p,p'-DDT Biotransformation by Bacterial Consortium, Single and Mixed Bacterial Cultures

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ABSTRACT

1,1,1-Trichloro-2,2-bis(p-chlorophenyl)ethane or *p,p′*-DDT is one of the most persistent pesticides known to date due to its chemical composition and properties. Microbial degradation is a potential mean of remediation to remove recalcitrant and toxic compounds such as DDT from the environment. This study was conducted to investigate the DDT transformation ability of indigenous soil bacteria in agricultural soil samples from Songkhla province, Thailand with known DDT levels between 0.19 - 9.84 ng/g soil dry weigh (ppb). Twelve bacterial consortia with 53 isolates were screened from agricultural soil samples of Songkhla province inoculated in mineral salts yeast extract medium (MSYM) supplemented with 100 ppm of *p,p′*-DDT (DDT_{100}). Bacterial consortium PD7, which exhibited a combination of high *p,p′*-DDT transformation of 94.72% and maximum growth within 72 hrs, was chosen for further transformation studies. From the single isolate and mixed culture transformation studies, it was found that increasing DDT transformation were obtained with increasing the number of appropriate isolates involved in the transformation processes. Highest DDT transformation obtained from single isolate, mixed culture of two, three, four and five isolates were observed from isolate PD7-5, Mixed PD7-[3/5], Mixed PD7-[3/4/5], Mixed PD7-[2/3/4/5] and Mixed PD7-[1/2/3/4/5] with 50.63, 57.08, 78.75, 78.52 and 81.57%, respectively. Interestingly, PD7-1, PD7-2 and PD7-3 contributed less significant role on the overall transformation process compared with that obtained by PD7-4 and PD7-5. Moreover, PD7-1 and PD7-2 showed the inhibitory effect on the overall mixed culture studies. Based on 16S rRNA sequences analysis, all five bacterial isolates were identified to be *Curtobacterium citreum* (PD7-1), *Rhodococcus pyridinivorans* (PD7-2), *Niabella* sp. (PD7-3), *Bacillus anthracis* (PD7-4) and *Shinella zoogloeoides* (PD7-5).

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Jaka Darma Jaya

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
DBP	4,4′-dichlorobenzophenone
DDA	bis(4-chlorophenyl)-acetic acid
DDD	Dichlorodiphenyl-dichloroethane
DDE	Dichlorodiphenylchloroethylene
DDM	bis(4-chlorophenyl) methane
DDMS	1-chloro-2,2-bis(4-chlorophenyl)ethane
DDMU	1-chloro-2,2-bis(4-chlorophenyl)ethylene
DDNU	2,2-bis(4-chlorophenyl)ethylene
DDOH	2,2-bis(4-chlorophenyl)ethanol
DDT	Dichlorodiphenyl-trichloroethane
DNA	Deoxyribonucleic acid
GC-ECD	Gas Chromatography - Electron Capture Detector
MR-VP	Methyl Red Voges Proskauer
MSYM	mineral salt yeast extract medium
NA	Nutrient agar
OD	Optical density
PCR	Polymerase chain reaction
PCPA	*p*-chlorophenylacetic acid
ppb	part per billion
ppm	part per million
RNA	Ribonucleic acid
TSI	Triple Sugar Iron
US EPA	United State Environmental Protection Agency
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Background and Rationale

1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane or dichlorodiphenyl-trichloroethane (DDT), discovered as a pesticide in 1939, is the most widely known pesticide of the 20th century. It is also one of the most persistent environmental pollutants because of its toxicity and hydrophobicity, resulting in its bioaccumulation (Kamanavalli and Ninnekar, 2004). Its low solubility, tendency to partition preferentially into lipophilic phase and presence of chlorine atoms make DDT highly ecotoxic especially to higher organisms, with the major mode of toxicity being the attack on central nervous system, interruption of the respiratory system functions, DNA damage in blood cells and disruption of synthesis and metabolism of endogenous hormones (Gautam and Suresh, 2007).

Although the use of DDT has been banned by several developed countries in the 1970s, the developing countries continue to use this pesticide in their agriculture and public health program. These have resulted in DDT contamination of various areas around the world, including soil and sediment in Thailand (Kumblad *et al.*, 2001; Thapinta and Hudak, 2000).

Due to the extremely slow transformation of DDT in natural environments, enhancement of the transformation or mineralization process of DDT by microorganisms has been gaining popularity in the last decades. Biotransformation and biodegradation are two important processes involved in the remediation of DDT contaminated areas. Biotransformation is the chemical modifications of toxic/persistent pollutant by microorganism such as bacteria or fungi, which resulted in the reduction of toxicity or persistency of the metabolites. If this modification ends in compounds like CO_2, NH^{3+} or H_2O, the biotransformation process is referred to as mineralization. Whereas biodegradation referred more to the biotransformation process of pollutant breakdown (decomposition) resulting in simple

metabolite products which may be utilized by the microorganism for growth. A range of bacteria and white rot fungi have been demonstrated to enhance the transformation process in both pure culture and natural environment (Huang *et al.*, 2007). In some cases, biotransformation of DDT using single isolate usually yields low transformation result compared with those of bacterial consortium under the same conditions (Bidlan and Manonmani, 2002). Therefore, it is important to study and understand the transformation capability and relationships of each bacterial species within a microcosm in order to predict the biotransformation of various contaminants in the environment. Thus, in this study we aim to screen for indigenous soil bacteria with DDT transformation abilities and investigate their role and function by comparing DDT transformation of bacterial consortium, single and mixed isolates.

Review of literatures

1. DDT as environmental pollutant

1.1 Properties and chemistry

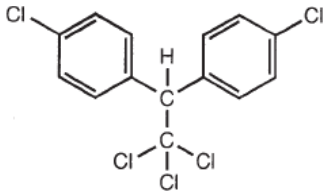
DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane) is a pesticide that belongs to the group of organochlorines compound, which has been extensively used worldwide since the Second World War. Even though its use is banned or restricted in many countries because of its deleterious effects, it is still commonly found in the environment, especially in soil, due to its strong adsorption to solid particles, which results in a high persistency (EPA, 1986).

DDT is a highly hydrophobic, colorless, crystalline solid, with a weak chemical odor. It is nearly insoluble in water but has a good solubility in most organic solvents, fats, and oils (Table 1). DDT is classified by the US Environmental Protection Agency (US EPA) as a moderately toxic chemical (LD_{50} of 50 - 500 mg/kg), with a rat LD_{50} of 113 mg/kg. DDT has been shown to have potent insecticidal properties. Its mode of action is by opening sodium ion channels in insect neurons, causing the neuron to fire spontaneously (WHO, 1979). Therefore, the persistency and toxicity of DDT can be attributed to its chemical and physical

properties, i.e. molecular structure which contains aromatic moieties and chlorine atom, high boiling and melting point and also its low solubility in water.

DDT does not occur naturally, it is produced by the chemical reaction between chloral (C_2HCl_3O) and chlorobenzene (C_6H_5Cl) in the presence of sulfuric acid as a catalyst. Commercial DDT is actually a mixture of several closely related compounds, with *p,p′*-DDT generally comprising 77% of the formulation, *o,p*-DDT 15%, and the related compounds making up the balance. The major metabolites and breakdown products of DDT in the environment are dichlorodiphenyldichloroethylene (DDE), which is produced by the dehydrohalogenation of DDT, and dichlorodiphenyl-dichlorodiphenyl-dichloroethane (DDD), which is produced by the reductive dechlorination. Both DDE and DDD are found in small amounts in commercial DDT samples, such as Anofex, Cezarex, Chlorophenothane, Clofenotane, Dicophane, Dinocide, Gesarol, Guesapon, Guesarol, Gyron, Ixodex, Neocid, Neocidol, and Zerdane (WHO, 1979).

Table 1. Chemical and physical properties of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane (DDT).

	Property
Molecular formula	$C_{14}H_9C_{l5}$
Structure	
Molar mass	354.49 g/mol
Density	1.55 g/cm³
Melting point	108.5–109°C
Boiling point	185–187°C (at 7 Pa)
Vapor pressure	2.3×10^{-5} Pa at 20°C
Water solubility	0.025 ppm at 25°C
Adsorption partition coefficient (K_d)	243,000

Source : Kalloyanova and El-Batawi (1991); Foght *et al.* (2001)

1.2 Effects of DDT in ecosystems

DDT had been widely used throughout the world to control arthropod disease-vectors and agricultural pests. DDT is a hydrophobic and lipophilic compound that easily accumulates in sediment or enters the food chain, and it is thus potentially harmful to human health as well as ecosystems (Huang *et al.*, 2007). As a result of this and the above mentioned chemical and physical characteristics of the compound, DDT residues and its metabolites, especially 1,1-dichloro-2,2,-bis-chlorophenylethane (DDD) and dichlorodiphenylchloroethylene (DDE), have been reported in soil, sediment, both aquatic and terrestrial animals, and agricultural crops in many parts of the world (Table 2).

Table 2. DDT concentrations in different environmental samples.

Sample	Country	Concentration (ppb)	References
Sediment	Spain	9 - 94	Cal *et al.* (2007)
Soil	China	5.942 - 1039	Li *et al.* (2007)
Lake Fish	Thailand	33 - 170	Kumblad *et al.* (2001)
Agricultural soil	Thailand	1.57 - 599.40	Thapinta and Hudak (2000)
Breast milk	Australia	7	Mueller *et al.* (2007)
Children	Spain	330	Espinosa *et al.* (2008)
Birds	Argentina	1068.98 - 6479.84	Cid *et al.* (2007)
Hair	Greece	23.2	Tsatsakis *et al.* (2007)
Rice grain	India	2 - 40	Babu *et al.* (2002)

1.3 Effects of DDT on humans

DDT is one of the most persistent pesticides, which can remain for more than 30 years in soil and accumulates in living organisms through the food chain (bioaccumulation or biomagnification) including human. The exposure pathway of DDT to humans and other animals are through inhalation, ingestion, and dermal adsorption. By estimate, 86% of daily intake of organochlorine compounds including DDT occurs via food consumption. On the other hand, when DDT is solubilized in oil

or organic solvents, it is readily absorbed through the skin and constitutes as considerable hazard. The metabolism of DDT involves various mechanisms, such as oxidation and hydrolysis. They have strong tendency to penetrate cell membranes and store themselves in the body fat and lipid-rich cell, i.e. the central nervous system, liver, kidneys and myocardium. In these organs, they can cause damage to the function of important enzyme systems and disrupt the biochemical activity of the cell. Furthermore, there are reports that this type of compound in our environment may also disrupt normal reproduction and development through inhibition of androgen receptor function (Kelce *et al.*, 1995). The average DDT content for people 5 - 24 years old, 25 - 44 years olds and over 45 years old in Thailand are 8.2, 12.4 and 6 ppm, respectively (Kaloyanova and El-Batawi, 1991).

2. Fate and transformation of DDT in the environment

The fate of pesticides in the environment is determined by both biotic and abiotic factors (Fig. 1). The rate at which different pesticides are biotransformed varies widely. Pesticides such as DDT and dieldrin have proven to be recalcitrant, which remain in the environment for a very long time and are known to accumulate in the food chains for decades after their application to soil. Pesticides which are more readily biodegradable, such as the organophosphates, are now used in preference to the more persistent chlorinated pesticides (Aislabie and Jones, 1995).

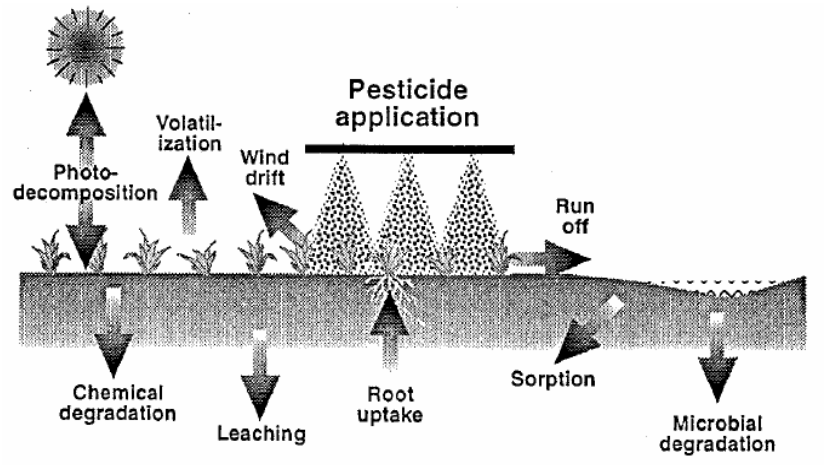


Figure 1. The fate of pesticide in the environment.
Source: Aislabie and Jones (1995)

Reduction of DDT concentrations in soils occurs through physical means such as adsorption, volatilization and erosion, and through chemical means such as abiotic reduction reactions involving biological reductants, nonbiological chemical reductants and photochemical reactions. These physicochemical processes can contribute to overall DDT losses, and in practice it may be difficult to apportion the contributions of abiotic and biotic transformations *in situ* (Foght *et al.*, 2001).

Accumulation of DDT in non-target species are one of the main reasons for placing severe limitations on its use (banned in several countries since 1970s). Morisawa *et al.* (2002) reported the half-life of DDT in various samples with results indicating that DDT can persist in the environment for up to 10 years (Table 3).

Table 3. Half-life of DDT in the environment, agricultural and animal products.

	Element	Half-life (year)
Environment	Air	8.3 - 9.4
	Seawater	1.9 - 2.2
	Soil	2.0 - 10
	Sediment	4.7 - 6.4
Farm Products	Brown rice	10.2
	Potato	3.5
	Orange	8.9
	Spinach	4.4
	Radish	4.8
	Green pepper	2.9
Animal Products	Beef	2.0
	Pork	2.3
	Chicken	3.0
	Chicken egg	7.7
	Cow milk	5.1
Marine Products	Fish	5.7
	Shellfish	7.3
Dietary Intake	-	6.1

Source : Morisawa *et al.* (2002)

Pesticides are transformed in the environment principally by the action of microorganisms or their enzymes (biotransformation). In soil, the initial attack on DDT appears to be centered on the aliphatic trichloroethyl group of the molecule and proceeds in either one of two directions, depending on the prevailing environmental parameters. Under aerobic conditions, DDT undergoes dehydrochlorination to yield DDE. In contrast, under anoxic conditions, transformation of DDT to DDD by reductive dechlorination is considered to be the dominant reaction (Aislabie *et al.*, 1997).

3. Factors affecting DDT biotransformation

Biological reduction of DDT in soils is primarily influenced by factors that affect bioavailability (e.g., adsorption) and microbial activity (e.g., soil temperature, pH, moisture, and organic matter content).

3.1 Bioavailability

The term "bioavailability" describes the ease with which a chemical (nutrient, substrate or toxicant) can be accessed by biological processes. In soils, bioavailability is reduced by physical limitations such as adsorption to mineral surfaces or soil organic matter (to various degrees of reversibility), and by limited aqueous solubility of the chemical, reducing its diffusion in pore water (Foght *et al.*, 2001).

In soils, biotransformation of pesticides can be limited by the poor availability of the chemical. A number of factors influence availability, though effects are primarily due to either low solubility and/or strong adsorption to the soil matrix. The chemical structure of a pesticide, its size and functional groups all determine the water solubility of the compound. Some pesticides are highly soluble, and others are applied as their water-soluble salts or amides to enhance dispersal. Less soluble pesticides are applied as an emulsion or suspension of fine solids in an aqueous carrier, which can aid biotransformation. For growth at the expense of pesticides that have low solubility in water, microorganisms may require some physiological adaptations such as surfactant production (Aislabie and Jones, 1995).

Studies have found that soils high in organic matter, such as peats have significantly lower concentrations of bioavailable DDT than sandy soils, due to

adsorption to soil organic matter. Hydrophobic adsorption onto the surface of humic materials and trapping within a matrix of humic macromolecules have been proposed as important mechanisms of DDT binding, thus reducing the bioavailability of DDT. Soil microorganisms themselves can play a role in binding residues to soil organic matter, mediated through enzymatic or chemical reactions involving microbial products (Foght *et al.*, 2001). With time, residues may become bound and resistant to chemical extraction, may be reversibly adsorbed to soil matter, or may diffuse into pores less than 100 nm in diameter where bacteria, fungi, or root hairs cannot penetrate (Alexander, 2000). Soil temperature affects the binding kinetics of DDT to soil particles, as do soil moisture (Cornelissen *et al*., 1997). Flooding of soils can significantly increase the amount of soil-bound DDT residues, likely by increasing diffusion rates of DDT into micropores or other sites that make the compounds subsequently inaccessible to microbes (Nair *et al*., 1992; Xu and Yongxi, 1996).

Bioavailability diminishes with time and although indigenous soil microorganisms may achieve significant initial rates of transformation of DDT freshly added to soil, the rate decreases substantially with time despite the continued presence of extractable concentrations of DDT in the soil, so that aged DDT persist. For example, long-term monitoring of soil contaminated with DDT, aldrin and its epoxide (dieldrin), heptachlor and its epoxide, and chlordane (Fig. 2), revealed that their disappearance were almost imperceptibly slow after several years (7 years) indicating that those pesticides had become poorly available to the indigenous microorganisms because of soil adsorption (Alexander, 2000).

In fact, there is considerable debate about whether slow adsorption processes result in irreversibly adsorbed residues. In this way, soil appears to remove or diminish the toxicity of a compound before the actual disappearance of the compound, through reduced bioavailability, sequestration, and adsorption. DDT toxicity decreased with the length of time the compounds remained in the soil. Similarly, some studies found that organic compounds incubated in sterile soils became progressively less bioavailable to microorganisms with time, and these compounds became increasingly difficult to remove through conventional solvent extraction methods (Foght *et al.*, 2001).

