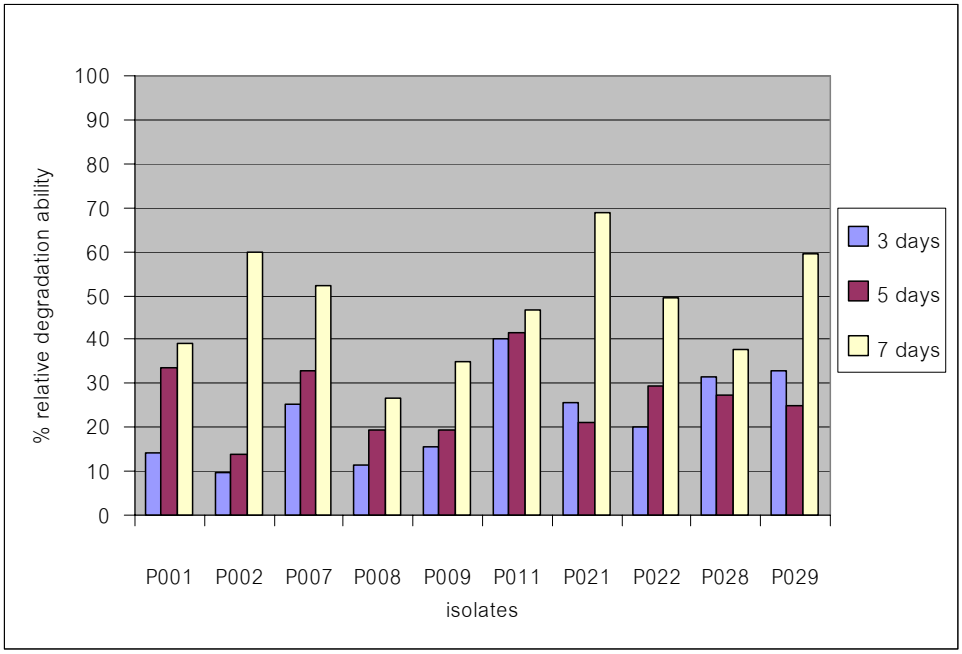


Figure 15 Relative degradation ability percentage at 3, 5 and 7 day of "P" isolates that belonged to genus *Bacillus* in group IA identified as or closed to *B. megaterium* compared with control.

Figure 15 showed that every isolates were capable to degrade carbofuran throughout the incubation periods. The isolates capable of degrading carbofuran more than 50% within 7 days were isolate P002, P007, P021 and P029. Isolate P021 showed highest ability of degradation. Relative degradation ability of isolate P021 was 68.8% in 7 days, while isolate P011 showed the rapid degradation at 40.1% degradation in day 3 and 41.6% in day 5 was the best in decrement in day 3 and 5.

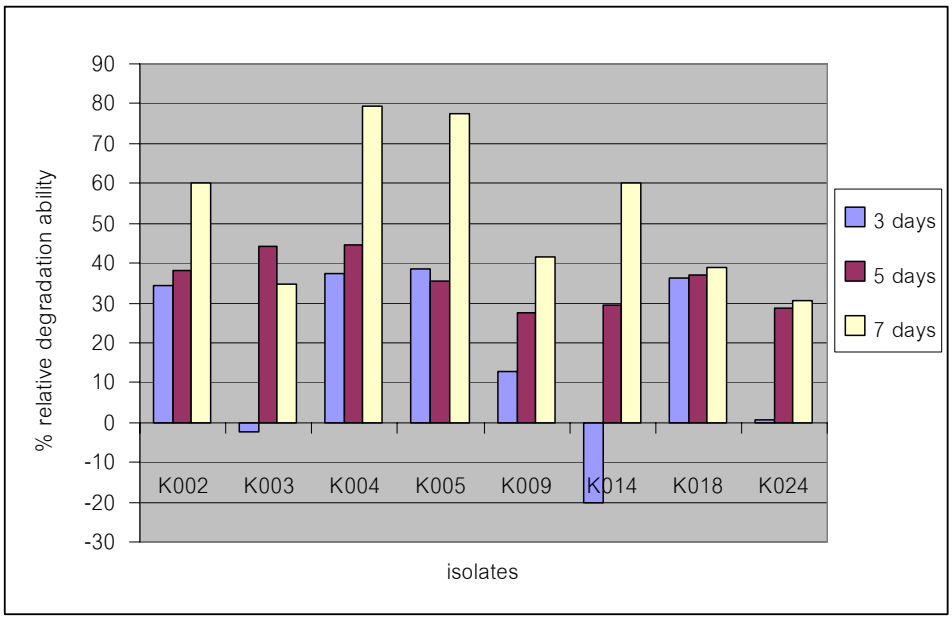


Figure 16 Relative degradation ability percentage at 3, 5 and 7 day of "K" isolates that belonged to genus *Bacillus* in group IA identified as *B. megaterium* compared with control.

Figure 16 showed that the effective isolates capable in degrading carbofuran more than 50% within 7 days were isolate K002, K004, K005 and K014. At day 3, isolate K014 could not degrade carbofuran but the degradation increased at day 5 and finally showed the relative degradation ability 60.2% at day 7. Isolate K004 was the best in decrement in day 3 and 7 which reduced carbofuran 37.5% in 3 days and 79.6% in 7 days.

It is not known why the negative results occurred in the culture of isolate K003 and K014 at day 3 of inoculation. Probably resulted from the technical problems resulted from electrical fluctuation or error in spiking in the medium culture.

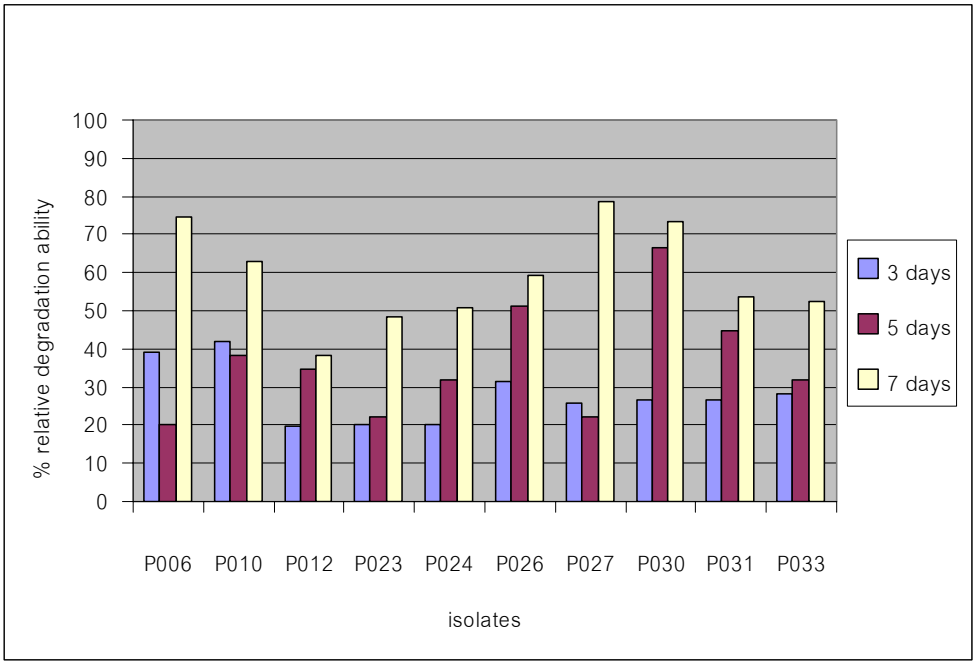


Figure 17 Relative degradation ability percentage at 3, 5 and 7 day of "P" isolates that belonged to genus *Bacillus* in group IA identified as *B. thuringensis* compared with control.

Figure 17 showed that the isolates capable of degrading carbofuran more than 50 % within 7 days were isolate P006, P010, P024, P026, P027, P030, P031 and P033. Isolate P027 showed highest ability of degradation among this group. Relative degradation ability of isolate P027 was 78.8% in 7 days, while isolate P010 showed the rapid degradation at 42.1% degradation in day 3. Isolate P030 was the best in decrement in day 5 which its decreasing ability at the period between day 3 and 5 was 40%.

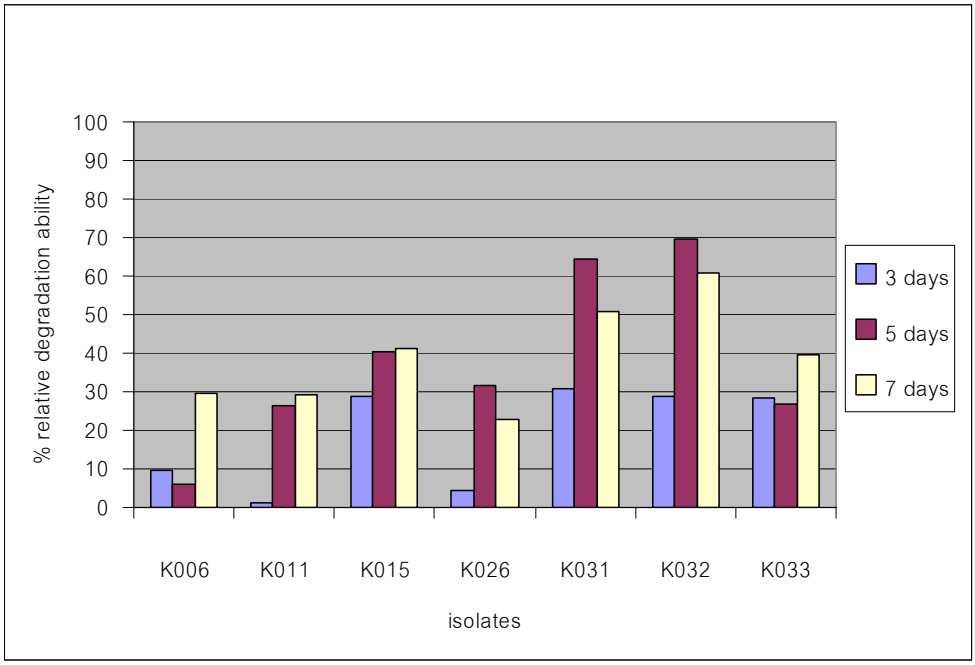


Figure 18 Relative degradation ability percentage at 3, 5 and 7 day of "K" isolates that belonged to genus *Bacillus* in group IA identified *B. thuringensis* compared with control.

Two isolates capable of degrading carbofuran more than 50 % within 7 days was isolate K031 and K032. Isolate K032 showed highest ability of degradation among this group. Relative degradation ability of isolate K032 was 60.9% in 7 days, while K031 showed the rapid degradation at 30.6% degradation in day 3. K032 was the best in decrement in day 5 which its decreasing ability at the period between day 3 and 5 was 40.9%.

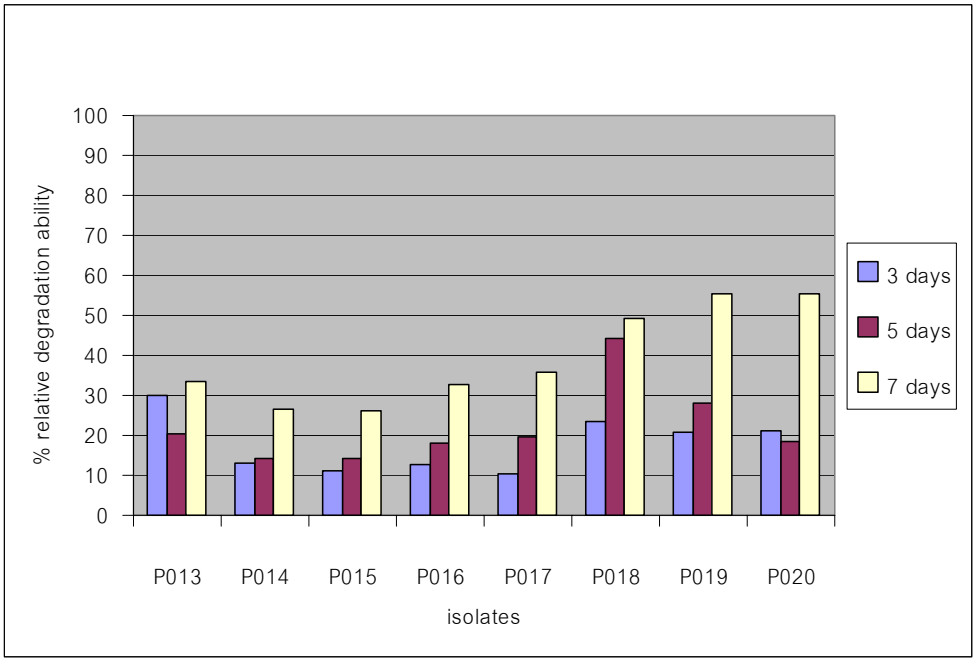


Figure 19 Relative degradation ability percentage at 3, 5 and 7 day of "P" isolates that belonged to genus *Bacillus* in group IB identified as *B. amyloliquefaciens* compared with control.

Figure 19 showed that the isolates capable of degrading carbofuran more than 50 % within 7 days were isolate P019 and P020. Isolate P019 and P020 showed the same highest ability of degradation among the group. Relative degradation ability of isolate P019 and P020 were 55.5 and 55.5% in 7 days, while isolate P013 showed the rapid degradation at 30.1% degradation in day 3. Isolate P018 was the best in decrement in day 5 which its decreasing ability at the period between day 3 and 5 was 20.9%.

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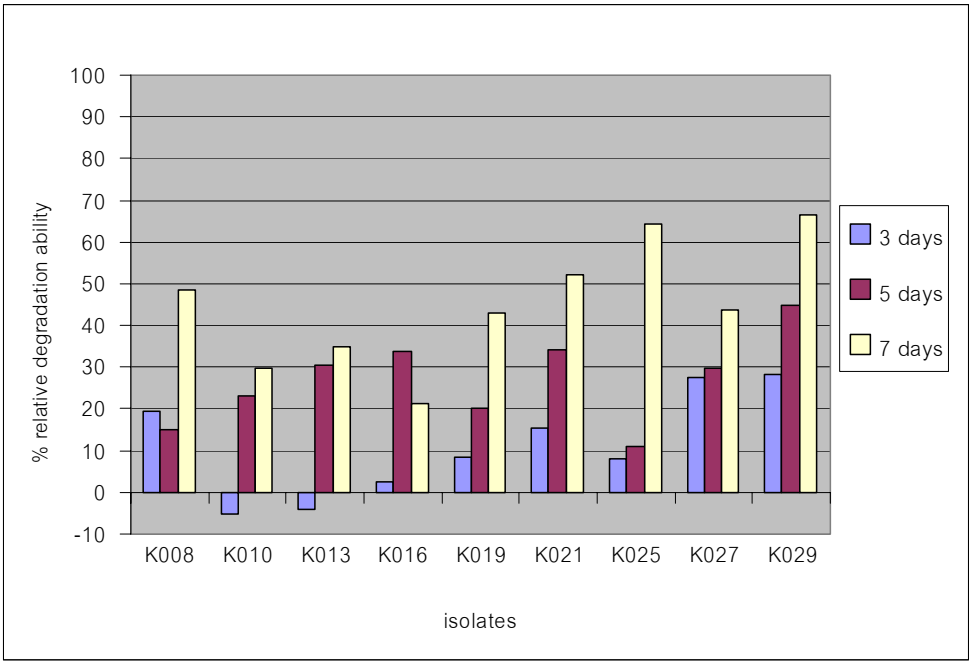

Figure 20 Relative degradation ability percentage at 3, 5 and 7 day of "K" isolates that belonged to genus *Bacillus* in group IB (*B. amyloliquefaciens* and *Bacillus* sp.