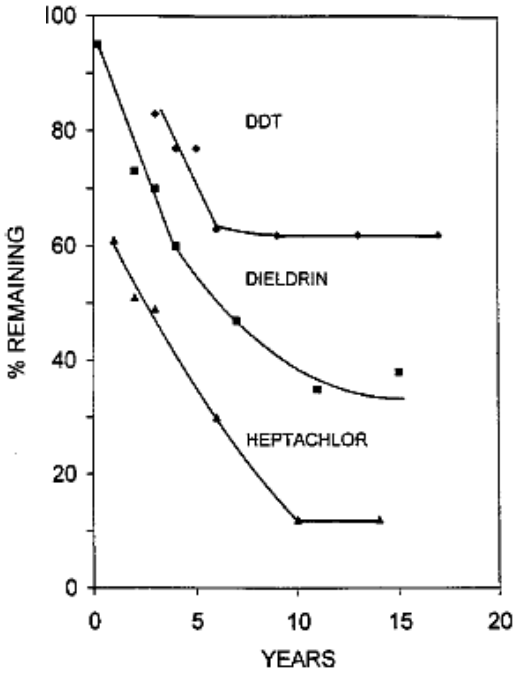


Figure 2. Degradation of DDT, dieldrin and heptachlor in soil.
Source: Alexander (2000)

3.2 Soil properties

Soil microbial biomass is intimately associated with soil organic matter, a complex and heterogeneous mixture of humic substances, polysaccharides, lignins, carbohydrates, lipids, proteins, and organic acids (Table 4). The presence and quality of soil organic matter positively influence biotransformation by sustaining an active microflora and providing a carbon source for co-metabolism of DDT (Gaultier *et al.*, 2007). Similarly, the presence of natural inducers (e.g., lignins and terpenes) may also stimulate DDT biotransformation. Conversely, soil organic matter has an indirect effect on biotransformation of organochlorine pesticide by mean of influencing microbial activity. Szeto and Price (1991) reported longer persistency of organochlorine residues in temperate soils with high organic matter content (e.g., 27 to 56%) than in silky loam (3.7% to 6.5% organic matter) or sandy loam soils with low organic matter (1.0 to 1.8%). In addition, free radicals in solution derived from soil humic and fulvic acids may be inhibitory to DDT-degrading microbial populations (Fujimura *et al.*, 1994).

Table 4. The effect of soil organic content on microbial activity.

Soil organic carbon (%W/W)	Microbial activity (μg fluorescein/g soil)
0 - 0.99	0.16 ± 0.37
1.00 - 1.99	0.38 ± 0.60
2.00 - 2.99	0.38 ± 0.46
3.00 - 3.99	0.32 ± 0.58
> 4	0.47 ± 0.52

Source: Gaultier *et al.* (2007)

Soil pH levels may also affect transformation and dissipation of DDT. Andrea *et al.* (1994) reported that acidic soil (pH 4.5 to 4.8) inhibited volatilization and mineralization of DDT. They also found that low pH greatly reduced the ability of microorganisms to release bound DDT from soils. Studies about the effect of pH on DDT stability showed that soil pH values above pH 7 resulted in significant conversion of ^{14}C-DDT to DDE in both moist and dry soils. This conversion was attributed to microbes in the moist (but not saturated) soils, and to chemical reactions in the dry soils at high pH. Besides affecting general microbial activity and contaminant adsorption, soil moisture affects DDT metabolism by influencing aeration (Nair *et al.*, 1992). As discussed previously, the major residue under aerobic conditions is DDE, whereas under anaerobic conditions such as those produce by flooding (i.e. water-saturated soil), DDD is the major product that accumulates (Boul, 1996; Xu and Yongxi, 1996).

Soil temperature directly affects microbial activity (and hence DDT biotransformation) and indirectly affects soil DDT concentrations through volatility losses. Nair *et al.* (1992) observed that up to 30% of radiolabel added as ^{14}C-DDT was recovered in the volatile fraction from soils exposed to solar irradiation for 42 days.

3.3 DDT concentrations and presence of co-contaminants

Low concentrations, combined with poor bioavailability, can contribute to the persistence of those contaminants that serve as growth substrates and

require induction of degradative enzymes (Boethling and Alexander, 1979), but low concentrations should be immaterial for DDT as degradation is co-metabolic.

Soils may be impacted by more than one contaminant. For example, soil at a pesticide mixing facility was contaminated to depth with DDT, arsenic, toxaphene and xylene. Such co-contaminants can complicate biotransformation: (1) by providing more readily utilizable substrates for the microflora, thus diverting enzymatic activity from the contaminant of concern; (2) by having specific or nonspecific toxic effects on the soil microflora; or (3) by affecting the solubility or adsorption of the contaminant of concern, decreasing its bioavailability or increasing its mobility through co-solvation transport. Conversely, co-contaminants theoretically could enhance biodegradation by serving as a co-metabolic carbon and energy source or an inducer of DDT degradation (Foght *et al.,* 2001).

The presence of arsenic in soil inhibits intrinsic breakdown of DDT to DDD. The model for DDD levels in soil shows a response curve where DDD increases with the level of DDT but declines as arsenic concentrations increase for a given concentration of DDT (Fig. 3) (Zwieten *et al.*, 2002).

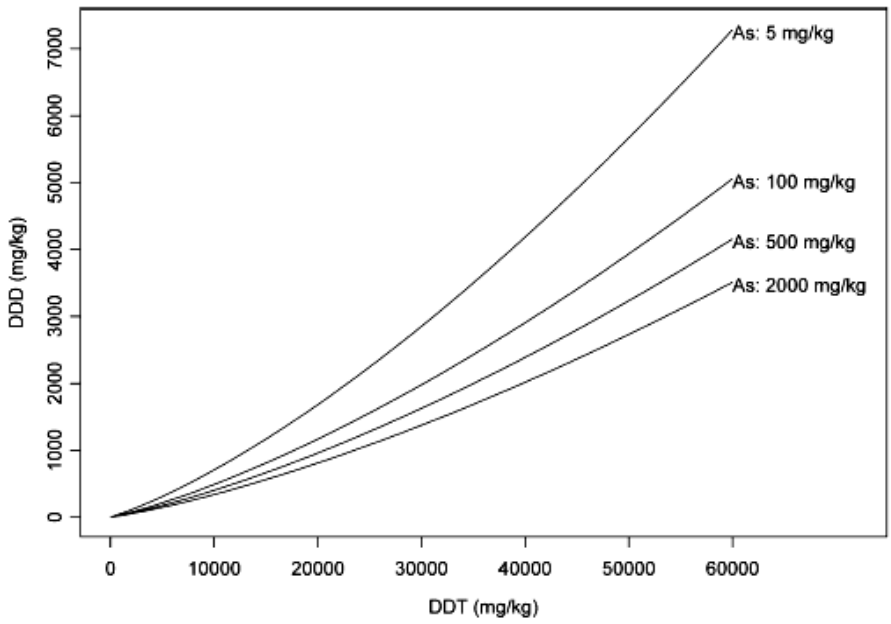


Figure 3. Inhibition of DDT degradation in the presence of arsenic.
Source: Zwieten *et al.* (2002)

4. Microbial transformation of DDT

Microbes play an important role to transform pesticide pollutant in environment. Transformation of DDT also depends on the presence and numbers of microbes in the contaminated soil with their required transformation ability. These microbes may be resident in the soil or they may be isolated from elsewhere and introduced to soil (Table 5). Microbes potentially useful for the biotransformation of DDT in soil include the bacteria and fungi that metabolize DDT via reductive dechlorination, ligninolytic fungi, and the chlorobiphenyl-degrading bacteria which carry out ring cleavage of DDT under aerobic conditions. Alternatively, microbes with the ability to transform DDT can be constructed using molecular techniques and introduced into the contaminated soil (Golovleva *et al.,* 1988).

Although there is evidence for the existence of DDT-metabolizing microbes in soils, and some have been isolated, their prevalence is unknown. From laboratory experiments, it is clear that the transformation of DDT to DDD occurs readily in spiked soils under certain conditions. The process may be attributed directly to microbial activity, either bacterial or fungal (Wedemeyer, 1966; Subba-Rao and Alexander, 1985), or indirectly to the generation of anaerobic conditions and/or the production and release of biomolecules that act as reductants, such as iron porphyrins (Zoro *et al.*, 1974).

4.1 Biotransformation of DDT under anaerobic conditions

Contaminated area usually constitutes lack of oxygen, thus indigenous bacteria need to grow and survive in anaerobic conditions. Reports have indicated that DDT is reductively dechlorinated to DDD under anaerobic conditions and then further metabolized. For example, pure cultures of *Escherichia coli* and *Enterobacter aerogenes* incubated with DDT produced trace amounts of 1-chloro-2,2-bis(4-chlorophenyl)ethylene (DDMU), 1-chloro-2,2-bis(4-chlorophenyl)ethane (DDMS), 2,2-bis(4-chlorophenyl)ethylene (DDNU), 2,2-bis(4-chlorophenyl)ethanol (DDOH), bis(4-chlorophenyl)-acetic acid (DDA), and 4,4′-dichlorobenzophenone (DBP) (Langlois *et al*., 1970; Wedemeyer, 1967). Under anaerobic condition, DBP was not further metabolized (Aislabie *et al*., 1997). In contrast, under aerobic soil conditions DDT is dehydrochlorinated to yield predominantly DDE (Foght *et al.,* 2001).

Table 5. Examples of microorganisms able to transform DDT.

Type	Source*	Reference
Bacteria		
Proteus vulgaris	Mouse intestine	Barker *et al.* (1965)
Enterobacter aerogenes	NA	Wedemeyer (1967)
Bacillus spp., *Enterobacter aerogenes*	NA	Langlois *et al.* (1970)
Pseudomonas sp.	NA	Francis *et al.* (1977)
Ralstonia eutropha A5	Soil	Nadeau *et al.* (1994); Hay and Focht (2000)
Pseudomonas acidovorans M3GY	Genetically engineered	Hay and Focht (1998)
Terrabacter sp. strain DDE 1	Soil	Aislabie *et al.* (1999)
Serratia marcescens	Soil	Bidlan and Manonmani (2002)
Rhodococcus sp., *Klebsiella* sp.	Sewage	Cruz *et al.* (1999)
Cyanobacteria	Soil	Megharaj *et al.* (1998)
Pseudomonas fluorescens	NA	Santacruz *et al.* (2005)
Mycobacterium spp.	Soil	Yagi *et al.* (1999)
Pseudomonas sp.	Soil	Kamanavalli and Ninnekar (2004)
Fungi		
Saccharomyces cerevisiae	Bakers yeast	Kallman and Andrews (1963)
Aspergillus flavus, *Thanatephorus cucumeris*	NA	Subba-Rao and Alexander (1985)
Phanerochaete chrysosporium, *Pleurotus ostreatus*, *Phellinus weirii*, *Polyporus versicolor*	NA	Bumpus and Aust (1987)
Phlebia strigoso-zonata, *Basidiomycete*	Rotting wood	Katayama *et al,* (1993)
Phanerochaete chrysosporium	NA	Bumpus *et al.* (1993)

* NA = Not available

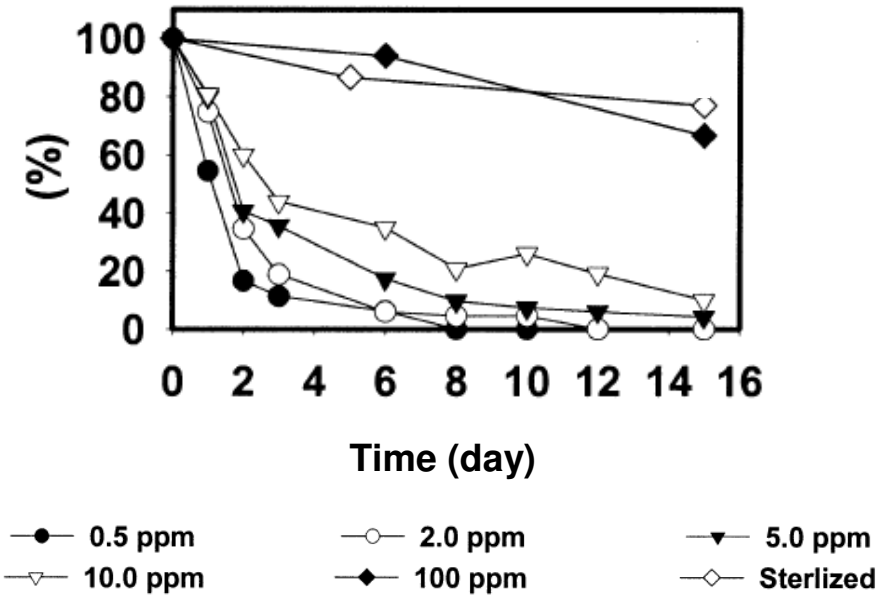


Figure 4. Anaerobic degradation of *p,p′*-DDT under different initial concentration at 30°C by bacterial consortium from river sediment.

Source: Chiu *et al.* (2004)

Studies have shown anaerobic degradation of DDT in soil, estuarine and river sediment (Chiu *et al.*, 2004; Cruz *et al.*, 1999; Huang *et al.*, 2007). The use of anaerobic mixed culture can degrade and transform *p,p′*-DDT to *p,p′*-DDD. The degradation rate of sterilized culture was much less than that in non-sterilized culture. The results indicated that the dissipation of DDT was attributed to microbial degradation with more than 95% of the initial DDT (0.5 - 10 ppm) degraded after 15 days incubation (Fig. 4).

4.2 Biotransformation of DDT under aerobic conditions

Although most articles reported reductive dechlorination of DDT under anaerobic conditions as the major mechanism of DDT biotransformation, aerobic DDT degradation have also been described, involving aromatic-degrading bacteria, i.e. promotion of ring cleavage reactions (Nadeau *et al.*, 1994; Hay and Focht, 1998, 2000; Bidlan and Manonmani, 2002).

DDT metabolizing microbes have been isolated from a range of habitats, including animals, soil, rotting wood, sewage, and activated sludge. Biodegradation of DDT primarily is co-metabolic, as the microbes involve do not derive any nutrient or energy for growth from the process, and require an alternate carbon source for growth. Co-metabolic reaction of DDT degrading bacteria required O_2 utilize nutrient. Aislabie *et al.* (1997) reported that only the first step in the process, that is the reductive dechlorination of DDT to DDD, took place without an additional substrate. All other degradative reactions, until the formation of benzhydrol, proceeded exclusively under co-metabolic conditions.

It is often difficult to isolate microbes attacking a compound co-metabolically from the environment. A technique known as analogue enrichment in which a structural analogue is substituted for the compound of interest is therefore adopted. The DDT-metabolizer "*Pseudomonas* sp.", for example, was isolated from sewage when 1,1-bis(*p*-chlorophenyl)ethane and bis(*p*-chlorophenyl)methane, a structural analogue of DDT, was supplied for growth (Francis *et al.,* 1977). This organism grew on both DDT structural analogue and was able to co-metabolize DDT. A study reported *Alcaligenes eutrophus* A5 could metabolize both *o,p′*- and *p,p′*-DDT isomers when incubated in high density of resting cell cultures. The mechanism for attack presumably involves the same enzymes that are specific for 4-chlorobiphenyl degradation, which in DDT appears to be oxidized by a dioxygenase to yield a dihydrodiol-DDT derivative that undergoes *meta*-cleavage, ultimately yielding 4-chlorobenzoic acid (Nadeau *et al.*, 1994).

There are many reports of bacteria having the ability to degrade DDT under aerobic condition, e.g., *Serratia marcescens* DT-1P (Bidlan and Manonmani, 2002), *Alcaligenes eutrophus* A5 (Nadeau *et al.*, 1994), *Mycobacterium* spp. (Yagi *et al.*, 1999), *Pseudomonas putida* and *Enterobacter* sp. (Subba-Rao and Alexander, 1985). *Serratia marcescens* DT-1P was able to degrade nearly 40% of the initial DDT of 5 to 15 ppm in 24 hrs after incubation, with complete degradation within 5 days (Bidlan and Manonmani, 2002). Increasing DDT concentration to 20 ppm or above resulted in partial biodegradtion even after 8 days. Addition of 50 ppm DDT resulted in an inhibitory effect to microbial degradation of the compound (Fig. 5).

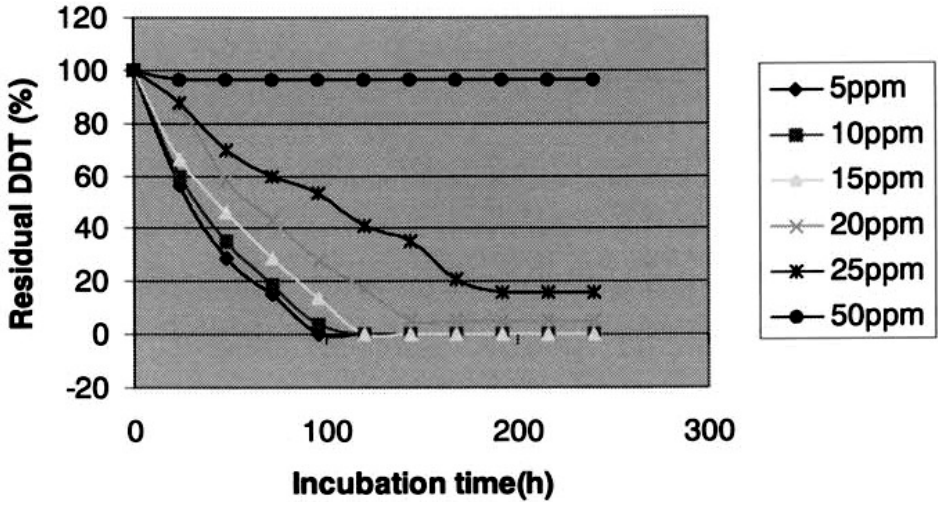


Figure 5. Aerobic degradation of different concentration of DDT by *Serratia marcescens* DT-1P at ambient temperature (26-30^{o}C)

Source: Bidlan and Manonmani (2002)

5. Mechanisms of DDT biotransformation

5.1 Reductive dechlorination

Under reducing conditions, reductive dechlorination is the major mechanism for the microbial conversion of both the *o,p′*-DDT and *p,p′*-DDT isomers of DDT to DDD. The reaction involves substitution of an aliphatic chlorine atom for a hydrogen atom and requires transition metals and metal complexes acting as reductants (Fig. 6) (Holliger and Schraa, 1994).