Figure 20 showed that isolate K010 and K013 were the worst in degradation at day 3 but the degradation rate was apparently increased in day 5 and finally 29.9 and 34.8% in day 7, respectively. The effective isolates capable of degrading carbofuran more than 50 % within 7 days were isolate K021, K025 and K029. Relative degradation ability of isolate K029 was 66.5% in 7 days, while isolate K027 and K029 showed the rapid degradation at 27.4 and 28.4% degradation in day 3, respectively. Isolate K029 was the best in decrement in day 5 which its decreasing ability at the period between day 3 and 5 was 16.5%.

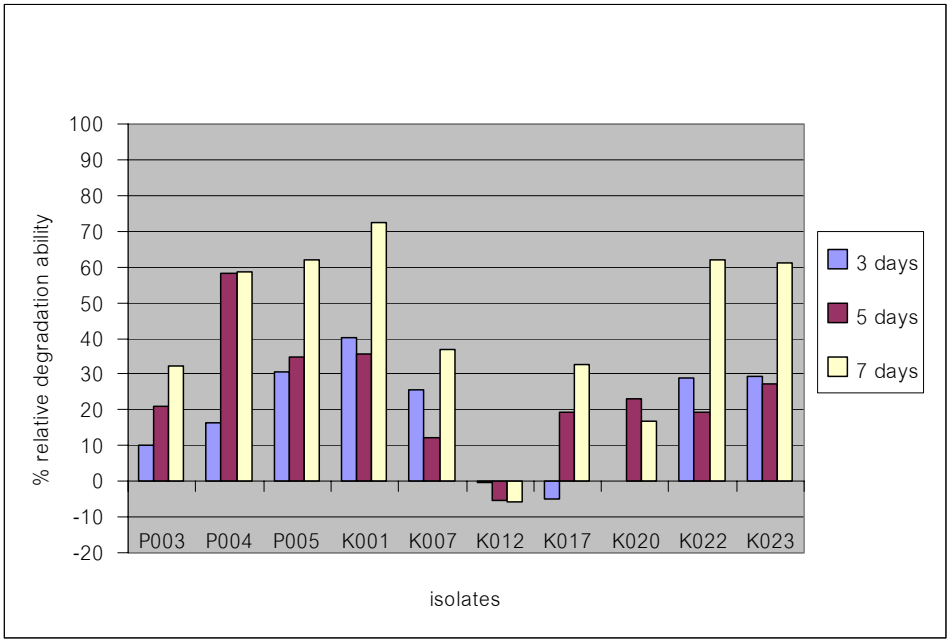

Figure 21 Relative degradation ability percentage at 3, 5 and 7 day of the isolates that belonged to genus *Bacillus* in group II and group III.

Figure 21 showed that the isolates capable of degrading carbofuran more than 50 % within 7 days were isolate P004, P005, K001, K022 and K023. Isolate K001 showed highest ability of degradation among this group. Relative degradation ability of isolate K001 was about 72.6% in 7 days, while isolate K001 showed the rapid degradation at 40.2% degradation in day 3. Isolate P004 was the best in decrement in day 5 which its decreasing ability at the period between day 3 and 5 was 42.1%.

It is not known why the negative results occurred in the culture of isolate K012 at day 3, 5 and 7 of inoculation and isolate K017 at day 3 of inoculation. Probably resulted from the technical problems resulted from electrical fluctuation or error in spiking in the medium culture.

From 62 isolates, there were 28 isolates capable to degrade carbofuran more than 50% within 7 days. Those effective isolates were shown in Table 14.

Table 14 Twenty eight isolates which capable to degrade carbofuran more than 50% within 7 days.

Isolate	Percentage of relative degradation ability		
	3 days	5 days	7 days
B. megaterium			
P002	9.5	13.7	60.0
P007	25.1	32.8	52.1
P021	25.6	21.3	68.8
P029	32.9	24.9	59.4
K002	34.5	38.3	60.3
K004	37.5	44.7	79.6
K005	38.6	35.6	77.7
K014	0	29.5	60.2
B. thuringiensis			
P006	39.1	20.0	74.5
P010	42.1	38.1	63.1
P024	20	31.9	51.0
P026	31.3	51.0	59.2
P027	25.6	22.1	78.8
P030	26.5	66.5	73.2
P031	26.6	44.9	53.8
P033	28.2	31.8	52.3
K031	30.6	64.4	50.7
K032	28.8	69.6	60.9
B. amyloliquefaciens			
P019	20.6	28.0	55.5
P020	21.1	18.4	55.5
K021	15.2	34.2	52.2
K025	7.8	11.1	64.3
Unidentified *Bacillus* sp.			
K029	28.4	44.8	66.5
Bacillus sp. closely to *B. brevis*			
P004	16.3	58.4	58.4
P005	30.6	34.6	62.1
K023	29.3	27.4	61.0
Unidentified *Bacillus* sp			
K001	40.2	35.7	72.6
B. sphaericus			
K022	29	19.4	61.8

Table 14 showed that *Bacillus* group IA, consisted of 8 isolates of *B. megaterium* and 10 isolates of *B. thuringiensis*, was the predominant of carbofuran degrading bacteria in soils. In 2002, Bhadbhade *et al* reported that *B. megaterium* was able to degrade organophosphate pesticide, Monocrotophos.

Bacillus sp. closely to *B. brevis*, isolate P004 and *B. thuringiensis*, isolate K031 were the effective isolates which carbofuran degradation rate between day 3 and 5 were 42.1 and 33.8%, respectively. Whereas *B. thuringiensis*, isolate P027 and *B. amyloliquefaciens*, isolate K025 were the effective isolates which carbofuran degradation rate between day 5 and 7 were 56.7 and 53.1%, respectively. Therefore isolate P004, P027, K025 and K031 were taken to study the effect of pH, light and inoculum size on degradation of carbofuran further.

Effect of pH, Light and Inoculum Size on Degradation of Carbofuran in Minimal Medium

From the carbofuran degradation of all 62 cultures isolated from carbofuran treated soils, 2 of *B. thuringiensis*, isolate P027 and K031, 1 of *B. brevis*, isolate P004 and 1 of *B. amyloliquefaciens*, isolate K025, were selected to study the effect of pH, light and inoculum size on carbofuran degradation at day 5 of incubation.

Effect of pH on Carbofuran Degradation in Minimal Medium

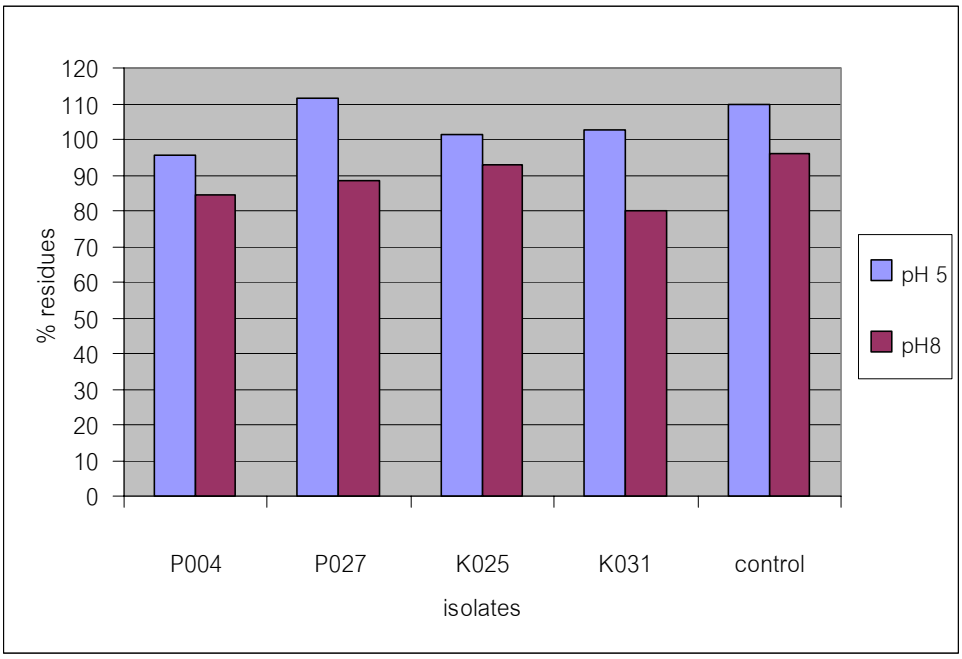

Figure 22 Effect of pH 5 and 8 on carbofuran degradation in minimal medium inoculated with isolate P004, P027, K025, K031 and control (no inoculation) at day 5.

From the figure above, higher pH increased the rate of degradation in every treatment. At pH8, isolate K031 was the isolate with highest degradation rate, it reduced carbofuran at 15.98% compared with control in 5 days of incubation. While at pH 5, isolate P004 showed 14.63% reduction compared with control in 5 days of incubation.

Figure 22 presented the carbofuran residues in the medium at 5 days of incubation based on the comparison with the detectable concentration in the control treatment at day 5. At pH 8, the degradation of carbofuran was higher than at day 5 in every isolate.

Effect of Light on Carbofuran Degradation in Minimal Medium

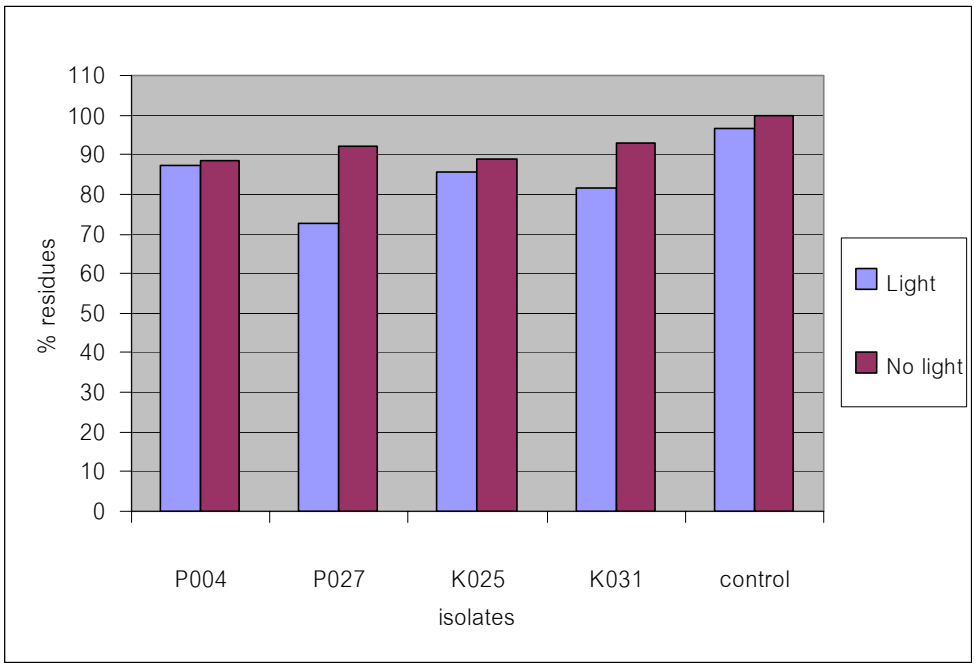

Figure 23 Effect of light on carbofuran degradation in minimal medium inoculated with isolate P004, P027, K025, K031and control (no inoculation) at day 5.

Figure 23 showed the effect of sunlight to the carbofuran degrading ability of isolate P004, P027, K025 and K031. It is noted that medium exposed to light showed slightly different in degradation compared to no light treatment. The differential percentage between light and no light control treatment was 3.26%. Percent reduction of isolate P004, P027, K025 and K031 between light and no light condition were 1.12, 20.88, 4.49 and 11.96%, respectively.

Effect of Inoculum Size on Carbofuran Degradation in Minimal Medium

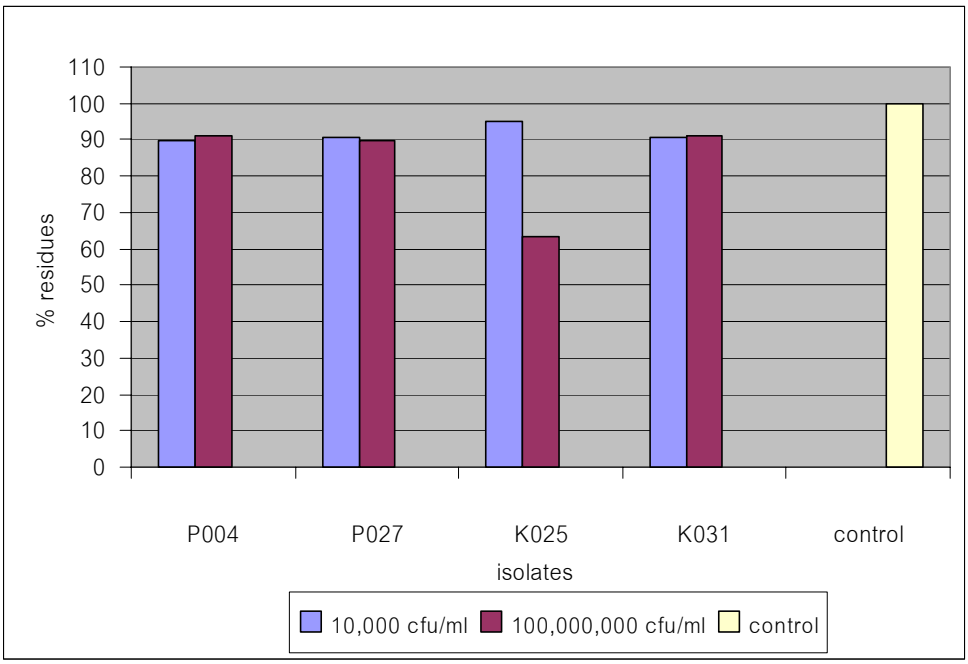

Figure 24 Effect of inoculum size on carbofuran degradation in minimal medium inoculated with isolate P004, P027, K025, K031and control (no inoculation) at day 5.