In most cases, the process involves single electron transfer, removal of a chlorine ion, and formation of an alkyl radical. This is not an energy-yielding process for the organism, unlike other reductive reactions (e.g., sulfate reduction), as DDT is not serving as the terminal electron acceptor in a bioenergetic pathway. As mentioned previously, reductive dechlorination of DDT can also occur in the absence of viable organisms, catalyzed by biomolecules like hematins; DDD can also be reductively dechlorinated by this mechanism, but not for DDE (Baxter, 1990).

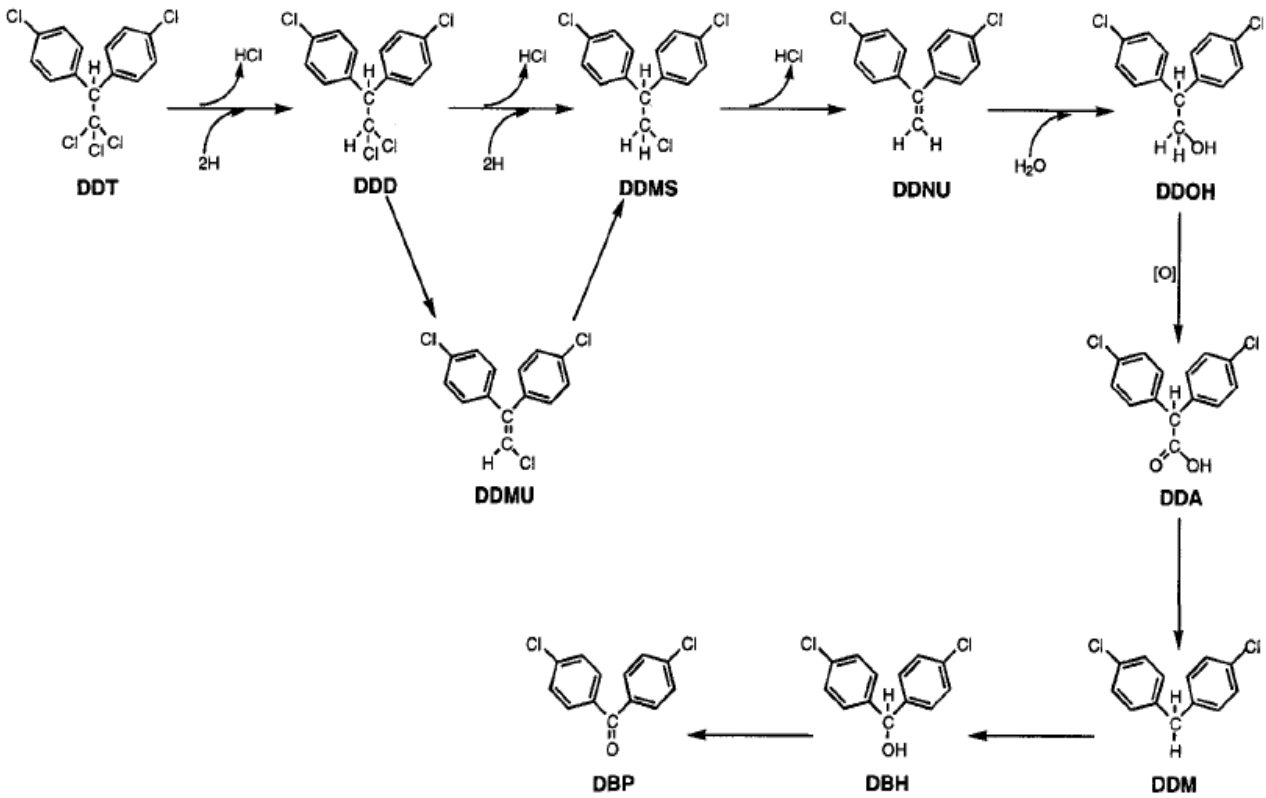


Figure 6. Proposed pathway for bacterial metabolism of DDT via reductive dechlorination.

Source: Wedemeyer (1967); Langlois *et al.* (1970)

Under anaerobic conditions, DDD may be further metabolized. For example, pure cultures of *Escherichia coli* and *Enterobacter aerogenes* (formerly *Aerobacter aerogenes*) incubated with DDT produced trace amounts of 1-chloro-2,2-bis(4-chlorophenyl)ethylene (DDMU), 1-chloro-2,2-bis(4-chlorophenyl)ethane (DDMS), 2,2-bis(4-chlorophenyl)ethylene (DDNU), 2,2-bis(4-chlorophenyl)ethanol (DDOH), bis(4-chlorophenyl)-acetic acid (DDA), and 4,4′-dichlorobenzophenone (DBP) (Langlois *et al*., 1970; Wedemeyer, 1967), in addition to the major metabolite DDD. The proposed pathway for the anaerobic transformation of DDT by bacteria (Fig. 6) shows that DDD is reductively dechlorinated to DDNU, which is successively oxidized to DDOH and DDA, the latter of which in turn is decarboxylated to bis(4-

chlorophenyl) methane (DDM). DDM is oxidized to DBP, which is not further metabolized under anaerobic conditions (Pfaender and Alexander, 1972).

Study of the transformation of DDT by *Pseudomonas aeruginosa* 640X (Golovleva and Skryabin, 1981) has shown that this strain either mineralizes DDT or degrades it significantly to produce (nonchlorinated) phenylacetic, phenylpropionic, and salicylic acids. However, all steps after the first reductive dechlorination of DDT to DDD until the formation of benzhydrol were found to require co-metabolic substrates, and the extent of co-metabolism depended on the nature of those substrates and on the aeration conditions. Strain 640X was subsequently genetically modified by introduction of a plasmid encoding naphthalene and salicylate degradation, creating *P. aeruginosa* strain BS827 that degraded kelthane (dicofol) to unknown products (Golovleva *et al*., 1988).

Alternating anaerobic and aerobic incubation conditions can enhance DDT biodegradation by promoting reductive dechlorination of DDT to DPB with subsequent aerobic aromatic ring cleavage. Pfaender and Alexander (1972) observed that cell-free extracts of a *Pseudomonas* sp. metabolized ^{14}C-DDT to DDD, DDMS, DDNU, and DBP under anaerobic conditions. When these metabolites were subsequently incubated under aerobic conditions with fresh *Pseudomonas* sp. inoculum, *p*-chlorophenylacetic acid (PCPA) was identified as a product. This metabolite was found to be susceptible to further degradation by an *Arthrobacter* sp., producing *p*-chlorophenylglycoaldehyde. Because PCPA was not produced under strict anaerobic conditions, these *in vitro* studies implied that the ring cleavage reactions required molecular oxygen. However, DDT was not metabolized by the *Pseudomonas* sp. under strictly aerobic conditions either, demonstrating the advantage of using alternating anaerobic-aerobic conditions to achieve significant DDT degradation in this system.

Anaerobic bacterial dechlorination of DDE (which is primarily an aerobic transformation product of DDT) to DDMU was reported in pure culture (Massé *et al.,* 1989) and this transformation has since been described in soil and anaerobic marine sediments under methanogenic and sulfidogenic conditions (Quensen *et al.*, 1998).

5.2 Oxidative transformation of DDT via ring cleavage reactions

Microbes that transform DDT co-metabolically can be difficult to isolate from the environment because there is no direct means of enriching or selecting for that property. Instead, a technique known as analogue enrichment, in which a structural analogue is substituted for the compound of interest, has proven useful. Using this method, a "*Hydrogenomonas*" sp. (*Pseudomonas* sp.) capable of co-metabolizing DDT was isolated from sewage by providing diphenylmethane, a structural analogue of DDT, as a growth substrate during enrichment (Foght *et al*., 2001). Similarly the ability to degrade 4-chlorobiphenyl, another structural analogue of DDT, was used to select bacteria capable of co-metabolizing DDT (Nadeau *et al*., 1994).

Some of the 4-chlorobiphenyl degrading bacteria were also subsequently shown to degrade DDT through novel pathways. Cultures of *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) A5 slowly metabolized both the 4,4 *p*- and 2,4 *o*-DDT isomers (Nadeau *et al.*, 1994), presumably using 4-chlorobiphenyl degradation enzymes. This mechanism was suggested because the initial attack on DDT appeared to involve a dioxygenase. The product was a dihydrodiol-DDT intermediate that then underwent *meta*-cleavage to produce 4-chlorobenzoic acid, representing extensive aerobic metabolism of DDT. In contrast, strain B-206, a 4-chlorobiphenyl-degrading Gram-negative bacterium, produced phenolic metabolites from DDT, DDE, DDD, and DDMU but no ring cleavage products.

Recent studies have shown that degradation of DDE by *Pseudomonas acidovorans* M3GY (Hay and Focht, 1998) and *Terrabacter* sp. DDE-1 (Aislabie *et al.*, 1999) also proceed via *meta*-ring cleavage (Fig. 7) under aerobic conditions when the bacterium is induced with biphenyl. In fact, the ring cleavage pathways for degradation of DDT, DDE and DDD appear to be analogous based on detection of similar metabolites. Thus, despite reports to the contrary, compound such as DDE are susceptible to *in vitro* aerobic microbial degradation (Megharaj *et al.,* 1998). This is a significant observation, as DDE has previously been considered a dead-end metabolite of DDT under aerobic conditions.

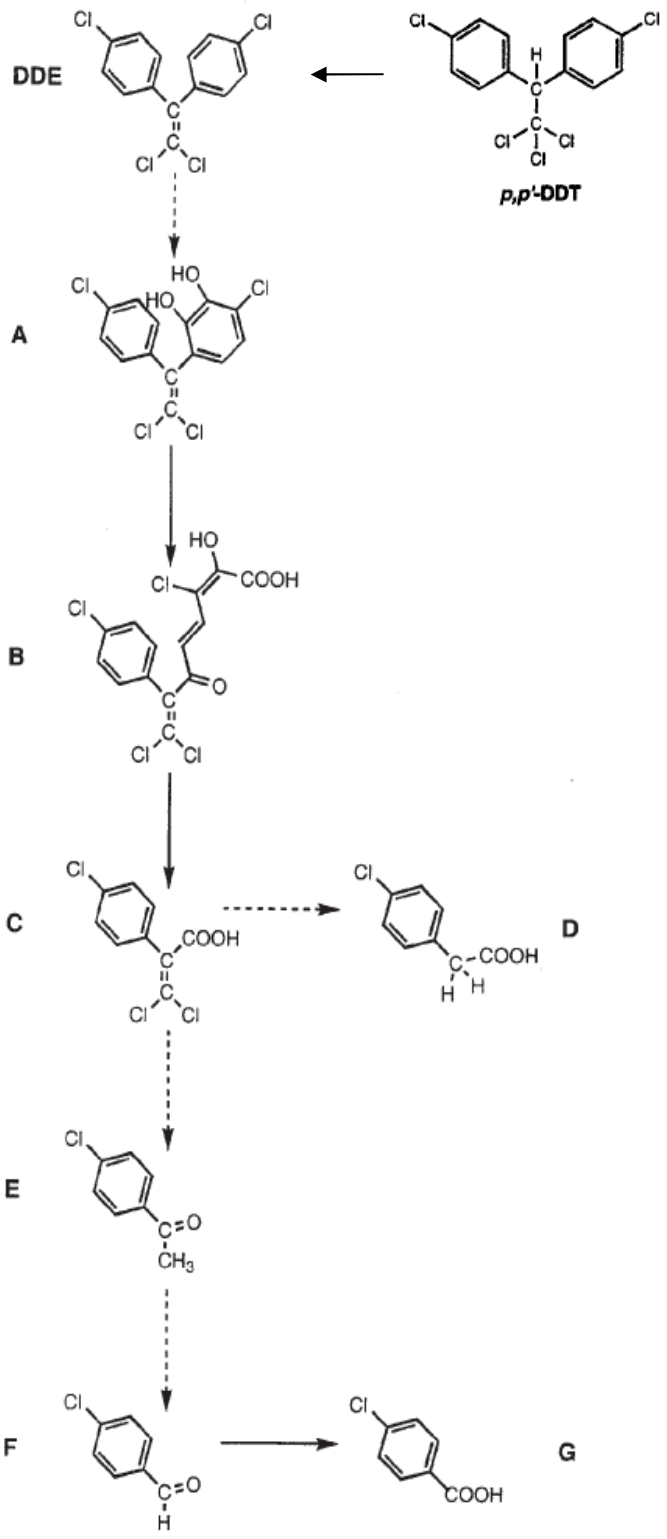


Figure 7. Proposed *meta*-ring cleavage pathway for the degradation of DDE.

Source: modified from Hay and Focht (1998); Nadeau *et al.* (1994); Aislabie *et al.* (1999)

5.3 Mineralization of DDT by ligninolytic fungi

Ligninolytic fungi have been shown to possess biodegradative capabilities for a broad spectrum of environmentally persistent compounds, including DDT. This capability has been attributed to their ability to degrade lignin. The majority of work on DDT degradation by ligninolytic fungi has been carried out by Aust and colleagues during the 1980s using a white rot fungus *Phanerochaete chrysosporium*. Degradation of ^{14}C-labelled DDT in cultures of *P. chrysosporium* deficient in nutrient nitrogen (N) showed that 50% of DDT was transformed during this period with 10% being mineralized and the remainder appeared as metabolites including dicofol, FW-152, and DBP (Bumpus and Aust, 1987). These results led to the proposal of a DDT degradation pathway involving oxidation to dicofol followed by dechlorination to FW-152 and subsequent breakdown via DBP (Fig. 8).

This pathway was thought to be controlled by the ligninase system as mineralization and dicofol production were observed only after lag phase during which ligninase production was established. DDD was the only product during this initial phase and was subsequently degraded. It was therefore concluded that DDD was produced by a mechanism distinct from the ligninase system, but degraded by it, while DDE was not produced. Moreover, it was discovered that C-source greatly influenced the extent of DDT mineralization by *P. chrysosporium*. Starch and cellulose supported much greater mineralization of ^{14}C-DDT than other complex carbohydrates or sugars. Mineralization only occurred in the presence of an utilizable C-sources and $^{14}CO_2$ release stopped when available carbohydrate was exhausted.

The involvement of the ligninase system is important as it suggests the organisms and conditions that may facilitate DDT transformation. As previously described, DDT mineralization coincided with ligninase production. DDT mineralization began immediately when added to ligninolytic culture, but if the culture was initiated from spores, the onset of degradation and ligninolytic activity was exhibited after about 4 days. Inhibitors of ligninase such as EDTA and tetramethylethylenediamine also inhibited DDT mineralization. Although some xenobiotic compounds are oxidized by purified lignin peroxidases, this does not happen with DDT, indicating that DDT is co-oxidized by a mediator molecule such as veratryl alcohol rather than directly by the enzyme (Foght *et al*., 2001).