Figure 24 showed that only isolate K025 showed its inoculum size influenced the degradation rate. At density of 10^8cfu/ml, the degradation rate increased 34 % compared with those inoculated at density 10^4cfu/ml. In the mean time, when inoculated with P004, P027 and K031, there were no different degradation between 10^4 and 10^8cfu/ml inoculation

Metabolites Produced from Carbofuran Degradation Process.

B. thuringiensis (isolate P027) and *B. megaterium* (isolate K004) which showed high efficiency in carbofuran utilization were studied on metabolites structure after degradation at 7 days of inoculation. The isolates were cultured in minimal medium supplemented with 20 ppm filtered steriled carbofuran paralleled with uncultured control. The samples were determined by GC-MS at Laboratory Center for Food and Agricultural Products (LCFA). The chromatograms of control, isolate P027 and isolate K004 were demonstrated as Figure 29, 30 and 31, respectively.

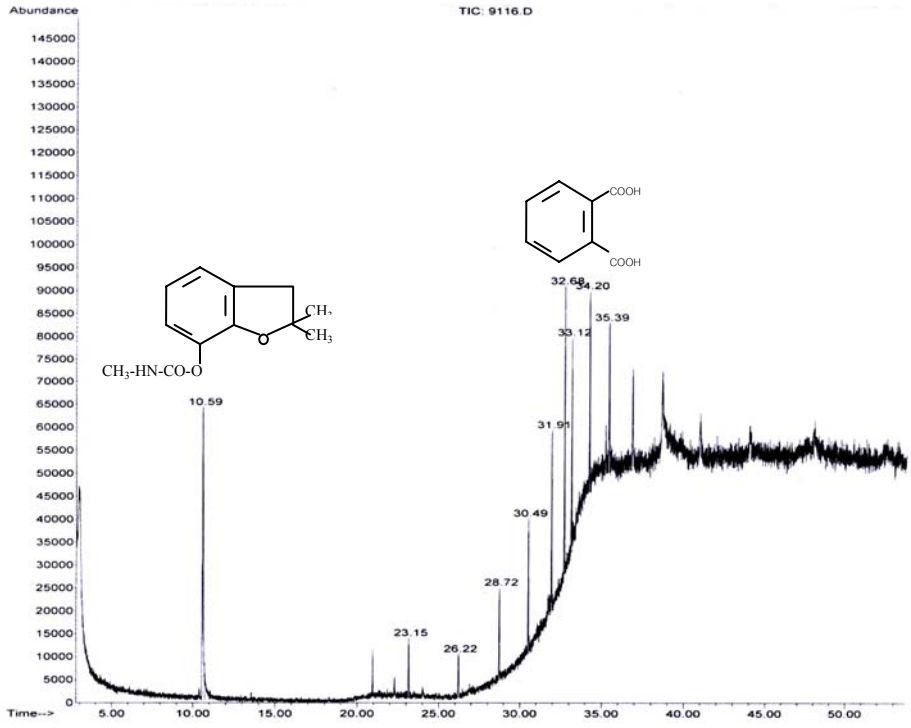

Figure 25 The chromatogram of unknown substances in control sample at day 7.

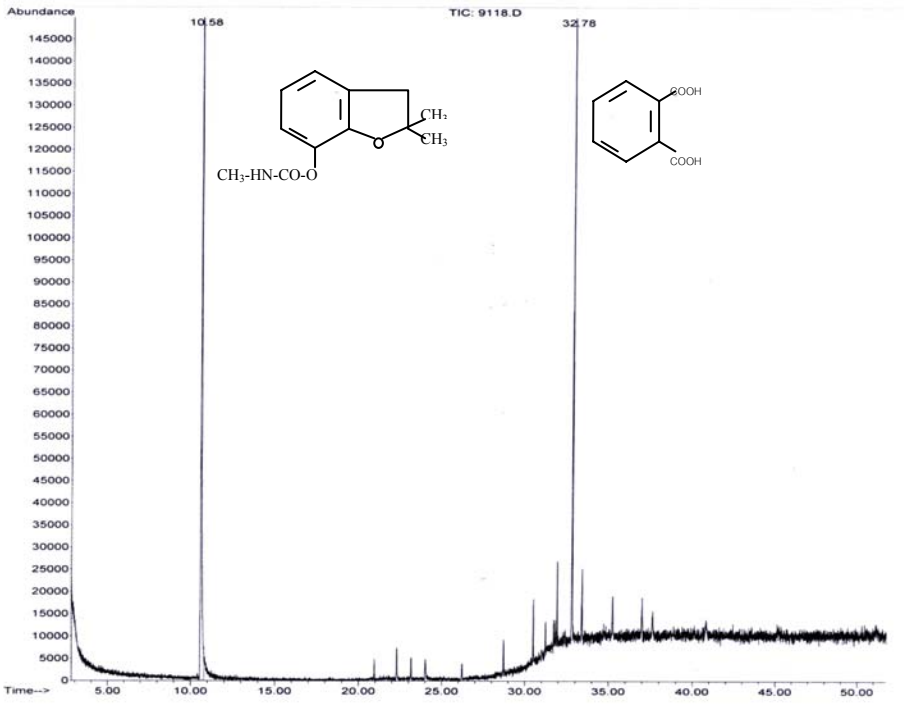

Figure 26 The chromatogram of unknown substances in P027 sample at day 7.

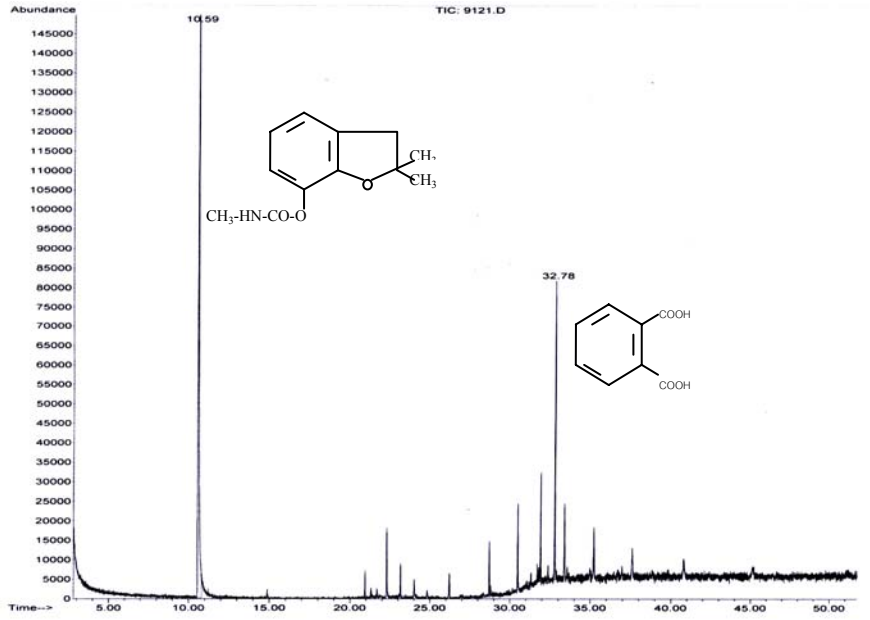

Figure 27 The chromatogram of unknown substances in K004 sample at day 7.