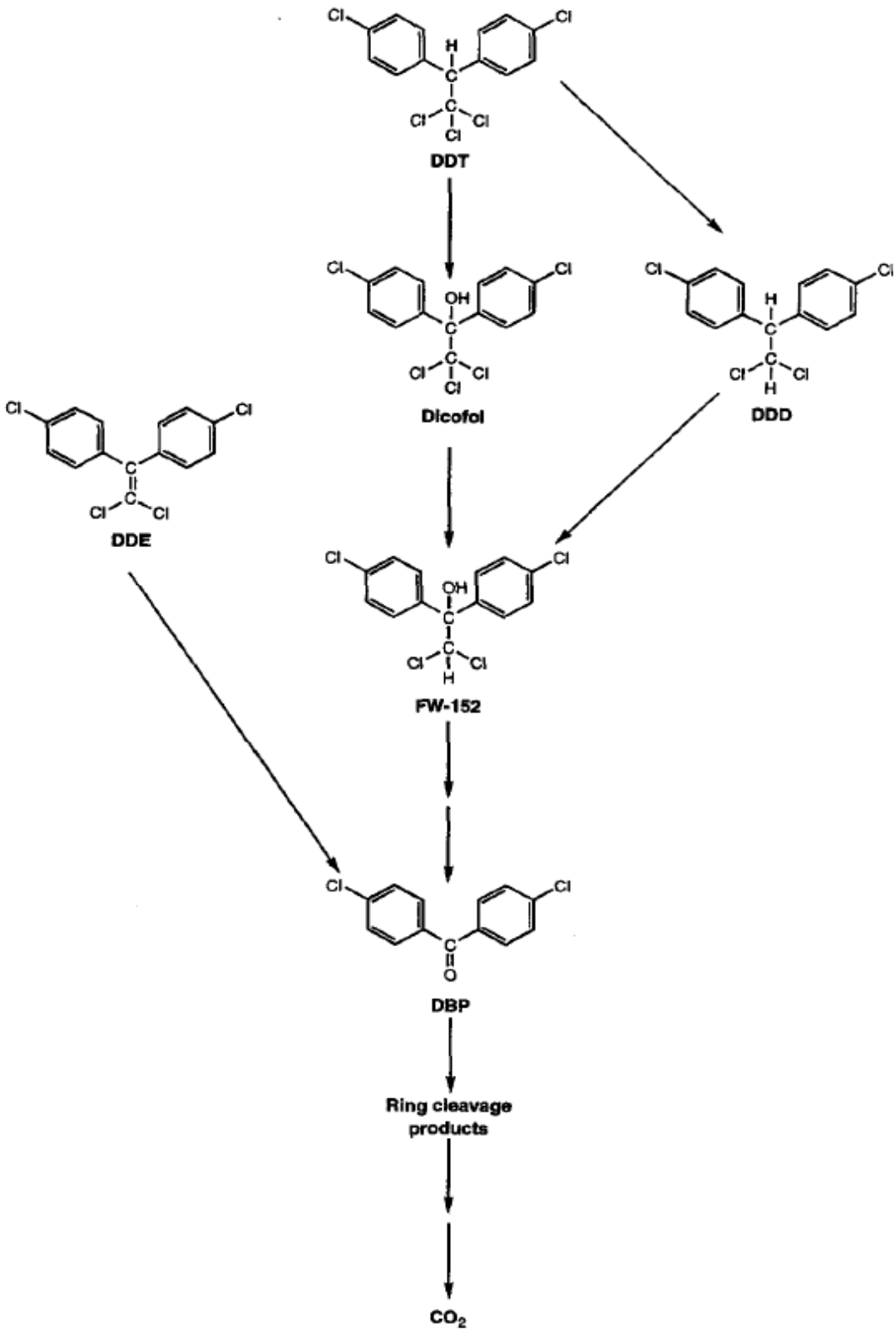


Figure 8. Proposed pathway for DDT degradation by *Phanerochaete chrysosporium*. Source: Bumpus and Aust (1987)

6. DDT biotransformation by bacterial consortium, single and mixed isolates

There have been studies on DDT biotransformation by both pure and mixed bacterial cultures. Most pure culture study was aimed at understanding the role of each bacterial species in DDT biodegradation, whereas mixed culture studies were to optimize DDT degradation. Furthermore, study has been performed at understanding the biodegradation ability of natural bacterial consortium (Bidlan and Manonmani, 2002). All in all microbial application may eventually improve the bioremediation and biodegradation processes.

6.1 Study on biotransformation process by single bacterial isolates

Many studies reported bacterial strain capable of DDT transformation, e.g. *Alcaligenes euthrophus* (Nadeau *et al.,* 1994), *Pseudomonas* sp. (Cruz *et al.,* 1999; Kamanavalli and Ninnekar, 2004; Huerta *et al*., 2007), *Aeromonas* sp., *Enterobacter* sp., *Arthrobacter* sp., *Dermabacter* sp., *Lactobacillus* sp. (Huerta *et al.*, 2007), *Pseudomonas fluorescens* (Santacruz *et al.*, 2005).

Proteus vulgaris, isolated from the intestinal microflora of a mouse, was one of the first pure cultures observed to reduce DDT to DDD (Barker *et al.,* 1965). Study of the transformation of DDT by *Pseudomonas aeruginosa* 640X (Golovleva and Skryabin, 1981) has shown to be able either mineralizes or degrades DDT significantly to produce (nonchlorinated) phenylacetic, phenylpropionic, and salicylic acids. However, all steps after the first reductive dechlorination of DDT to DDD until the formation of benzhydrol were found to require co-metabolic substrates, and the extent of co-metabolism depended on the nature of those substrates and on the aeration conditions.

Furthermore, Nadeau *et al.* (1994) investigated DDT mineralization by single isolate of *Alcaligenes euthrophus* A5. The study showed the involvement of dioxygenase, in which the oxidation product is subsequently subjected to ring fission to eventually yield 4-chlorobenzoic acid as a major stable intermediate. Similar metabolite was observed by Kamanavalli and Ninnekar (2004) in their study with *Pseudomonas* sp. In this latter study, bacterial strain capable of degrading DDT was isolated from insecticide-contaminated soil by biphenyl enrichment technique. The microorganism degraded DDT through the intermediate formation of 2,3-dihydroxy

DDT, which undergoes *meta*-ring cleavage, ultimately yielding 4-chlorobenzoic acid as a stable metabolite.

Modification of DDT biodegradation has been performed using the bacteria grown in microniches created in the porous structure of green bean coffee (Fig. 9) (Huerta *et al.*, 2007). Five bacteria isolated from coffee beans, identified as *Pseudomonas aeruginosa*, *P. putida*, *Stenotrophomonas maltophilia*, *Flavimonas oryzihabitans* and *Morganella morganii*. *P. aeruginosa* and *F. oryzihabitans*, were selected for pesticide degradation according to their ability to grow on mineral media amended with: (a) glucose (10 g/l), (b) peptone (2 g/l), and (c) ground coffee beans (2 g/l). These three media were supplemented with 50mg/l of *p,p'*-DDT and endosulfan. It was found that, *F. oryzihabitants* and *P. aeruginosa* were able to grow with different carbon sources when supplemented with 50 ppm of DDT or endosulfan. In all cases, biomass production was higher than 0.3 g/l and reached a maximum between 24 and 48 hrs of incubation at 30^{o}C and 100 rpm. No significant endosulfan degradation by *F. oryzihabitans* was observed. In contrast, *P. aeruginosa* provided between 30 to 50% endosulfan degradation in all media after 7 days of incubation.

On the other hand, removal of DDT was higher than that of endosulfan, indicating that both bacteria preferentially degrade aromatic compounds. A DDT concentration of only 32 - 37% remained in the coffee bean culture medium after 7 days of incubation. Changes in the DDE concentration indicate its production during DDT biodegradation, as well as its degradation, in the medium with coffee bean addition. DDMU and 2,2-bis (*p*-chlorophenyl) ethanol (DDOH) metabolites were also identified by GC/MS in the culture broth.

The above experiment was performed under aerobic conditions. However, it is possible that inside the porous structure of the coffee bean, anaerobic microniches could be created, promoting the reductive dechlorination of DDE to DDMU. In addition, the DDOH peak in the chromatogram indicates further transformation of DDMU (Huerta *et al.*, 2007). The combination of anaerobic and aerobic environments enhances the mineralization of many electrophilic aromatic contaminants such as organochlorine and azo compounds, for which several strategies have been proposed (Field *et al.*, 1995).

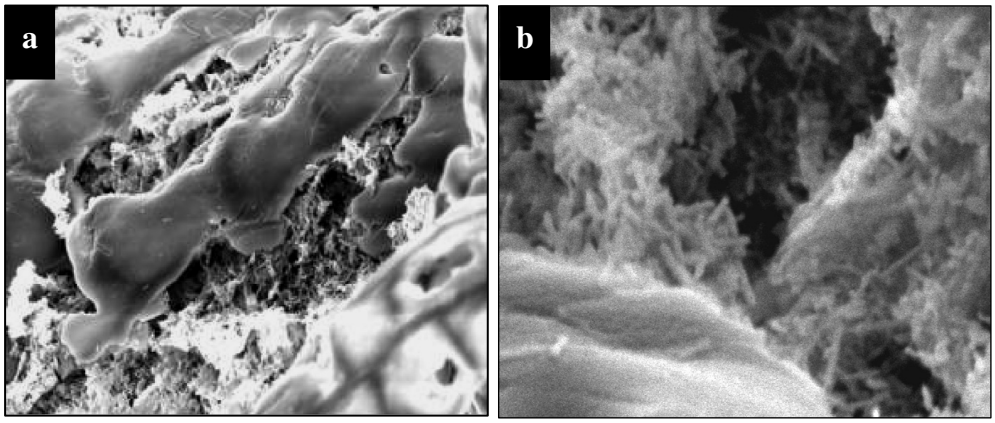


Figure 9. SEM photomicrographs showing bacterial colonization (a, 5000x) in microniches of green bean coffee (b, 500x). *F. Oryzihabitans* in coffee bean (2 g/l) and 50 ppm DDT, at 30^{o}C, 100 rpm, 30 days incubation.

Source : Huerta *et al.* (2007)

6.2 Study on biotransformation process by bacterial consortium and mixed bacterial culture

Since the rate of DDT biotransformation by single bacterial isolates are usually low, studies have been performed to investigate biotransformation process by mixed bacterial isolates, either in the form of natural bacterial mixture (consortium) or in form of defined mixed bacterial culture (Bidlan and Manonmani, 2002; Hii *et al*., 2008; Beunink and Rehm, 1988; Liz. *et al*., 2009; Ramirez *et al.,* 2008). Mixed culture study was usually aimed at optimizing DDT transformation.

Bidlan and Manonmani (2002) studied the roles of bacterial consortium from soil and its pure isolate of *Serratia marcescen* in DDT biodegradation (Fig. 10). The result showed that DDT degradation by bacterial consortium was higher than that of single isolate. In addition, microorganism was gradually acclimated with increasing concentrations of DDT from 5 to 25 ppm. After 6 months of enrichment in DDT contaminated soil, a microbial consortium was further enriched in shake flasks containing DDT as a sole source of carbon and shown to completely degrade 25 ppm of DDT within 144 hours (Fig. 10a). DDT was added as an acetone solution and degradation was determined by the release of chloride ions. The rate of DDT degradation by acclimated bacterial consortium gradually decreased

as DDT concentration increased. In all cases the stoichiometric release of 100% chloride was observed. In the case of DDT transformation by *S. marcescen* DT-1P, mineralization only occurred with concentration of 15 ppm or lower (Fig. 10b). The rate of DDT degradation by single isolate was significantly slower than that of bacterial consortium. Five ppm of DDT was shown to be completely degraded by the bacterial consortium in 48 hours while *S. marcescen* took DT-1P 96 hours to achieve the same level of degradation.

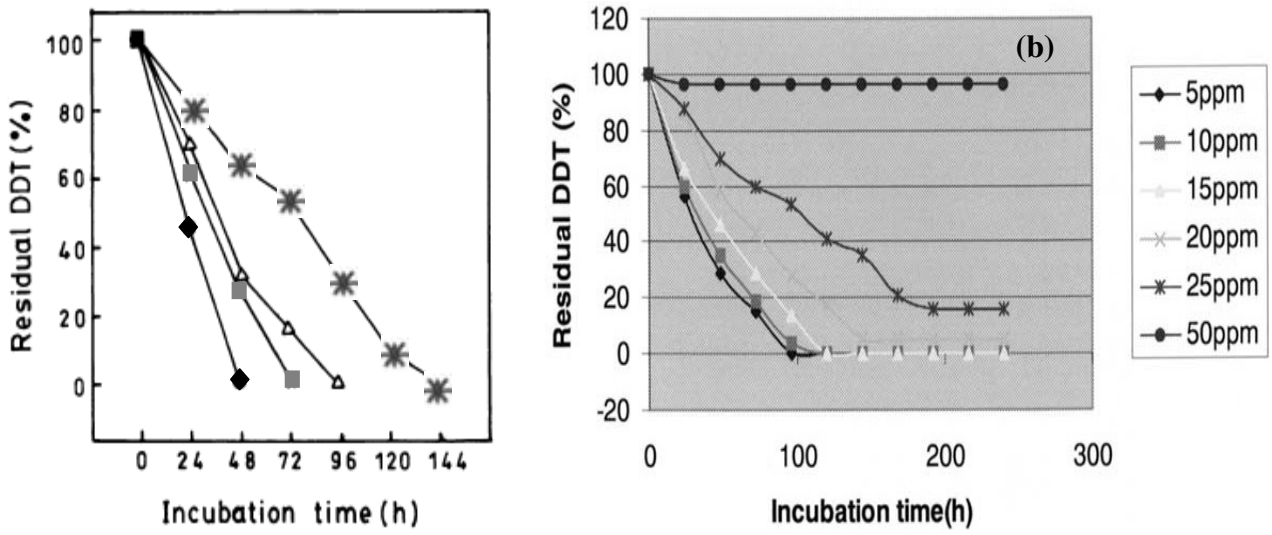


Figure 10. Comparison of DDT degradation by bacterial consortium (a) and single isolate of *Serratia marcescen* DT-1P (b).

Source: Bidlan and Manonmani (2002)

Chiu *et al.* (2004) reported the degradation of DDT by bacterial consortium screened from river sediment. The bacterial consortium was observed to degrade and transform *p,p′*-DDT to *p,p′*-DDD. The results indicated that DDT from 0.5 to 10 ppm was almost completely degraded (>90% degraded) after 15 days. The same as the above phenomenon observed by Bidlan and Manonmani (2002), who found that consortium activity was completely inhibited in the medium containing 100 ppm of DDT. At this concentration, no metabolites were detected, which suggests a limiting concentration value for DDT dechlorination where the dechlorination activity may be inhibited when the concentration exceeds this value.

Study on the degradation of other organochlorine pesticide by defined bacterial consortium (mixed culture) has also been performed (Murthy and Manonmani, 2007). The consortium was developed by long-term enrichment in contaminate soil and sewage samples, and acclimated with increasing concentrations of tech-HCH (technical grade) from 5 to 25 ppm. This defined consortium was found to degrade up to 25 ppm of tech-HCH when an inoculum level of 100 μg protein/ml was applied and cultivated at ambient temperature (26 - 28^{o}C), pH 7.5. The bacterial consortium was shown to consist of *Pseudomonas fluorescens* biovar I, II, and V, *P. diminuta*, *Burkholderia pseudomallei*, *P. putida*, *Flavobacterium* sp., *Vibrio alginolyticus*, *P. aeruginosa*, and *P. stutzeri*. Unfortunately, the study did not report the biodegradation of DDT by defined consortium (mixed culture).

7. Interaction between bacteria within bacterial community or consortium

7.1 Synchronous (symbiotic) interaction

In many cases, interaction between bacteria within a community will result in synchronous effect of growth and activity. Hii *et al.* (2008) reported that bacterial consortium exhibited higher degradation of total aliphatic hydrocarbons (TAHs; C_{12}-C_{34}) after 10 days of incubation in comparison with those by single bacterial isolate (*P. pseudoalcaligenes*) (Fig. 11). Moreover, a study on anaerobic and aerobic degradation of DDT by immobilized mixed bacterial culture showed that a degradation system with both conditions resulted in improvement of the degradation process (Beunink and Rehm, 1988). These results suggest the synchronous interaction between each bacterial member in the consortium which enabled the enhancement of the overall degradation.

Although DDT is metabolized through co-metabolic pathways by a number of facultative and obligate anaerobic microorganisms under suitable environmental conditions, no organism is known which utilizes DDT as sole source of carbon and energy. Nevertheless, a complete mineralization of DDT by sequential cooperation of microbial populations through co-metabolic reactions is possible (Focht and Alexander 1970; Pfaender and Alexander 1972). After reductive dechlorination of the aliphatic part of the molecule accompanied by the formation of 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane (DDD, TDE) and further dehalogenation

of DDD to 4,4'-dichlorodiphenylmethane (DDM) under subsequent oxidative conditions, hydroxylations and ring cleavage reactions were observed (La and Saxena 1982). This sequence of oxidative and reductive processes is necessary for the co-metabolic degradation of DDT. Initial oxidative reactions can lead to the formation of 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene (DDE) by dehydrochlorination or 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethanol) by hydroxylation. These products were observed to be metabolized very slowly (Lal and Saxena 1982).

Due to the necessity of this sequence of reductive and oxidative conditions for the microbial degradation of DDT, the degradation process needs to proceed via both aerobic and anaerobic bacteria. During the process, a system with entrapped microorganisms should be established, in which reductive dechlorinations and oxidative ring fission reactions proceed simultaneously. The above model-reactions of the dechlorination of DDT to DDD and the oxidative degradation of DDM have been studied by Beunink and Rehm (1988).

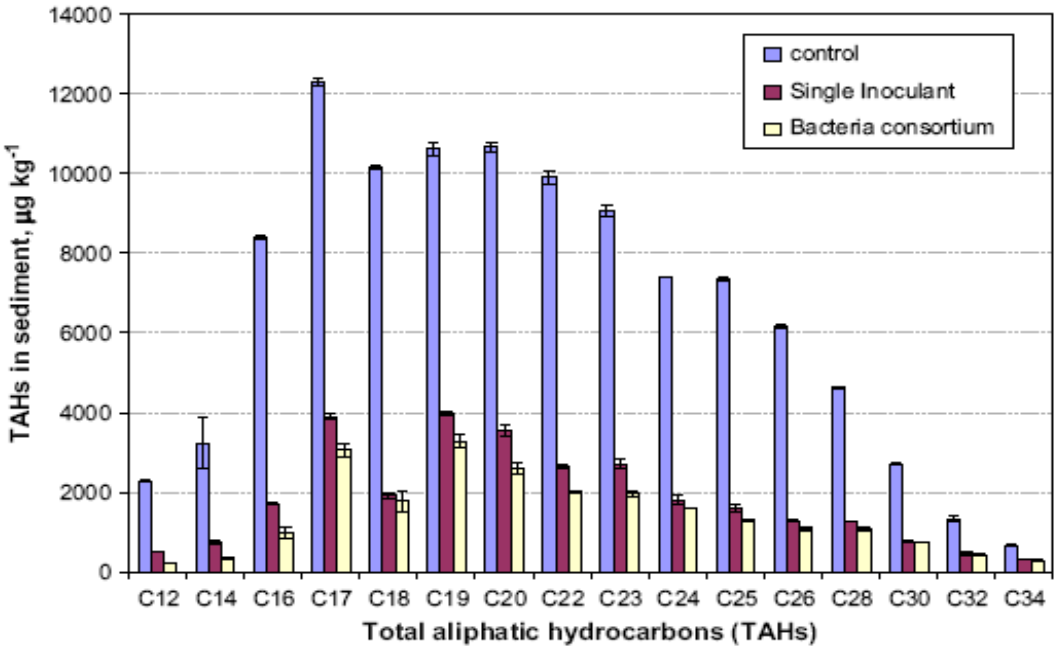


Figure 11. Total aliphatic hydrocarbons remaining in sediment after 10 days of biodegradation by single inoculant (*P. pseudoalcaligenes*) and bacterial consortium.

Source : Hii *et al.* (2008)

7.2 Antagonistic interaction

Another possible bacterial interaction which has been shown is the inhibitory effect on each bacterial activity within a consortium (Syakti *et al.*, 2004). The study involved two hydrocarboclastic strains isolated from contaminated marine sediment. One was identified as *Corynebacterium* sp., a gram-positive strain able to grow on alkanes, particularly on *n-eicosane* (C_{20}). The second was identified as *Sphingomonas* sp. 2MPII (DSMZ11572) and was able to use the polyaromatic hydrocarbon, phenanthrene (PHE) as its sole source of carbon and energy.

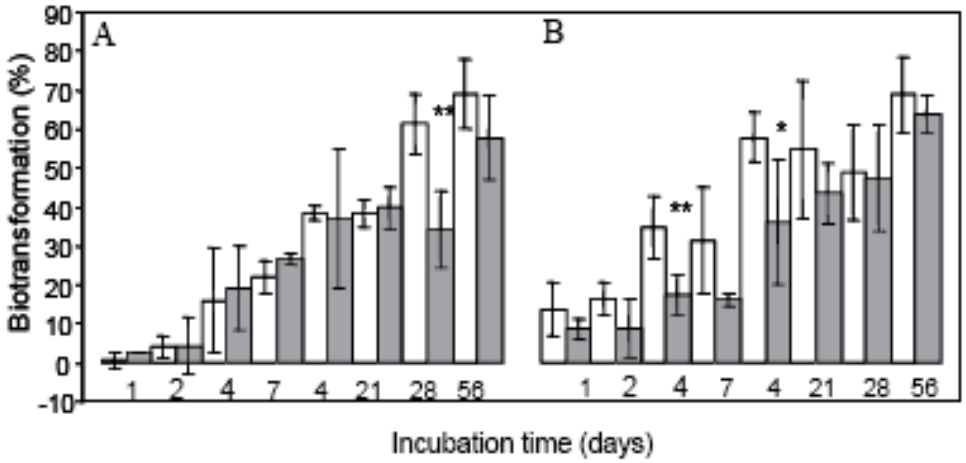


Figure 12. Percentages of *n*-eicosane (A) and phenanthrene biotransformation (B) by *Corynebacterium* sp. and *Sphingomonas* strain 2MPII, respectively, in pure (□) and mixed cultures (■) over 56 days.

Source : Syakti *et al.* (2004)

In pure cultures, *Corynebacterium* sp. was unable to grow on PHE, whereas strain 2MPII was unable to grow on C_{20}. Moreover, for the pure cultures on mixed substrates, no co-metabolism occurred. Indeed, *Corynebacterium* sp. could not utilize PHE in the presence of C_{20}, and strain 2MPII was unable to use C_{20} in the presence of PHE. However, when both strains were co-incubated and supplemented with a single substrate (C_{20} or PHE), the target substrate uptake rate was not affected but a decrease in degradation percentage (refer to as biotranformation) was observed, which could be due to catabolite repression (Fig. 12) (Syakti *et al.,* 2004).

Objectives

- Screening and identification of DDT transforming bacteria isolated from soil samples.
- Comparing the DDT transformation of bacterial consortium, single and mixed isolates.

Scope of research

The scope of this research includes screening of soil bacteria with the ability to grow in medium containing DDT and selecting bacterial consortium with the highest DDT transformation ability. Bacterial isolate was identified based on morphological, biochemical and 16S rRNA characteristics. Lastly, comparison of DDT biotransformation ability by bacterial consortium, single and mixed bacterial isolates was investigated.

CHAPTER II

RESEARCH METHODOLOGY

Materials and Equipments

1. Chemicals

The chemicals used in this research are analytical standardized from various suppliers as mentioned in the following list:

	Chemicals	Suppliers
1.	Aldrin [1,1,1 Trichloro 2,2 bis(4-chlorophenyl)ethane] or *p,p'*-DDT	Sigma Aldrich Chemie GmbH, Germany
2.	Ethyl Acetate	Lab Scan, Thailand
3.	Acetone	Lab Scan, Thailand
4.	PCR purification Kit	QIAGEN, Inc.
5.	Folin-Ciocalteu reagent	Merck, Germany
6.	DNAzol® Genomic DNA extraction reagent	Invitrogen, USA

2. Equipments

Scientific instruments used in this research consist of equipment as mentioned in the following list:

	List of equipments
1.	Gas Chromatography-Electron Capture Detector (GC-ECD) model Hewlettt-Packard 6890 and HP-35 capillary column
2.	GeneAmp PCR System 2400 Thermocycler
3.	Electrophoresis apparatus and power supply
4.	UV Transilluminator
5.	Microcentrifuge
6.	Microplate reader : model Zenyth 200rt

3. Soil samples

All 12 soil samples used in this study are sample stocks collected and prepared by Sonkong (2007) from soil in various agriculture area of Songkhla Province with history of continued farming activities for more than 30 years. Each samples were collected from 3 - 4 positions within the area. Samples were collected from the first 15 cm of soil surface and kept on ice or at 4^{o}C. Soil were air-dried and sieved through a 10 millimesh before homogenously mixed to acquire a good representation of soil sample from each sampling area. These samples were used for screening of *p,p′*-DDT degrading bacteria.

4. Cultivation and biotransformation medium

Mineral salt yeast extract medium (MSYM) used for screening and degradation studies consisted of (per 1 liter of distilled water) 0.20 g $MgSO_4.7H_2O$, 0.25 g NH_4NO_3, 0.675 g K_2HPO_4, 0.10 g $Ca(NO_3)_2$ and 0.01% yeast extract (modified from Nadeau, 1995). The medium pH was adjusted to 7.0 and sterilized by autoclaving at 121^{o}C for 15 min. Supplemented *p,p′*-DDT was filter-sterilized and added to the medium at a concentration of 20, 40, 60, 80 or 100 ppm**.**

p,p′-DDT was prepared as *p,p′*-DDT stock of $1x10^4$ mg/l (ppm) in acetone solutions. Starter culture medium (MSYM+DDT_{20}) was prepared by adding 0.2 ml of *p,p′*-DDT stock solution to 100 ml of sterilized MSYM to obtain 20 ppm final concentration.

Methods

Analytical methods

1. Measurement of bacterial growth by estimating total cell protein (Stoscheck, 1990)

Bacterial growth was measured by estimating total cell protein (Stoscheck, 1990). Cell was harvested from 1 ml culture broth by centrifugation at 10,000 rpm for 10 min. Supernatant was decanted and cell pellet was washed with 1 ml 0.85% NaCl solution. This step was repeated and cell pellet was then mixed with 1 ml 0.1 N NaOH and digested at 90^{o}C for 15 min. After the reaction mixture cooled to

room temperature, 100 µl of sample was pipetted into a microplate well. The samples were then mixed with 100 µl of Lowry's reagent and incubated at room temperature for 10 minutes. Fifty microliters of 0.2 N Folin-Ciocalteau reagent was added and further incubated at room temperature for 30 min. Protein concentration was analyzed spectrophotometrically at 750 nm in comparison to a standard bovine serum albumin (BSA) concentration (see Appendix 3).

Note: Lowry's reagent was prepared by mixing solution A, B and C in the ratio of 3:1:1. While Folin-Ciocalteu reagent was freshly prepared before analysis by diluting with distilled water in the ratio of 10:1 (0.2 N). Solution A was prepared by dissolving 20 g Na_2CO_3 in 260 ml distilled water, 0.4 g $CuSO_4.5H_2O$ in 20 ml distilled water and 0.2 g sodium potassium tartrate in 20 ml distilled water, and then those solutions were mixed together. Ten grams of sodium dodecyl sulfate (SDS) was dissolved in 100 ml water (solution B). NaOH 1 N solution was prepared as solution C.

2. Quantitative determination of *p,p'*-DDT by gas chromatography-electron capture detector (GC-ECD)

2.1 Extraction of *p,p'*-DDT from cultivation broth (modified from Bidlan and Manonmani, 2002)

Two mililiters of sample from cultivation broth was extracted three times with 6 ml of ethyl acetate. Organic phase was collected and then evaporated overnight to dryness. The residue was redissolved in 2 ml of acetone. A known quantity of the sample was injected into gas chromatography with electron capture detector (GC-ECD) for analysis.

2.2 *p,p'*-DDT analysis by GC-ECD

p,p'-DDT concentration was determined by gas chromatography-^{63}Ni micro electron capture detector (GC-ECD) using the following conditions: HP-35 capillary column (35% crosslinked methyl phenyl siloxane; 30 m x 0.25 µm ID); injector and detector temperatures of 250^{o}C and 320^{o}C, respectively; oven temperature programmed at 150^{o}C for 1 min and increasing at 20^{o}C/min to 250^{o}C then held for 4 min. The carrier and make up gasses were helium (2 ml/min) and nitrogen (60 ml/min), respectively. Injection was performed using the split injection

technique with 50:1 injection ratio. Standard curve was constructed by injecting 1 µl of *p,p′*-DDT at 1, 5, 10, 50 and 100 ppm. This standard curve was used to quantify *p,p′*-DDT concentration from both soil and culture broth samples (see Appendix 4).

Experimental methods

1. Screening of bacterial consortium and isolates with the ability to grow in the presence of *p,p′*-DDT

Mineral salt yeast extract medium (MSYM) was used to screen for *p,p′*-DDT degrading bacteria. Ten gram of soil sample was added to 100 ml MSYM supplemented with 20 ppm of *p,p′*-DDT (DDT_{20}). Culture was incubated at room temperature, 150 rpm for 2 days or until medium turbidity was observed. Sub-culturing was then performed with 10% inoculum into MSYM supplemented with 40, 60, 80 and 100 ppm of *p,p′*-DDT respectively. Culture was again incubated at the conditions previously described. To screen for single bacterial isolate, 100 µl of bacterial consortium grown at 100 ppm of *p,p′*-DDT was spread on MSYM+DDT_{100} agar and incubated at room temperature until bacterial colony was observed. All bacterial consortia and single isolates were stored as stock cultures in MSYM+DDT_{20} containing 20% glycerol at -20^{o}C.

2. Screening of bacterial consortium and isolates with the ability to transform *p,p′*-DDT

For starter culture, 100 µl of each stock culture was added to 10 ml MSYM+DDT_{20} and incubated at room temperature, 150 rpm until turbidity was observed (approximately OD_{600} = 0.5). Ten mililiter of sample was added to 100 ml MSYM+DDT_{100} and incubated at room temperature, 150 rpm until exponential phase of growth was reached. Samples were collected every 12 hours for the determination of growth and *p,p′*-DDT degradation (see Analytical methods 1 and 2). Bacterial consortium showing the highest ability of *p,p′*-DDT transformation was selected for further studies.

3. Comparison of *p,p′*-DDT transformation by bacterial consortium, single and mixed isolates

Selected bacterial consortium and individual isolates were prepared in MSYM+DDT_{20} as starter cultures. Ten mililiter of starter culture was inoculated into 100 ml MSYM+DDT_{100} and incubated at room temperature, 150 rpm for 10 days.

For mixed culture studies, the combination ratio of 1:1 (v/v) of each bacterial isolate (OD_{600} = 0.5 of the single isolate starter cultures) was inoculated into MSYM+DDT_{100} and incubated at the previously mentioned condition. Samples were collected every 12 hours up to 3 days and then every 24 hours from days 4 to 10. These samples were used for the determination of growth and *p,p′*-DDT concentration. Negative control of MSYM+DDT_{100} without inoculum was routinely used to observe the effects of abiotic factors such as light and evaporation on *p,p′*-DDT reduction. While MSYM+DDT_{100} inoculated with the selected bacterial consortium was considered as a positive control during single and mixed isolates studies.

4. Identification of *p,p′*-DDT transforming bacterial isolates

Isolated bacteria were identified based on their cells/colony morphology and biochemical properties. While 16S rRNA sequences analysis was performed on the isolates from the consortium with the highest ability of *p,p′*-DDT degradation.

4.1 16S rDNA sequence analysis

- DNA preparation and extraction

Colony grown on nutrient agar were picked up and resuspended in 10 µl water or BL buffer (40 mM Tris, 1% Tween 200, 0.5% Nonidet P-40, 1 mM EDTA:2Na, pH 8.0). The cell suspension was then incubated at 95^{o}C for 15 min.

- Amplification and purification of 16S rDNA

Amplification of 16S rDNA is performed in a reaction volume of 25 µl. PCR reaction consisted of 1 µl of the above suspension, 0.1 µl of Ex*T*aq DNA polymerase, 2 µl of dNTPs, 2.5 µl of Ex*T*aq DNA polymerase buffer, 1 µl of each primers Eu8f (AGAGTTTGATCCTGGCTCAG) and Eu1492r (GGCTACCTTGTTACGACTT) and water up to 25 µl. Negative controls consisted

of the PCR reaction mixture without DNA sample (substituted with sterile water and sterile water prepared in the same way as the samples) were included in each set of PCR amplification. PCR amplification composed of the following steps: initial denaturation at 95°C for 5 min and then performed with 25 cycles as follows: denaturation at 95°C for 1 min, annealing at 50°C for 30 sec and extension at 72°C for 1.5 min followed by final extension step of 5 min at 72°C then immediately cooled at 15°C. Amplification products (5 µl of reacted mixture) were separated by 0.7% agarose gel electrophoresis and 1.5 kb DNA band was visualized under UV light after staining with ethidium bromide.

One microliter of sodium acetate (pH 5.5, 3M) and 50 µl of 100% ethanol were added to the rest 20 µl of amplified DNA. The mixture was then centrifuged at 13,000 rpm at 4°C for 10 min and the supernatant was discarded. DNA was washed by adding 70% ethanol and centrifuged again at 13,000 rpm. Supernatant was then discarded completely and air-dried. DNA was resuspended in 15 µl of TE buffer and stored at -20°C.

- **Sequencing and phylogenetic analysis**

Sequencing was performed using the purified DNA as a template. The 1.5 kb rDNA amplicon was sequenced with following primers: Eu8f (AGAGTTTGATCCTGGCTCAG), Eu1092f (AAGTCCCGCAACGAGCGCA), Eu1492r (GGCTACCTTGTTACGACTT), Eu518r (GTATTACCGCGGCTGCTGG), Eu803r (CATCGTTTACGGCGTGGAC), Eu1093r (TTGCGCTCGTTGCGGGACT) and Eu1389r (ACGGGCGGTGTGTACAAG). Sequences were compared to 16S rDNA gene sequences database of GenBank via BLAST search (http://www.ncbi.nlm.nih.gov). The phylogenetic tree was constructed by Treeview software program.

CHAPTER III

RESULTS AND DISCUSSION

1. Screening of bacterial consortium and isolates with the ability to grow on *p,p′*-DDT supplemented medium

Soil samples from agricultural area in Songkhla province with history of continuous farming activities for many decades were used for bacterial screening. Based on previous studies by Sonkong (2007) and Jeenon (2008), these 12 agricultural areas were reported to contain both *p,p′*-DDT and γ-HCH in varying amount of 0.19 - 9.84 and 0.03 - 0.45 ng/g soil dry weigh (ppb), respectively (Table 6). Since there have been reports showing effective *p,p′*-DDT degradation under aerobic condition (Nadeau *et al*., 1993; Bidlan and Manonmani, 2002), soil were collected from the first 15 cm of the surface. This collecting strategy was chosen in order to obtain high diversities of aerobic/facultative anaerobic bacterial communities, which may be accustomed to the prolong exposure of pesticides, particularly *p,p′*-DDT.

To further increase the probability of isolating *p,p′*-DDT degrading soil bacteria, selective enrichment and acclimatization to the increasing concentration of *p,p′*-DDT from 20 to 100 ppm in the cultivation medium (MSYM) were performed. These technique was proven to be able to increase the ability of growth as well as degradation of *p,p′*-DDT in the cultivation medium. The observation have been made in studies with DDT (Bidlan and Manonmani, 2002) and HCH (Manonmani *et al.*, 2000), where acclimatization improved the degradation ability of DDT/HCH degrading consortium. The acclimated consortium showed a higher rate of degradation. Bhuyan *et al.* (1992) and Wada *et al.* (1989) have made similar observation, where γ-HCH degradation increased after every successive application of the compound.

From these procedures, all soil samples were shown to contain bacterial consortium tolerant to the supplemented *p,p′*-DDT. Selective enrichment and acclimatization process ultimately resulted in the survival of 12 bacterial consortia

with 53 morphologically distinct isolates (Table 7). The isolates were observed to be 29 Gram negative rod-shaped and 24 Gram negative coccoid-shaped bacteria. Thirteen isolates also showed evidence of producing extracellular polymeric-liked substance as seen when grown on MSYM+DDT_{100} agar (Fig. 13d - f). It was interesting that all isolates were observed to be Gram-negative bacteria. In many cases, Gram-negative bacteria were isolated from hydrocarbon-contaminated area (Bicca *et al.*, 1999; Bodour *et al.*, 2003). Gram-negative bacteria with DDT degrading ability such as

Table 6. Texture and organochlorine pesticide residues (γ-HCH and *p,p′*-DDT) of soil samples.