From Figure 25, 26 and 27, time course of 3 samples were compared with GC-MS spectrum data library. It obviously indicated that 2 peaks of unknown substances appeared coincidentally at similar of the 2 isolates retention time i.e.; 10.58-10.59 and 32.60-32.80 minutes. It was found that the substance at 10.58-10.59 minutes was carbofuran whose molecular weight is 321.3 g/mol as parent material and 32.60-32.80 minutes was 1, 2-benzenedicarboxylic acid or phthalic acid whose molecular weight is 166.14 g/mol as metabolite. The structure of 1, 2-benzenedicarboxylic acid was shown as Figure 28.

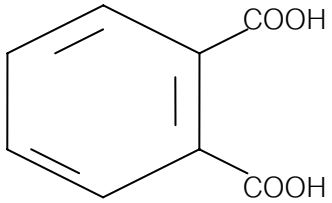

Figure 28 Structure formula of 1, 2-benzenedicarboxylic acid.

From the result, It clearly seen that 1, 2-benzenedicarboxylic acid was the metabolite of carbofuran degradation process at 7 days. The existence of 1, 2-benzenedicarboxylic acid in uncultured control is due to the chemical degradation process of carbofuran but in cultured samples showed the degradation occurred through chemical and microbial process.

Phthalic acid or 1,2- benzenedicarboxylic acid which its molecular formula is $C_6H_4(COOH)_2$ and molecular weight is 166.14 g/mol is used to produce dyes, perfumes, saccharin, phthalates and many others (Anonymous, 2006b). It forms white crystals which melt at 210°C (Anonymous, 2006b).

Phthalate esters can accumulate predominantly from food and cause cancer in animals. The common one, DEHP-di (2ethylhexyl) phthalate or bis (2ethylhexyl) phthalate, has been classified as possibly carcinogenic to humans. Some can also affect the liver, the kidney and irritate the eyes. Individual phthalates can harm the male reproductive tract, the female

reproductive tract, impair reproductive success and cause teratogenicity (malformation of the offspring) (Yarman, Ş. M., 2005).

Ramanand *et al*. (1988) reported that *Arthrobacter sp*. was able to mineralize carbofuran completely to CO_2 within 3-5 days in medium containing carbofuran but Lalah *et al*. (1996) reported that carbofuan-7-phenol and 3-keto carbofuran-7-phenol were the metabolites of the degradation in soil samples at 33 days. In the other hands, Perekh *et al*. (1994) reported that carbofuran was degraded to carbofuran phenol in 3-7 days and carbofuran phenol was not broken down further in minimal medium containing carbofuran. However, in 2004, Kim *et al*. reported that *Sphingomonas* sp. stain SB5 attacked carbofuran at furanyl ring structure and turned to 2-hydroxy-3-(3-methylpropan-2-ol) phenol as metabolite after incubation for 12 hours.

CONCLUSION

Carbofuran affected in reducing total number of culturable soil microorganisms. It was found that at high concentration, number of bacteria was decreased. Carbofuran at low concentration induced the number of carbofuran degrading bacteria, but in higher concentration carbofuran became toxic to growth which reduced number of bacteria.

Sixty two cultures characterized as *Bacillus* sp. were isolated from soil #A 31 isolates and soil # B 31 isolates. According to the morphological, biochemical and physiological properties, 62 isolates were grouped as group IA 35 isolates, group IB 17 isolates, group II 9 isolates and group III 1 isolate. In group IA, 35 isolates were identified as *B. megaterium* 17 isolates and *B. thuringiensis* 17 isolates. Only isolate P011 was not clearly identified but closed to *B. megaterium*. Fifteen of 17 isolates from group IB were identified as *B. amyloliquefaciens* and 2 isolates, K010 and K029, were still unidentified. However in group II, 6 isolates were *Bacillus* sp. that closed to *B. brevis*, 1 isolate closed to *B. circulans* and 2 isolates were still unidentified. One isolate in group III, isolate K022 was clearly identified as *B. sphaericus*.

Carbofuan degrading abilities of 62 isolates were examined. There were 28 isolates which capable to degrade carbofuran more than 50% within 7 days. These isolates were 8 isolate of *B. megaterium* (P002, P007, P021, P029, K002, K004, K005 and K014), 10 of *B. thuringiensis* (P006, P010, P024, P026, P027, P030, P031, P033, K031 and K032), 4 of *B. amyloliquefaciens* (P019, P020, K021 and K025), 3 of *Bacillus* sp. closed to *B. brevis* (P004, P005 and K023), *B. sphaericus* (K022) and 2 of unidentified *Bacillus* (K001 and K029). Among these isolate, *B. megaterium* K004 and *B. thuringiensis* P027 were the most effective isolates which capable to degrade up to 80% in 7 days while *B. thuringiensis* K032 and P030 were the effective isolates which capable to degrade up to 70% in 5 days.

Bacillus sp. closely to *B. brevis* P004, *B. thuringiensis* P027 and K031 and *B. amyloliquefaciens* K025 were taken to study the factor affecting the degradation of carbofuran i.e.

pH, light and inoculum size. The effect of pH showed that the higher pH increased the rate of degradation. At pH 8 and 5, *B. thuringiensis* K031 and *Bacillus* sp. P004 were the most effective isolates, respectively. But when compared with pH 7, it showed that the degradation rate was decreased. Light showed the increment of degradation, medium exposed with light slightly increased carbofuran reduction compared to unexposed medium. It is noted that inoculum size at density 10^8cfu/ml of *B. amyloliquefaciens* K025 increased the degradation rate remarkably when compared with density 10^4cfu/ml. In the mean time, there were no different in degradation rate between 10^4 and 10^8cfu/ml when inoculated with others.

B. thuringiensis P027 and *B. megaterium* K004 were studied for the metabolites produced from carbofuran degradation process at 7 days. It was found that 1, 2-benzenedicarboxylic acid or phthalic acid of which molecular weight is 166.14 g/mol was the metabolite in this degradation process. The existence of 1, 2-benzenedicarboxylic acid in uncultured control is due to the chemical degradation process of carbofuran but in cultured samples showed the degradation occurred through chemical and microbial process.

LITERATURE CITED

Anonymous. 1996. **Pesticide Information Profiles**. Extension Toxicology Network. Available Source: http://extoxnet.orst.edu/pips/carbofur.htm, March 30, 2005.

Anonymous. 2000. ***N*-methylcarbamates by High Performance Liquid Chromatography (HPLC)**. Available Source: http://www.epa.gov/epaoswer/hazwaste/test/pdfs/8318a.pdf, February 16, 2005.

Anonymous. 2006a. **Carbofuran**. Available Source: http://www.fao.org/ag/agp/agpp/pesticid/jmpr/download/97_eva/carbofur.pdf, April 12, 2006.

Anonymous. 2006b. **Phthalic Acid**. Wikipedia, the free encyclopedia. Available Source: http://en.wikipedia.org/wiki/1,2-benzenedicarboxylic_acid, February 26, 2006.

Attanandana, T. and J. Chancharoensook. 1999. **Soil and Plant Analysis**. 7th ed. Department of Agriculture, Kasetsart University (*in Thai*).

Barjac, H. D. 1981. Insect pathogens in the genus Bacillus, pp. 241-242. *In* R. C. W. Berkeley and M. Goodfellow, ed. **The Aerobic Endospore-forming Bacteria: Classification and Identification**. Academic Press. 365pp.