Soil	Area	γ-HCH concentration (ng/g soil dry wt) [(a)]	*p,p′*-DDT concentration (ng/g soil dry wt) [(b)]	Soil texture
1	Cabbage field	0.03	0.19	loam
2	Broccoli field	ND	0.80	loam
3	Broccoli field	ND	0.52	loam
4	Sediment from irrigation ditch	0.45	1.81	silty clay
5	Water spinach field	0.08	0.34	loam
6	Broccoli field	ND	0.84	loam
7	Chilli field	ND	6.27	loam
8	Yu Choy field	ND	0.95	loam
9	Chinese Parsley field	0.17	0.24	loam
10	Broccoli field	ND	9.84	laterite
11	Chinese Kale/ Broccoli field	0.22	0.62	loam
12	Lettuce field	ND	0.79	laterite

ND : Not detected

Source : (a) Jeenon (2008); (b) Songkong (2007)

Pseudomonas sp., *Serratia marcescens*, *Pseudomonas acidovorans*, *Escherichia coli*, *Ralstonia eutropha* have also been reported (Langlois *et al.*, 1970; Nadeau *et al.*, 1994; Hay and Focht, 1998; Bidlan and Manonmani, 2002; Kamanavalli and Ninnekar, 2004). The possible reason for this phenomenon is due to the cellular structure of Gram-negative bacteria, especially the outer membrane. This outer membrane composes of lipopolysaccharides, which acts as biosurfactant to stimulate the release of hydrophobic contaminant absorbed in soil organic matter and increase the bioavailability of the contaminant to microorganisms (Mercade *et al.*, 1996).

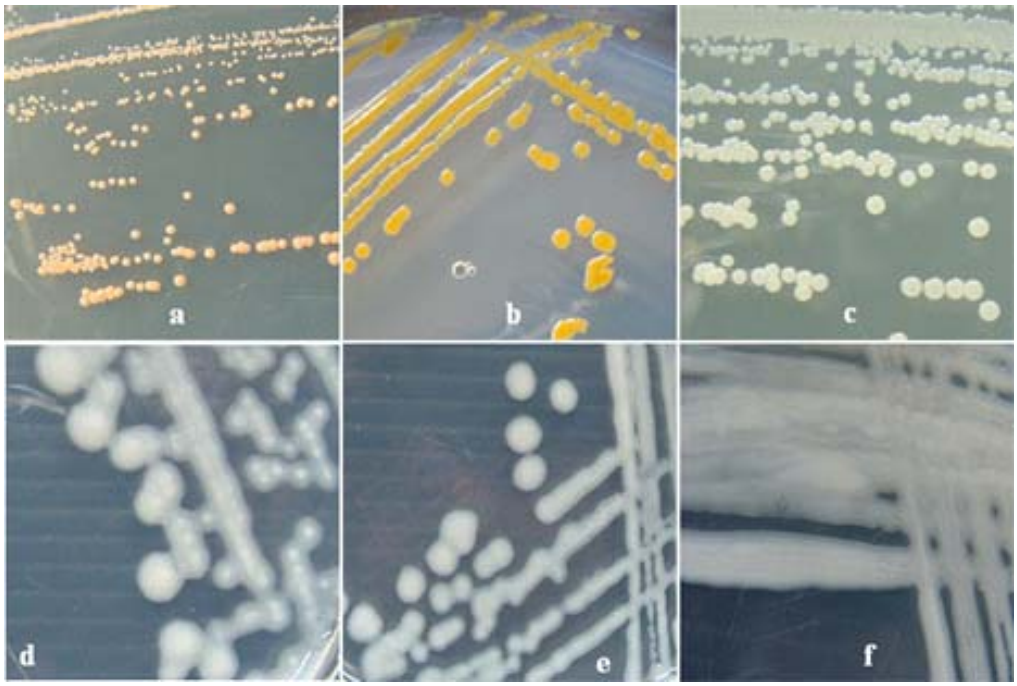


Figure 13. Colony morphologies of *p,p'*-DDT transforming bacterial isolates grown on MSYM+DDT_{100} agar. (a = PD7-2; b = PD7-3, c = PD7-5; d = PD3-4; e = PD5-6; f = PD6-4)

Table 7. Observed cell and colony morphologies of bacterial isolates grown in MSYM+DDT_{100}.

Consortium	Isolate	Cell morphology		Colony morphology					
		Gram staining	Shape	Color and Opacity	Shape	Elevation	Size*	Consistency	Surface
PD1	1	(-)	rod	red	round	convex	small	butyrous	smooth
	2	(-)	cocci	yellow	round	convex	small	butyrous	glistening
	3	(-)	cocci	white translucent	irregular	raised	small-medium	butyrous	smooth
	4	(-)	cocci	orange	round	convex	dot	butyrous	smooth
	5	(-)	rod	white transparent	round	raised	small-medium	butyrous	smooth
	6	(-)	cocci	white opaque	round	flat	large (spread out)	brittle	rough
PD2	1	(-)	rod	white translucent	irregular	convex	large	butyrous	glistening
	2	(-)	cocci	white translucent	round	convex	medium	butyrous	glistening
	3	(-)	rod	white	round	flat	small	butyrous	smooth
	4	(-)	rod	yellow	irregular	raised	small (filamentous)	butyrous	rough
PD3	1	(-)	cocci	yellow translucent	round	raised	medium	butyrous	rough
	2	(-)	rod	yellow	round	raised	small	mucoid	glistening
	3	(-)	cocci	white translucent	irregular	flat	medium (filametous)	butyrous	smooth
	4	(-)	rod	white transparent	irregular	raised	medium (filamentous)	mucoid	smooth
	5	(-)	rod	white translucent	round	convex	small-medium	butyrous	smooth
PD4	1	(-)	cocci	red	round	convex	puncti form	butyrous	smooth
	2	(-)	rod	yellow	round	convex	medium	brittle	rough
	3	(-)	rod	white opaque	irregular	raised	medium	brittle	rough
	4	(-)	rod	white opaque	round	convex	small-medium	butyrous	glistening
PD5	1	(-)	cocci	red	round	convex	medium	butyrous	smooth
	2	(-)	cocci	red	irregular	raised	medium	butyrous	smooth
	3	(-)	cocci	yellow	round	convex	medium	butyrous	glistening
	4	(-)	cocci	yellow	irregular	raised	medium	butyrous	glistening
	5	(-)	cocci	white-translucent	round	convex	medium	brittle	rough
	6	(-)	cocci	white-opaque	irregular	convex	large	mucoid	glistening

Note: * small < 1 mm
medium 1 - 2 mm
large > 2 mm

Table 7. (continued)

Consortium	Isolate	Cell morphology		Colony morphology					
		Gram staining	Shape	Color and Opacity	Shape	Elevation	Size*	Consistency	Surface
PD6	1	(-)	rod	red-pink	round	raised	small	butyrous	smooth
	2	(-)	rod	yellow	irregular	raised	medium	butyrous	glistening
	3	(-)	rod	yellow-orange	round	raised	medium	mucoid	smooth
	4	(-)	cocci	white opaque	irregular	raised	medium	mucoid	glistening
	5	(-)	cocci	white opaque	round	raised (rough)	medium	mucoid	glistening
	6	(-)	rod	white translucent	irregular	raised (rough)	spread out filamentous	mucoid	glistening
PD7	1	(-)	rod	yellow	irregular	convex	medium	butyrous	smooth
	2	(-)	rod	pink	round	convex	small	butyrous	smooth
	3	(-)	cocci	orange	round	convex	medium	butyrous	glistening
	4	(-)	rod	white opaque	irregular	Raised (rough)	medium	butyrous	rough
	5	(-)	rod	white opaque	irregular	raised	small	butyrous	rough
PD8	1	(-)	cocci	bright yellow	irregular	raised	medium	butyrous	glistening
	2	(-)	cocci	orange	round	raised	medium	mucoid	smooth
	3	(-)	rod	white translucent	round	convex	medium	butyrous	smooth
	4	(-)	rod	white opaque	irregular	raised (rough)	medium	mucoid	smooth
PD9	1	(-)	cocci	white opaque	irregular	raised	medium	mucoid	smooth
	2	(-)	rod	white opaque	round	raised	medium	butyrous	smooth
	3	(-)	cocci	white translucent	round	raised	medium	mucoid	smooth
	4	(-)	cocci	white translucent	irregular	raised	big spread out	mucoid	smooth
	5	(-)	rod	pink	round	raised	small puncti form	butyrous	smooth

Note: * small < 1 mm
medium 1 - 2 mm
large > 2 mm

Table 7. (continued)

Consortium	Isolate	Cell morphology		Colony morphology					
		Gram staining	Shape	Color and Opacity	Shape	Elevation	Size*	Consistency	Surface
PD10	1	(-)	rod	white opaque	round	raised	medium	butyrous	smooth
	2	(-)	rod	white	irregular	raised	medium	butyrous	smooth
PD11	1	(-)	cocci	white translucent	round	raised	medium	butyrous	rough
	2	(-)	cocci	white opaque	irregular	raised	medium	butyrous	rough
	3	(-)	rod	white	irregular	raised	medium	butyrous	smooth
PD12	1	(-)	rod	white opaque	round	raised	small	butyrous	rough
	2	(-)	rod	white opaque	irregular	raised	small	butyrous	rough
	3	(-)	rod	white opaque	round	convex	large	mucoid	glistening

Note: * small < 1 mm
medium 1 - 2 mm
large > 2 mm

In theory, the use of air-dried soil samples for bacterial screening instead of fresh (wet) soil samples may reduce the possibility of obtaining high bacterial diversity. However, it was found that there were still varying number of different isolates found in each soil samples ranging from 2 to 6 bacterial isolates. Soil sample 1, 5 and 6 which had low concentrations of *p,p′*-DDT residue from 0.19, 0.34 and 0.84 ng/g soil dry weight, respectively, comprised of six isolates each. Soil sample 3, 7 and 8 which had varying degree of *p,p′*-DDT residues of 0.52, 6.27 and 0.24 ng/g soil dry weight, respectively, consisted of five bacterial isolates each. Soil sample 2, 4 and 5 which had *p,p′*-DDT concentrations of 0.80, 1.81 and 0.95 ng/g soil dry weight, respectively, consisted of four bacterial isolates each. Soil sample 11 and 12, which contained *p,p′*-DDT concentrations of 0.62 and 0.79 ng/g soil dry weight, consisted of three bacterial isolates each. While soil sample 10, which had the highest *p,p′*-DDT concentration of 9.84 ng/g soil dry weight, consisted of only two bacterial isolates (Table 7). Most soil samples with low residue of *p,p′*-DDT consisted of three or more bacterial isolates member when cultivated in MSYM+DDT_{100} medium. The results obtained were in accordance with previous study which reported that toxic

contaminants in soil, including *p,p′*-DDT, may potentially inhibit the growth and activity of microorganism such as bacteria and fungi (Megaharaj *et al.*, 1999; Zwieten *et al.*, 2002; Bidlan and Manonmani, 2002). Interestingly, soil sample 7 which also had high concentration of *p,p′*-DDT (6.27 ng/g soil dry weight) still comprised of high number of bacterial isolates (five) when cultivated in MSYM+DDT_{100} medium.

Besides the selective enrichment method used in this study, another screening technique known as analogue enrichment has been shown to be successful in the isolation of *p,p′*-DDT degrading bacteria. Structural analogue compounds such as pentachloronitrobenzene, diphenyl methane, and 4-chlorobiphenyl were used to substitute for *p,p′*-DDT (Masse *et al.*, 1989; Nadeau *et al.*, 1994; Parson *et al.*, 1995; Focht and Alexander, 2000; Huerta *et al.*, 2007).

2. Study of *p,p′*-DDT transformation by bacterial consortium

Bacterial consortia screened from agricultural soils were tested for their *p,p′*-DDT transformation ability by cultivating in MSYM medium supplemented with 100 ppm *p,p′*-DDT at room temperature, 150 rpm for 10 days. Growth and *p,p′*-DDT transformation profiles of the bacterial consortia displayed significantly different trends (Fig. 14 and 15). From these growth and transformation activities, we can define the bacterial consortium into 3 groups, i.e., low, moderate and high DDT transformers. Low DDT transformers, which had *p,p′*-DDT transformation less than 50%, while moderate and high DDT transformers have *p,p′*-DDT transformation between 50 - 75% and more than 75%, respectively (Fig. 16).

All bacterial consortia exhibited various *p,p′*-DDT transformation ability of 37.03 - 100% from initial 100 ppm of *p,p′*-DDT (Fig. 16). After 10 days of incubation, bacterial consortium PD1, PD8 and PD9 were shown to exhibit low DDT transformation level (<50%) of 37.03, 43.52 and 47.39%, respectively. Whereas bacterial consortium PD2, PD3, PD4, PD5, PD6 and PD10 displayed moderate DDT transformation level (50 - 75%) of 50.70, 55.33, 66.34, 63.96, 71.55 and 58.63%, respectively. Interestingly both consortium PD7 and PD11 showed high DDT transformation level of 94.72 and 100%, respectively, with the results being non-significantly difference (P<0.05).

Supplementing 0.01% yeast extract to the cultivation medium help supported growth during the first day of cultivation, as shown by the increase of cellular mass in all bacterial consortia (Fig. 14). The major component of yeast extract is protein with the remaining being carbohydrate (4 - 13%) and lipid. Therefore, yeast extract may provide utilizable carbon and nitrogen sources to the bacterial consortia. Bacterial consortium PD1, PD8, PD9 and PD12, which have been grouped into the low DDT transformer category, also showed low cellular mass (less than 100 μg/ml total cell protein) (Fig. 14). The group of moderate DDT transformers, which achieved between 50 - 75% DDT transformation, had both high cellular mass (more than 100 μg/ml total cell protein) (bacterial consortium PD2, PD3, PD5 and PD6) and low cellular mass of less than 100 μg/ml total cell protein (bacterial consortium PD4 and PD10). The high *p,p'*-DDT degraders possessed both high (PD7 which achieved up to 193.83 μg/ml total cell protein) and low cellular mass (PD11 which achieved only 76.85 μg/ml total cell protein). Besides the amount of cellular mass, the rate of growth was of significantly differences as seen with bacterial consortium PD5 and PD6 which although had high cellular mass of more than 100 μg/ml total cell protein but required over 7 days before exponential phase of growth were observed (Fig. 14).

The growth phenomena of bacterial consortium PD5 and PD6 can be attributed to successive microbial population. This occurs as a response to the transformation pathways, which consisted of complex regulatory systems. The process may operate only when several substrates are simultaneously available, thus presenting the possibility in which transformation may be accomplished. The dramatic increase in growth by bacterial consortium PD5 and PD6 after day 7 of cultivation with no increase in the transformation rate was because of the availability of the transformation metabolites acting as substrate for specific bacterial members within the consortia. Microbial population succession are commonly found in the biotransformation of toxic contaminant (e.g. 2,4-D, DDT and Chlorophenol) as some bacteria could not utilize the initial compound but will assimilated the metabolites product generated after several days of incubation (Alcocer *et al.*, 2007; Neilson, 1995; Bidlan and Manonmani, 2002; Lerch *et al.*, 2009).

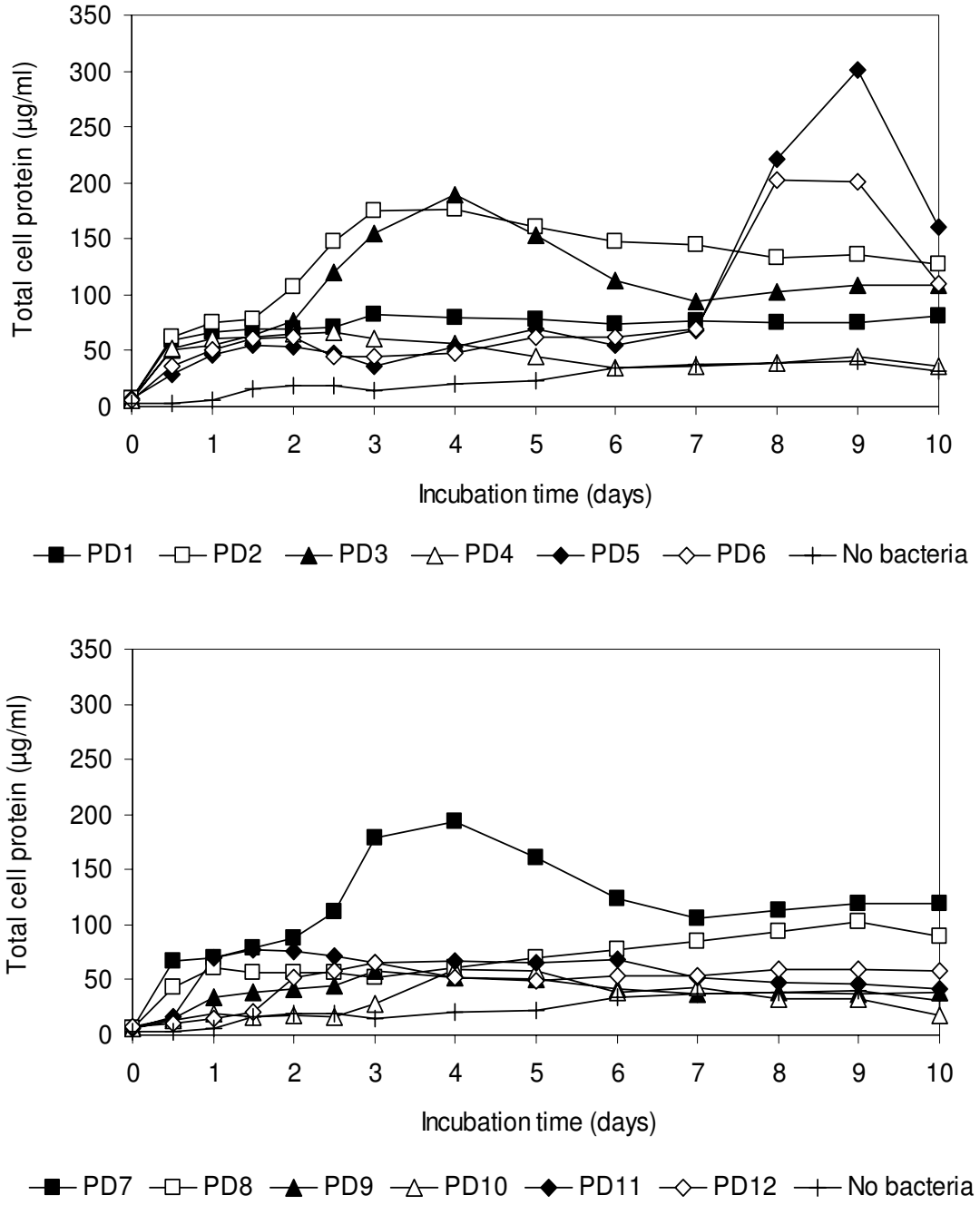
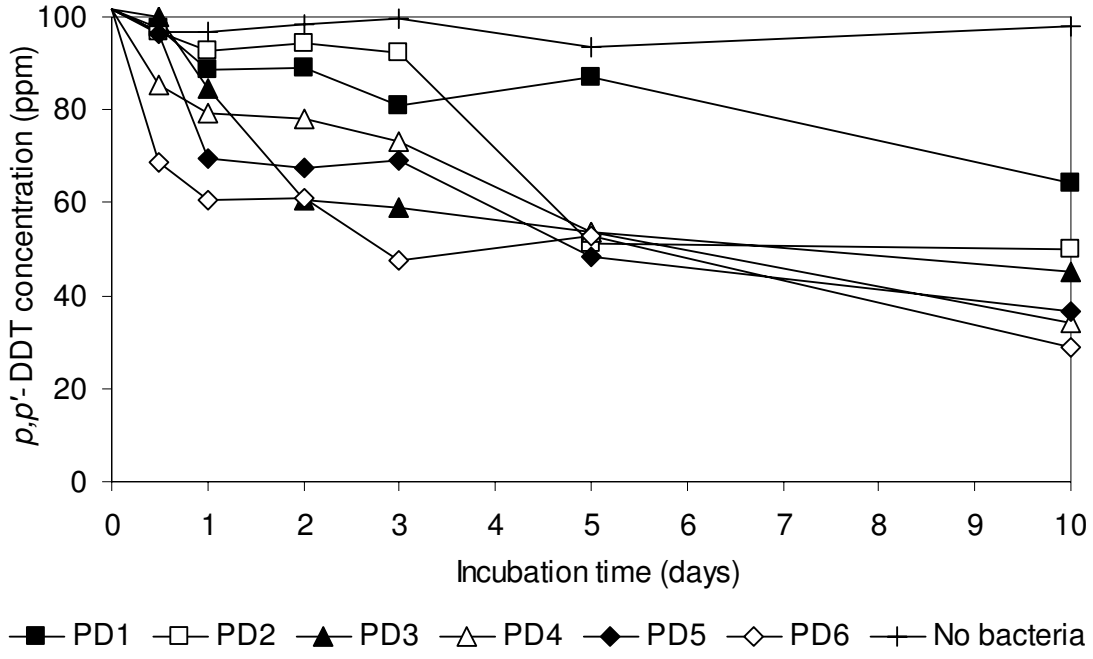


Figure 14. Growth profiles of individual bacterial consortium grown in MSYM+ DDT_{100} at room temperature, 150 rpm for 10 days.



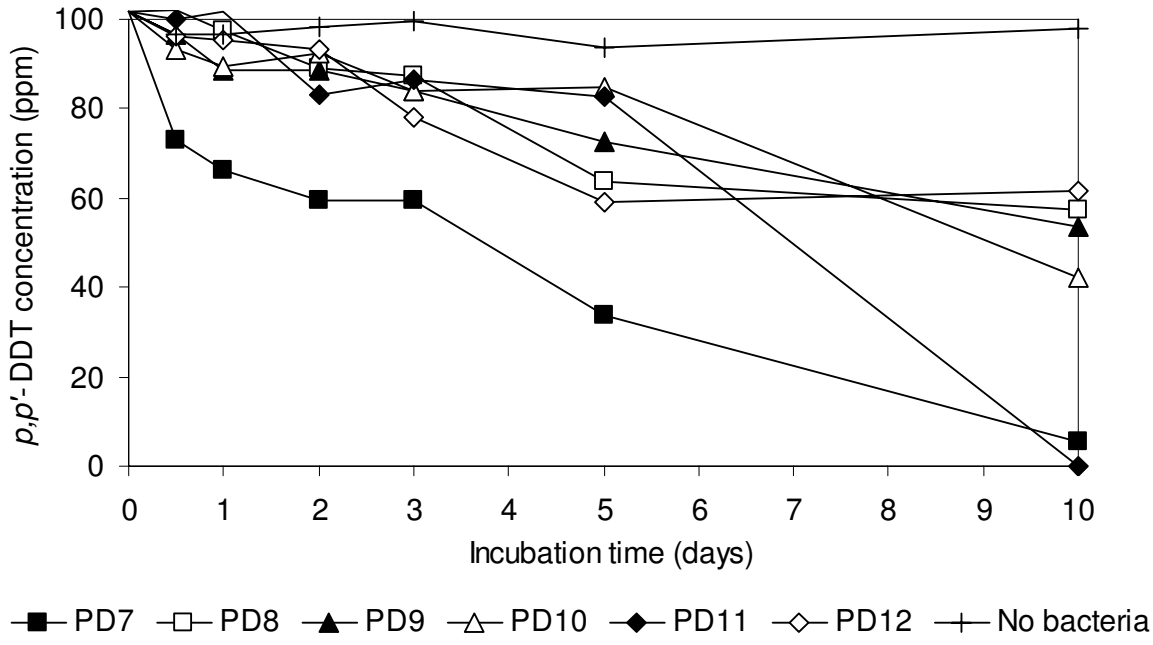


Figure 15. *p,p'*-DDT transformation profiles of individual bacterial consortium grown in MSYM+DDT_{100} at room temperature,150 rpm for 10 days.

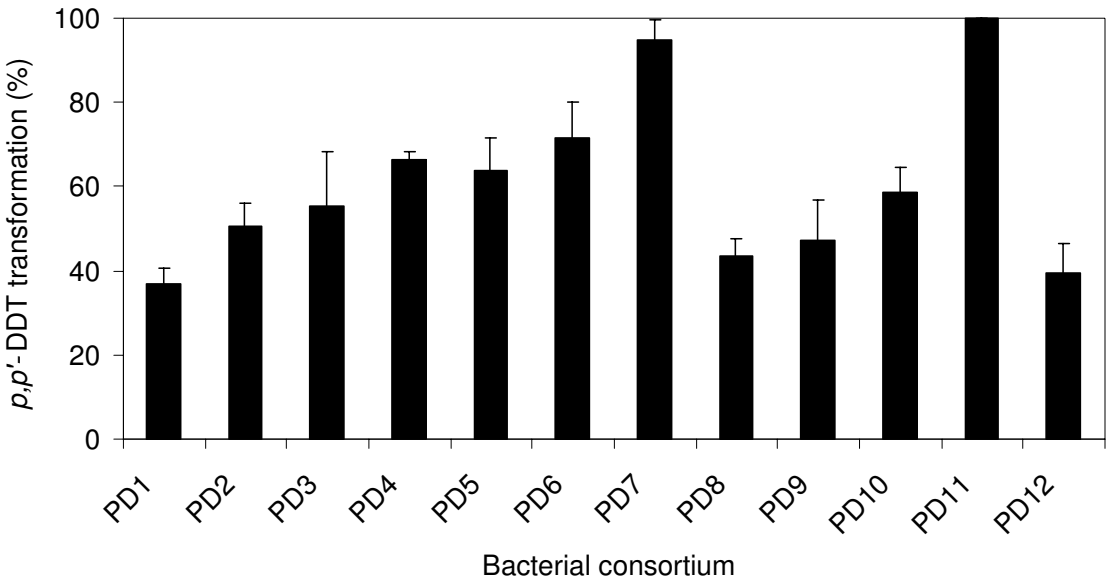


Figure 16. *p,p'*-DDT transformation levels by individual bacterial consortium after 10 days of incubation in MSYM+DDT_{100} at room temperature and 150 rpm. Bars represent the standard deviation from three determinations. (Negative control = No bacteria; Level of *p,p'*-DDT transformation = 3.47%)

The low level of growth suggests that bacteria had not utilized DDT as a carbon source. The reduction of *p,p'*-DDT observed in the cultivation medium with low growth level may be attributed to the accumulation, e.g., by intra- or extra-cellular synthesized biomaterials and adsorption on the cell surface rather than biotransformation or biodegradation. Accumulation of *p,p'*-DDT and methoxychlor from water sample were previously observed by pure culture of *Aerobacter aerogenes* and *Bacillus subtilis* (Johnson and Kennedy, 1973), where the amounts of insecticides were followed using ^{14}C-labeled insecticide solution.

Bacterial consortium PD7, PD10 and PD11 were shown to have high rate of DDT transformation after day 5 of incubation, while those of other bacterial consortia have reached constant or near constant levels (Fig. 15). The low DDT transformation by bacterial consortium such as PD1, PD8, PD9 and PD12 may be attributed to the co-metabolism phenomenon, where the bacteria could not utilized DDT as the sole source of carbon without any induction by another readily

metabolized carbon source. Co-metabolism appears to occur widely in nature because the compound considered recalcitrant may in fact undergo biodegradation. DDT is one example of a recalcitrant compound, which may undergo extensive degradation by microbial co-metabolism. DDT analogue, such as diphenylethane (DPE), has been frequently used as the inducer for co-metabolism (Focht and Alexander, 1970; Francis *et al*., 1977; Hay and Focht, 1998). On the other hand, bacterial consortium PD7, PD10 and PD11 showed significant DDT transformation throughout the cultivation period, thus suggesting that the bacterial members of those consortia were able to utilize *p,p′*-DDT or its metabolites and degrade them as well.

The growth and transformation results obtained in this study were significantly higher than that of previous reports. In other study, bacterial consortium was completely inhibited by the addition of 100 ppm *p,p′*-DDT under anaerobic condition (Chiu *et al*., 2004). While pure culture of *Serratia marcescens* DT-1P was also completely inhibited by the addition of only 50 ppm *p,p′*-DDT under aerobic condition, with less than 10 µg/ml of total cell protein generated (Bidlan and Manonmani, 2002).

Comparison of the two high *p,p′*-DDT transformers, bacterial consortium PD7 and PD11, showed approximately the same level of *p,p′*-DDT transformation. However, the rate of transformation, amount and rate of growth were significantly different. Bacterial consortium PD7 displayed high rate of *p,p′*-DDT transformation from day 1 of cultivation (Fig. 17a), while that of PD11 were very slow and required over 5 days before rapid *p,p′*-DDT transformation was observed (Fig. 17b). The growth profile of both consortia was also different. Although maximum growth by bacterial consortium PD7 took longer to reach than PD11 (5 days compared to 2 days), but bacterial consortium PD7 was able to generate higher growth of up to 193.83 µg/ml total cell protein within 5 days of incubation while PD11 generated only 75.35 µg/ml in day 2 of incubation. This phenomenon suggested that PD11 did not utilized *p,p′*-DDT as the sole source of carbon. The reduction of DDT concentration could be attributed to other mechanism such as bioaccumulation/biomagnifications into either cellular body or adsorption on the cell surface. Bioaccumulation and biomagnification were previously observed in study of *p,p′*-DDT reduction (Johnson and Kennedy, 1973). It was reported that *Aerobacter*

aerogenes and *Bacillus subtillis* accumulated *p,p'*-DDT and methoxychlor directly from the water. Based on this reasons, bacterial consortium PD7, which consisted of 5 isolates (Fig. 18), was selected for further DDT transformation studies and investigated for its single and mixed bacterial cultures ability to transform *p,p'*-DDT.

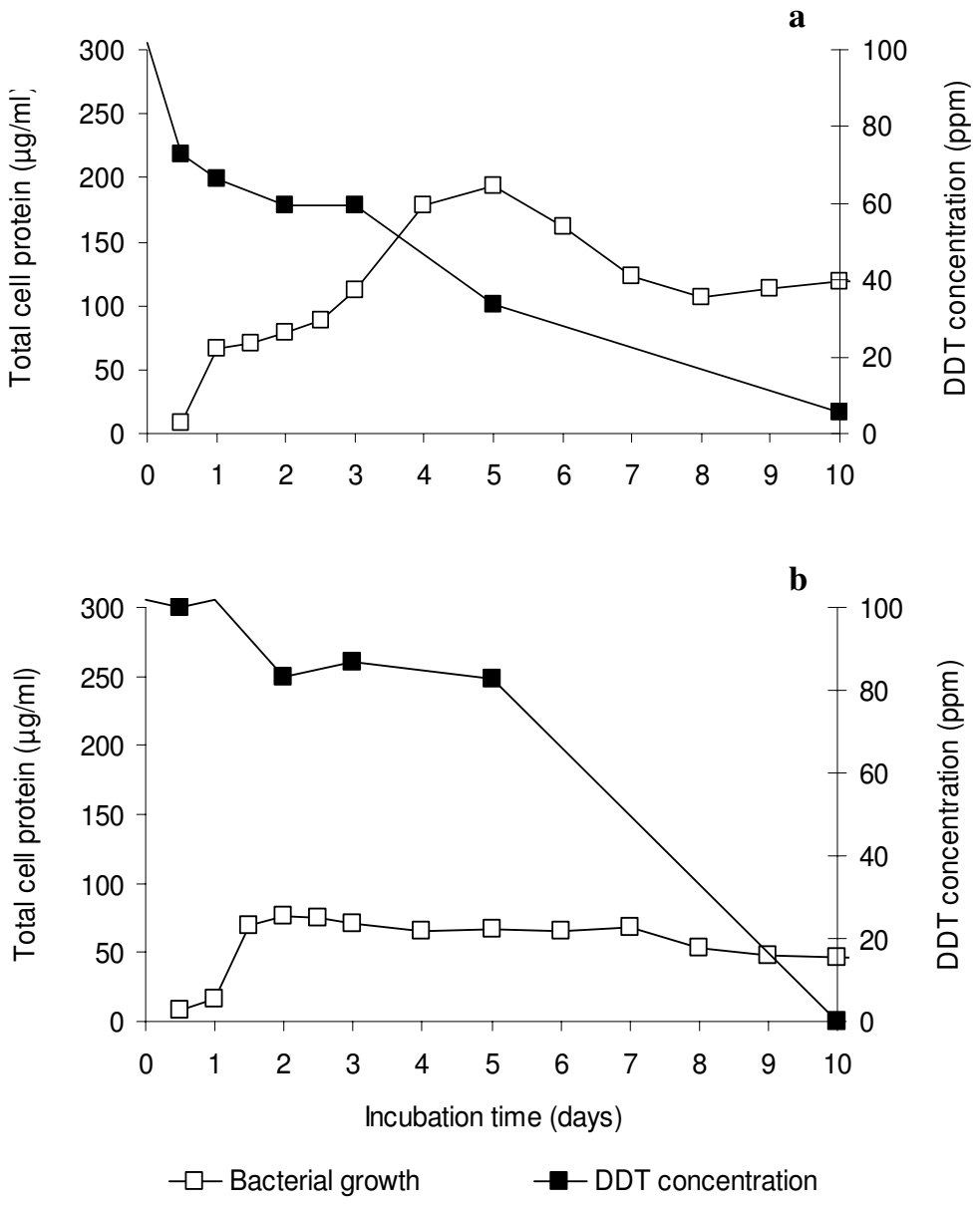


Figure 17. Comparison of growth and *p,p'*-DDT transformation profiles between bacterial consortium PD7 (a) and PD11 (b) in MSYM+DDT_{100} at room temperature, 150 rpm for 10 days.

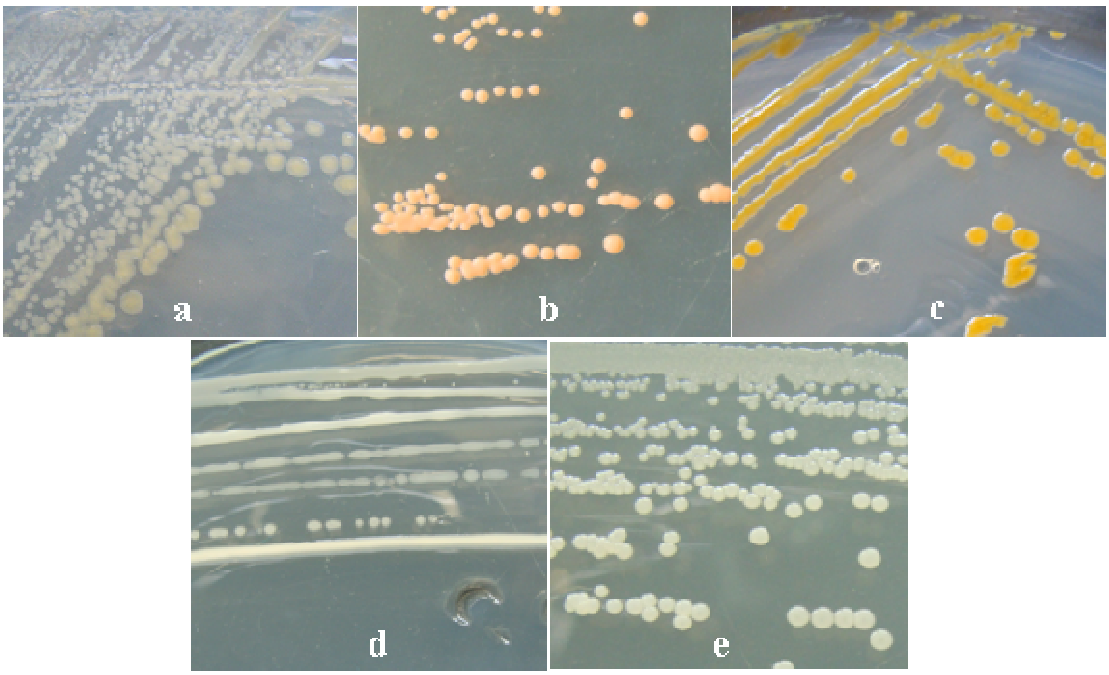


Figure 18. Five bacterial isolates from consortium PD7. (a = PD7-1; b = PD7-2, c = PD7-3; d = PD7-4; e = PD7-5)

3. Study of *p,p′*-DDT transformation by single bacterial culture

The selected bacterial consortium PD7 composed of five isolates, i.e. isolate PD7-1, PD7-2, PD7-3, PD7-4 and PD7-5. These isolates were used to investigate *p,p′*-DDT transformation by single and mixed bacterial culture.