Bhadbhade, B. J., S. S. Sarnaik and P. P. Kanekar. 2002. Biomineralization of an organophosphorus pesticide, Monocrotophos, by soil bacteria. **Journal of Applied Microbiology**. 93: 224-234.

Biros, F. J. 1970. **Pesticides Identification at the Residue Level**. American Chemical Society, USA.

Bohmont, B. L. 2000. **The Standard Pesticide User's Guide**. 5th ed. Prentice-Hall, Inc.,USA.

Chapalmadugu, S. and G. R. Chaudhry. 1993. Isolation of a constitutively expressed enzyme for hydrolysis of carbaryl in *Pseudomonas aeruginosa*. **J. Bacteriol**. 175: 6711-6716.

Chaudhry, G. R. and A. N. Ali. 1988. Bacterial metabolism of carbofuran. **Appl. Environ. Microbiol**. 54: 1414-1419.

Chaudhry, G. R., A. Mateen, B. Kaskar, M. Bloda and S. Riazuddin. 2002. Purification and biochemical characterization of the carbamate hydrolase from *Pseudomonas* sp. 50432. **Biotechnol. Appl. Biochem**. 36: 63-70.

Chaudhry, G. R., A. Mateen, B. Kaskar, M. Sardessai, M. Bloda, A. R. Bhatti and S. K. Walia. 2002. Induction of carbofuran oxidation to 4-hydroxycarbofuran by *Pseudomonas* sp. 50432. **FEMS Microbiology Letters**. 214: 171-176.

Cooperative extension offices of Cornell Univesity, Michigan State University, Oregon State Univesity and University of California at Davis. 1993. **Carbofuran**. Extension Toxicology Network. Available Source: http://ace.ace.orst.edu/info/extoxnet/pips/carbofur.htm, September, 1993.

Das, A. C. and D. Mukherjee. 1998. Insecticidal effects on soil microorganisms and their biochemical processes related to soil fertility. **World Journal of Microbiology & Biotechnology**. 14: 903-909.

Das, A. C. and D. Mukherjee. 2000. Soil application of insecticides influences microorganisms and plant nutrients. **Applied Soil Ecology**. 14: 55-62.

Das, A. C., A. Chakravarty, P. Sukul and D. Mukherjee. 2003. Influence and persistence of phorate and carbofuran insecticides on microorganisms in rice field. **Chemosphere**. 53: 1033-1037.

Desaint, S., A. Hartmann, N. R. Perekh and J. C. Fournier. 2000. Genetic diversity of carbofuran-degrading soil bacteria. **FEMS Microbiology Ecology**. 34: 173-180.

Edwards, C. A. 1973. **Environmental Pollution by Pesticides**. Plenum Publishing Company Ltd., Great Britain.

Feng, X., L-T Ou and A. Ogram. 1997. Plasmid-mediated mineralization of carbofuran by *Sphingomonas* sp. strain CF06. **Appl. Environ. Microbiol**. 63: 1332-1337.

Getz, M. E. 1980. **Paper and Thin Layer Chromatographic Analysis of Environmental Toxicants**. Heyden and son Ltd, England.

Gordon, R. E. 1989. Bacillus, pp. 109-126. *In* W. M. O'Leary, ed. **Practical Handbook of Microbiology**. CRC Press Inc., USA. 681p.

Hashimoto, M., M. Fukui, K. Hayano and M. Hayatsu. 2002. Nucleotide sequence and genetic structure of a novel carbaryl hydrolase gene (*ceh A*) from *Rhizobium* sp. strain AC100. **Appl. Environ. Microbiol**. 68: 1220-1227.

Hutson, D. H. and T. R. Roberts. 1994. **Environmental Fate of Pesticides**. A Wiley-Interscience Publication, New York.

Karpouzas, D. G., J. A. W. Morgan and A. Walker. 2000. Isolation and characterization of 23 carbofuran-degrading bacteria from soils from distant geographical areas. **Letters in Applied Microbiology**. 31: 353-358.

Khan, S. U. 1980. **Pesticides in the Soil Environment**. Elsevier Scientific Publishing Company, USA.

Kim, I. S., J. Y. Ryu, H. G. Hur, M. B. Gu, S. D. Kim and J. H. Shim. 2004. *Sphingomonas* sp. strain SB5 degrades carbofuran to a new metabolite by hydrolysis at the furanyl ring. **J. Agric. Food Chem**. 52: 2309-2314.

Krieger, R. 2001. **Handbook of Pesticide Toxicology**. 2nd ed. Academic Press, USA.

Lalah, J. O., S. O. Wandiga and W. C. Dauterman. 1996. Mineralization, volatilization and degradation of carbofuran in soil samples from Kenya. **Bull. Environ. Contam. Toxicol**. 56: 37-41.

Land Development Department. 2006. **Soil Profile Database System**. ArcIMS viewer-soil view. Available Source: http://giswebldd.ldd.go.th/viewer.cfm, May 20, 2006. *(in Thai)*

Matsumura, F. 1980. **Toxicology of Insecticides**. 3rd ed. Plenum Press, USA.

Meher, H.C., N.P. Agnihotri and C.L. Sethi. 1985. Persistence of carbofuran in soil and pea (*Pisum Sativum*) crop and its bioefficiency against soil nematodes. **Indian J. Nematol**. 15(2): 155-158.

Milne, G. W. A. 1995. **CRC Handbook of Pesticides**. CRC Press, Inc., USA.

Mohapatra, S. and M. P. Awasthi. 1997. Enhancement of carbofuran degradation by soil enrichment cultures, bacterial cultures and by synergistic interaction among bacterial cultures. **Pestic. Sci**. 49: 164-168.

Office of Agriculture Regulation. 1994. **Registration of Agricultural Toxic Substances of Thailand**. vol 4. Department of Agriculture, Ministry of Agriculture and Cooperatives (*in Thai*).

Office of Agriculture Regulation. 2000-2003. **Imported Pesticides of Thailand**. Available Source: http://www.doa.go.th/toxic.pdf, October, 2004 (*in Thai*).

Parekh, N. R., A. Walker, S. J. Roberts and S. J. Welch. 1994. Rapid degradation of the triazinone herbicide metamitron by a *Rhodococcus* sp. isolated from treated soil. **J. Appl. Bacteriol**. 77: 467-475

Parekh, N. R., D. L. Suett. S. J. Roberts, T. McKeown, E. D. Shaw and A. A. Jukes. 1994. Carbofuran-degrading bacteria from previously treated field soils**. J. Appl. Bacteriol**. 76: 559-567.

Ramanand, K., M. Sharmila and N. Sethunathan. 1988. Mineralization of carbofuran by a soil bacterium. **Appl. Environ. Microbiol**. 54: 2129-2133.

Roberts, M. S., L. K. Nakamura and F. M. Cohan. 1996. *Bacillus vallismortis* sp. nov., a close relative of *Bacillus subtilis*, isolated from soil in Death Valley, California. **Int. J. Syst. Bacteriol**. 46: 470-475.

Sakong, P. and S. Meepansakul. 2001. **Determinating Organochlorine and Carbamate Pesticide Residue in Tomatoes and Soils**. Available Source: http://www.qru.ac.th/org/research/rajabhat/ ris/11104.htm, December 20, 2005 (*in Thai*).

Shelton, D. R. and J. S. Karns. 1998. Pesticide bioremediation: genetic and ecological considerations, pp. 181-250. *In* P.C. Kearney and T. Roberts, ed. **Pesticide Remediation in Soils and Water**. John Wiley and Sons Ltd., New York.

Siriphontangmun, S. 1991. **Effect of Carbofuran Insecticide on Growth and Yields of Thai Supersweet Composite 1DMR Sweet Corn (*Zea Mays* L. var *saccharata* Bailey)**. M.S. thesis, Kasetsart University. *(in Thai)*

Sneath, P. H. A. 1986. Endospore forming Gram-positive rods and cocci, pp. 1104-1207. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe and J. G. Holt, ed. **Bergey's Manual of Systematic Bacteriology**. Williams and Wilkins Company, USA. 1599p.

Taumcharearn, T. and J. Sriplakij. 1995. Carbofuran analysis by HPLC method. **Toxic newsletter**. 22(2): 90-92 (*in Thai*).

Thaveechai, N., W. Kositratana, S. Vudhivanich, A. Paradornuwat, P. Hamelink and S. Chauevongskul. 1999. **Production of Pathogen-Free Ginger**. 1st ed. Plant Pathogen, Department of Agriculture, Kasetsart University, Bangkok (*in Thai*).

Tomasek, P. H. and J. S. Karns. 1989. Cloning of a carbofuran hydrolase gene from *Achromobacter* sp. strain WM111 and its expression in gram-negative bacteria. **J. Bacteriol**. 171: 4038-4044.

Topp, E., R. S. Hanson, D. B. Ringelberg, D. C. White and R. Wheatcroft. 1993. Isolation and characterization of an N-methylcarbamate insecticide-degrading methylotrophic bacterium. **Appl. Environ. Microbiol**. 59: 3339-3349.

Wongsuwan, D., S. Wansiri, T. Nuanplub, P. Vayuautkae, K. Kumudom. 1992. **Watermelon**. 4th ed. Mitrsiam Press, Bangkok (*in Thai*).

Yarman, Ş. M. 2005. **Petkim Petrochemical Co. (PVC Plant)**. International POPs Elimination Project. Available Source: http://www.ipen.org, June 20, 2006.

APPENDIX

Appendix A

Culture medium formula used to grow microorganisms

1. Minimal medium

K_2HPO_4	0.1 g
$MgSO_4.7H_2O$	0.2 g
$CaSO_4$	0.04 g
$FeSO_4.7H_2O$	0.002g
Agar	15 g
Distilled water	1 l

Autoclave 121°C, 15 minutes

2. Nutrient Agar (NA)

Beef extract	3 g
Peptone	5 g
Agar	15 g
Distilled water	1 l

Autoclave 121°C, 15 minutes

3. Plate Count Agar (PCA)

Tryptone	5 g
Yeast extract	2.5 g
Dextrose (Glucose)	1 g
Agar	15 g
Distilled water	1 l

Autoclave 121°C, 15 minutes

4. Tryptic Soy Agar (TSA)

Tryptone (Pancreatic digest of casein)	15 g
Soytone (enzymatic digest of soybean meal)	5 g
NaCl	5 g
Agar	15 g
Distilled water	1 l

Autoclave 121°C, 15 minutes

Appendix B

Carbofuran residual percentage

Appendix table B1 Carbofuran residual percentage at 3, 5 and 7 day of "P" isolates that belonged to genus *Bacillus* in group IA (*B. megaterium*).

Isolate	Carbofuran residual percentage		
	3 days	5 days	7 days
P001	92.6	66.4	59.0
P002	97.5	86.3	38.7
P007	80.7	67.2	46.4
P008	95.7	80.6	71.0
P009	91.2	80.8	63.0
P011	64.6	58.5	51.8
P021	80.2	78.8	30.2
P022	86.4	70.6	48.9
P028	73.9	72.6	60.3
P029	72.3	75.2	39.3
control	107.8	100.0	96.9

Appendix table B2 Carbofuran residual percentage at 3, 5 and 7 day of "K" isolates that belonged to genus *Bacillus* in group IA (*B. megaterium*).

Isolate	Carbofuran residual percentage		
	3 days	5 days	7 days
K002	70.7	61.7	38.5
K003	110.3	55.6	63.2
K004	67.4	55.3	19.8
K005	66.2	64.4	21.6
K009	94.0	72.3	56.8
K014	129.5	70.5	38.6
K018	68.6	63.0	59.3
K024	106.9	71.2	67.1
control	107.8	100.0	96.9

Appendix table B3 Carbofuran residual percentage at 3, 5 and 7 day of "P" isolates that belonged to genus *Bacillus* in group IA (*B. thuringensis*).

Isolate	Carbofuran residual percentage		
	3 days	5 days	7 days
P006	65.7	80.0	24.8
P010	62.4	61.9	35.8
P012	86.5	65.4	60.0
P023	86.3	77.7	50.1
P024	86.3	68.1	47.5
P026	74.0	49.0	39.6
P027	80.2	78.0	20.6
P030	79.2	33.5	26.0
P031	79.1	55.1	44.8
P033	77.4	68.2	46.2
control	107.8	100.0	96.9

Appendix table B4 Carbofuran residual percentage at 3, 5 and 7 day of "K" isolates that belonged to genus *Bacillus* in group IA (*B. thuringensis*).

Isolate	Carbofuran residual percentage		
	3 days	5 days	7 days
K006	97.2	94.0	68.3
K011	109.3	73.5	68.7
K015	76.6	59.6	57.2
K026	112.7	68.2	74.8
K031	74.8	35.6	47.8
K032	76.8	30.4	37.9
K033	77.4	73.4	58.6
control	107.8	100.0	96.9

Appendix table B5 Carbofuran residual percentage at 3, 5 and 7 day of "P" isolates that belonged to genus *Bacillus* in group IB.

Isolate	Carbofuran residual percentage		
	3 days	5 days	7 days
P013	75.4	79.6	64.5
P014	93.8	85.7	71.3
P015	95.9	86.0	71.7
P016	94.1	81.8	65.1
P017	96.7	80.3	62.4
P018	82.6	55.8	49.2
P019	85.6	72.0	43.2
P020	85.1	81.7	43.1
Control	107.8	100.0	96.9

Appendix table B6 Carbofuran residual percentage at 3, 5 and 7 day of "K" isolates that belonged to genus *Bacillus* in group IB.

Isolate	Carbofuran residual percentage		
	3 days	5 days	7 days
K008	86.7	84.8	50.0
K010	113.4	77.1	68.0
K013	112.4	69.5	63.2
K016	105.1	66.4	76.3
K019	98.8	79.8	55.3
K021	91.4	65.8	46.3
K025	99.4	88.9	34.6
K027	78.3	70.2	54.4
K029	77.2	55.2	32.5
Control	107.8	100.0	96.9

Appendix table B7 Carbofuran residual percentage at 3, 5 and 7 day of the isolates that belonged to genus *Bacillus* in group II and III.

Isolate	Carbofuran residual percentage		
	3 days	5 days	7 days
P003	96.8	79.2	65.5
P004	90.3	41.6	40.3
P005	74.8	65.4	36.7
K001	64.5	64.3	26.6
K007	80.1	88.0	61.3
K012	108.3	105.5	102.7
K017	113.1	80.7	65.3
K020	107.8	77.0	80.5
K022	76.6	80.6	37.0
K023	76.3	72.6	37.8
Control	107.8	100.0	96.9