The profiles of growth and DDT transformation of each bacterial isolates after 10 days of incubation displayed similar trend of increasing within the first five days and exhibited slower transformation rate from day 5 to day 10 (Fig. 19 and 20). The exception was for isolate PD7-5, which still showed the increase of growth after day 5 of incubation with no significant increase in *p,p′*-DDT transformation. In this stage, isolate PD7-5 may be encountering a substrate selection situation where more than one substrate is available, since some metabolites product such as DDE, which was less stable and toxic, were generated in the medium. This was a possible reason of just slightly increase of *p,p′*-DDT degradation was achieved by PD7-5 from day 5 to day 10, while growth showed significant increase at the same period (Fig. 19 and 20). However, the DDT transformation profiles of bacterial

consortium PD7 showed continuous increase from inoculation until day 10 of incubation with growth entering stationary phase by day 3 of cultivation (Fig. 19).

All bacterial isolates displayed *p,p′*-DDT transformation of 27.80% to 50.63% from the initial 100 ppm *p,p′*-DDT after 10 days of incubation in MYSM+DDT_{100} medium at room temperature and 150 rpm (Fig. 20 and 21). Bacterial isolate PD7-1, PD7-2, PD7-3, PD7-4 and PD7-5 showed DDT transformation levels of 30.12, 27.80, 39.40, 49.60 and 50.63%, respectively (Fig. 20). The transformation levels of all five bacterial isolates were quite lower than that of bacterial consortium PD7 (more than 90% DDT degradation). The possible reason was a synchronous (symbiotic) interaction between the isolates within the bacterial community, which worked together to increase the overall level of DDT transformation.

Similar results were observed in the studies of *p,p′*-DDT degradation by soil consortium and single *Serratia marcescens* DT-1P culture (Bidlan and Manonmani, 2002), immobilized mixed bacterial cultures (Beunink and Rehm, 1988), and the biodegradation of blended crude oil by marine sediment Hii *et al.* (2008). Bacterial consortium generally showed higher *p,p′*-DDT degradation ability in comparison with that of bacterial isolate. For example, soil consortium were shown to completely degrade 25 ppm *p,p′*-DDT within 144 hours under aerobic condition at room temperature, while only 80 % was achieved by single isolate under the same condition (Bidlan and Manonmani, 2002).

In this study, bacterial isolate PD7-5 also exhibited higher growth as indicated by higher total cell protein which was associated with higher *p,p′*-DDT transformation level in comparison with other single isolates. On the other hand, bacterial isolate PD7-4 showed even higher *p,p′*-DDT transformation level, but did not show higher growth in comparison with that of other isolates (Fig. 19 and 20). These results may be associated with their colony morphological features. Isolate PD7-4 produced polymeric-liked substance as observed during growth on MSYM+DDT_{100} agar (Table 6 and Fig. 18d). This characteristic may help accumulate DDT in their matrix rather than to transform. Therefore, it is one possible reason why isolate PD7-4, which has high *p,p′*-DDT transformation level in comparison with those of other isolates, did not display high growth profile as well.

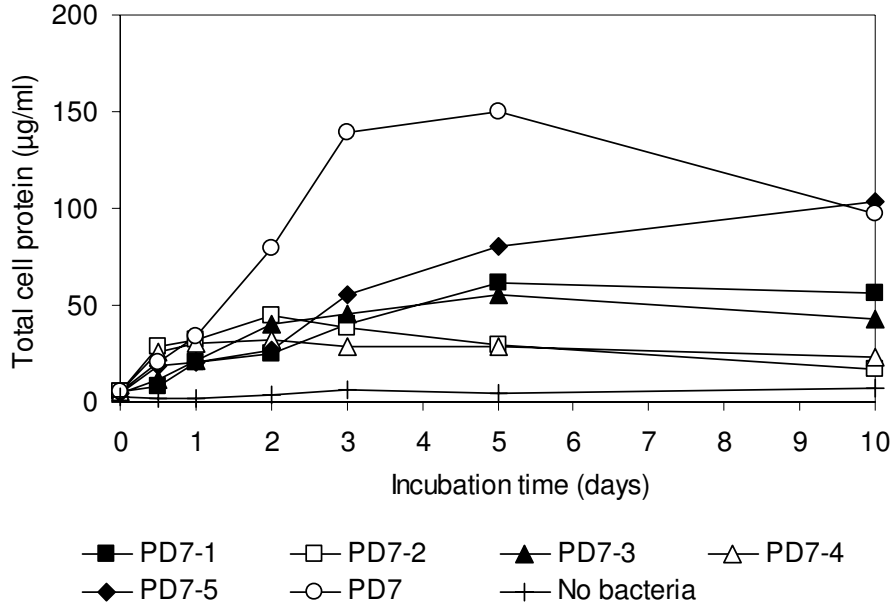


Figure 19. Bacterial growth profile of bacterial consortium PD7 and its isolates (PD7-1 to PD7-5) grown in MYSM+DDT_{100} at room temperature, 150 rpm for 10 days.

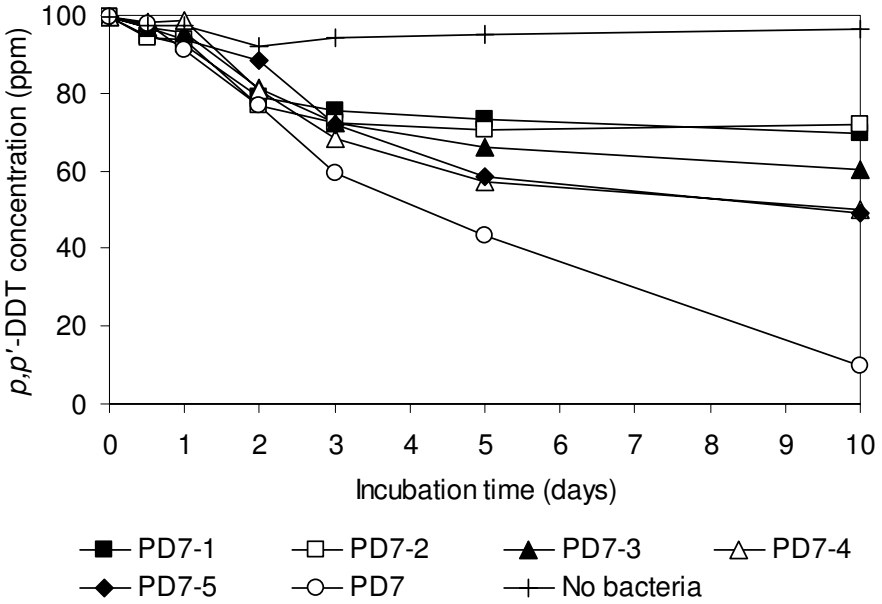


Figure 20. *p,p'*-DDT transformation profile of bacterial consortium PD7 and its isolates (PD7-1 to PD7-5) grown in MYSM+DDT_{100} at room temperature, 150 rpm for 10 days.

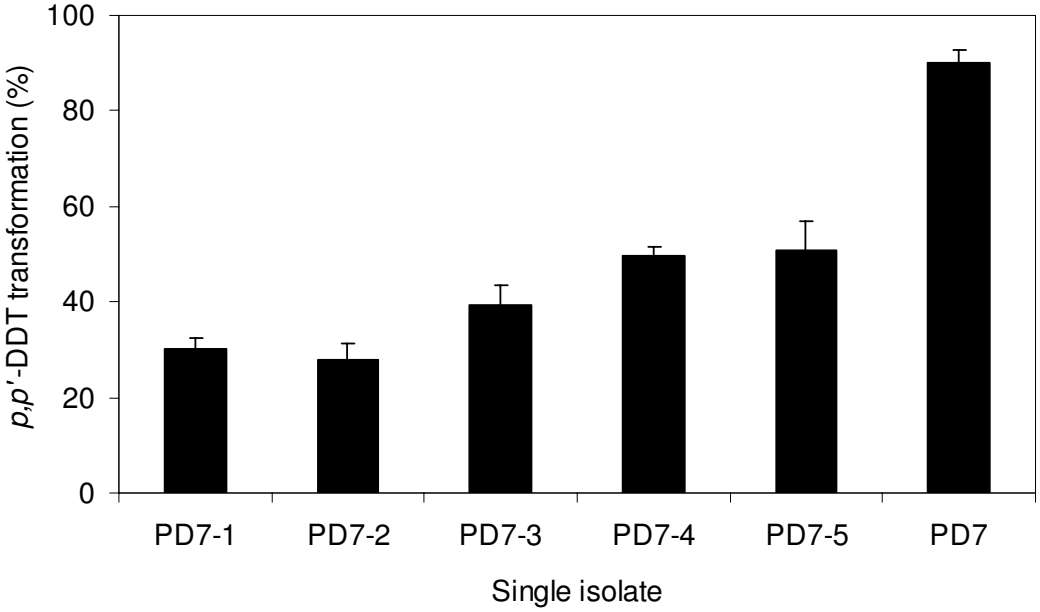


Figure 21. *p,p′*-DDT transformation levels by individual bacterial isolates grown in MSYM+DDT_{100} at room temperature, 150 rpm for 10 days. Bars represent the standard deviation from three determinations. (Negative control = No bacteria; Level of *p,p′*-DDT transformation = 2.81%)

4. Study of *p,p′*-DDT transformation by mixed two bacterial cultures

Although many studies have isolated and investigated DDT transformation ability of isolated cultures, no study have reported the role of single isolates within bacterial community in the form of defined mixed culture. Thus, studies to investigate the role of each isolate in mixed bacterial cultures were performed. In mixed cultures studies, bacterial isolates were grown as starter culture in MYSM+DDT_{20} until turbidity was observed (OD_{600} = 0.5). Mixed bacterial cultures were designed by combining starter culture of single isolate with the same cell density (OD_{600} value of approximately 0.5) in the ratio of 1:1 (v/v). All mixed bacterial culture inoculum concentrations were calculated to be approximately equivalent to that of single isolates and bacterial consortium inoculum concentrations. This mixed bacterial cultures was then cultivated in MYSM+DDT_{100} at room temperature, 150 rpm for up to 10 days.

Mixed two bacterial cultures exhibited rapid growth and *p,p′*-DDT transformation rates after inoculation with no apparent lag phase (Fig. 22 and 23). Growth entered stationary phase within 2 days of inoculation. However, mixed two bacterial cultures PD7-[1/2], [1/3], [1/5] and [2/5] continuously exhibited increase in growth up to day 10, even when no transformation of *p,p′*-DDT was longer observed (Fig. 22). The possible reason for the above observations can be attributed to the co-metabolism phenomenon, where bacteria could not directly utilized *p,p′*-DDT as the sole source of carbon. Thus, only low levels of growth were observed during cultivation. While mixed two cultures PD7-[1/2], [1/3], [1/5] and [2/5] continuously grew without further transformation indicating that these mixed bacterial cultures obtained and utilized metabolites product of *p,p′*-DDT as the easier metabolizable carbon source for their growth. From this concept, bacterial isolate PD7-1 seem to have a significant role in *p,p′*-DDT co-metabolism process in term of cellular growth since most mixed two bacterial cultures which include PD7-1 displayed increase in growth from the start of inoculation until day 10 of incubation.

All mixed two bacterial cultures exhibited *p,p′*-DDT transformation. However, the transformation levels were not significantly different in comparison with those of single isolates ($P<0.05$) and were still lower than that of bacterial consortium PD7 (Fig. 23 and 24). The *p,p′*-DDT transformation profiles by mixed two bacterial cultures exhibited higher rate than that of single isolates, although the transformation levels were approximately similar (Fig. 20 and 23). Transformation of *p,p′*-DDT by all mixed two bacterial cultures appeared to be rapid within 1 - 2 days after inoculation and gradually slow to stationary level all through the study. The association of bacterial growth and transformation of contaminant via co-metabolism has also been observed in other studies. There have been reported that DDE, which is primarily an aerobic catabolites product of *p,p′*-DDT, was metabolized to DDMU by pure bacterial culture (Massé *et al.,* 1989) and also by soil and marine sediments consortia (Quensen *et al.*, 1998).

Transformation level by mixed two bacterial cultures ranged between 28.2 - 57.1% of the initial 100 ppm *p,p′*-DDT (Fig. 24). The best *p,p′*-DDT transformation ability was achieved by mixed two bacterial cultures PD7-[4/5] at 57.1%, while the lowest *p,p′*-DDT transformation ability was by PD7-[1/2] at 28.2%.

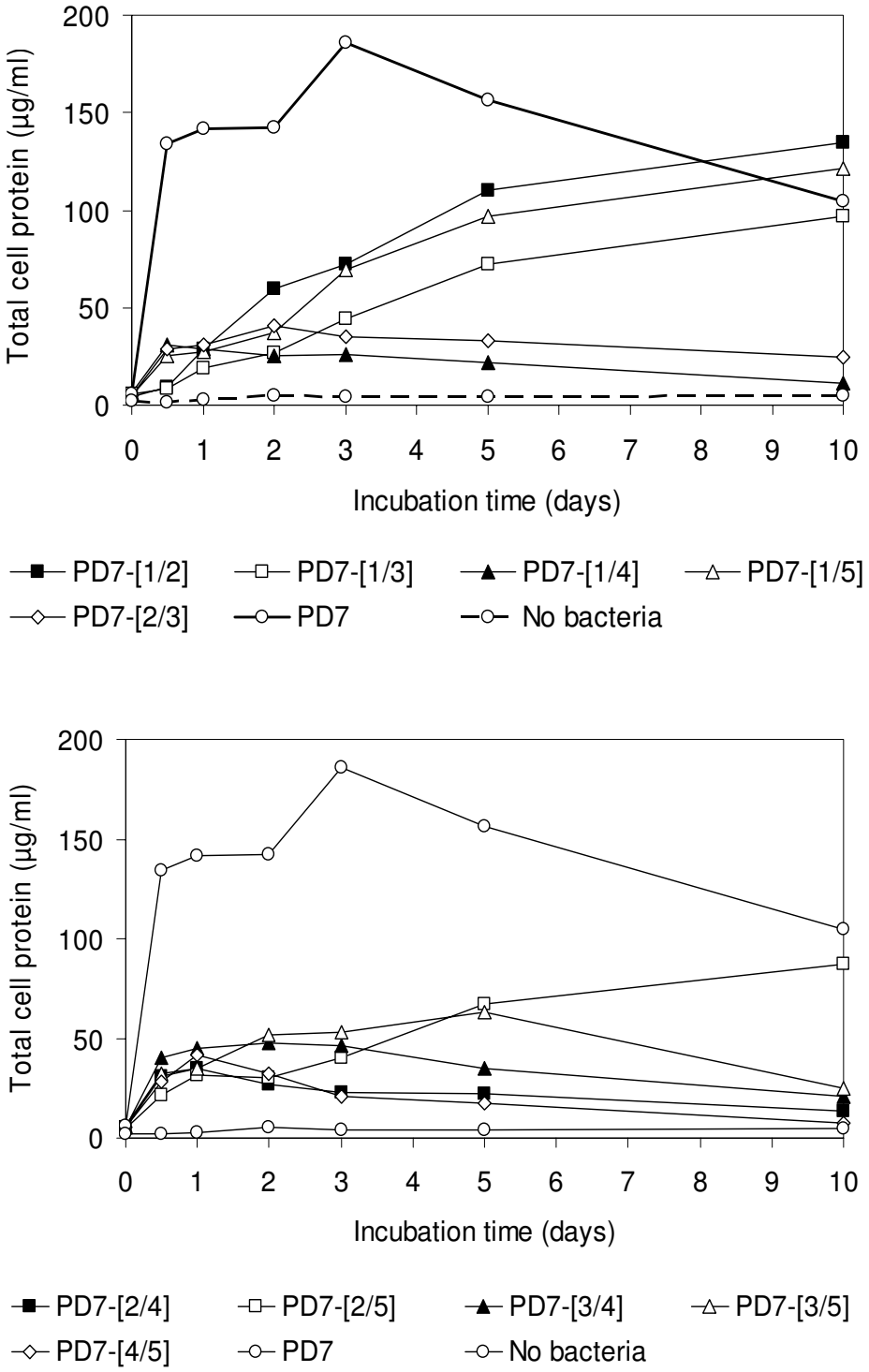


Figure 22. Bacterial growth profiles of mixed two bacterial cultures grown in MSYM+DDT_{100} at room temperature, 150 rpm for 10 days.

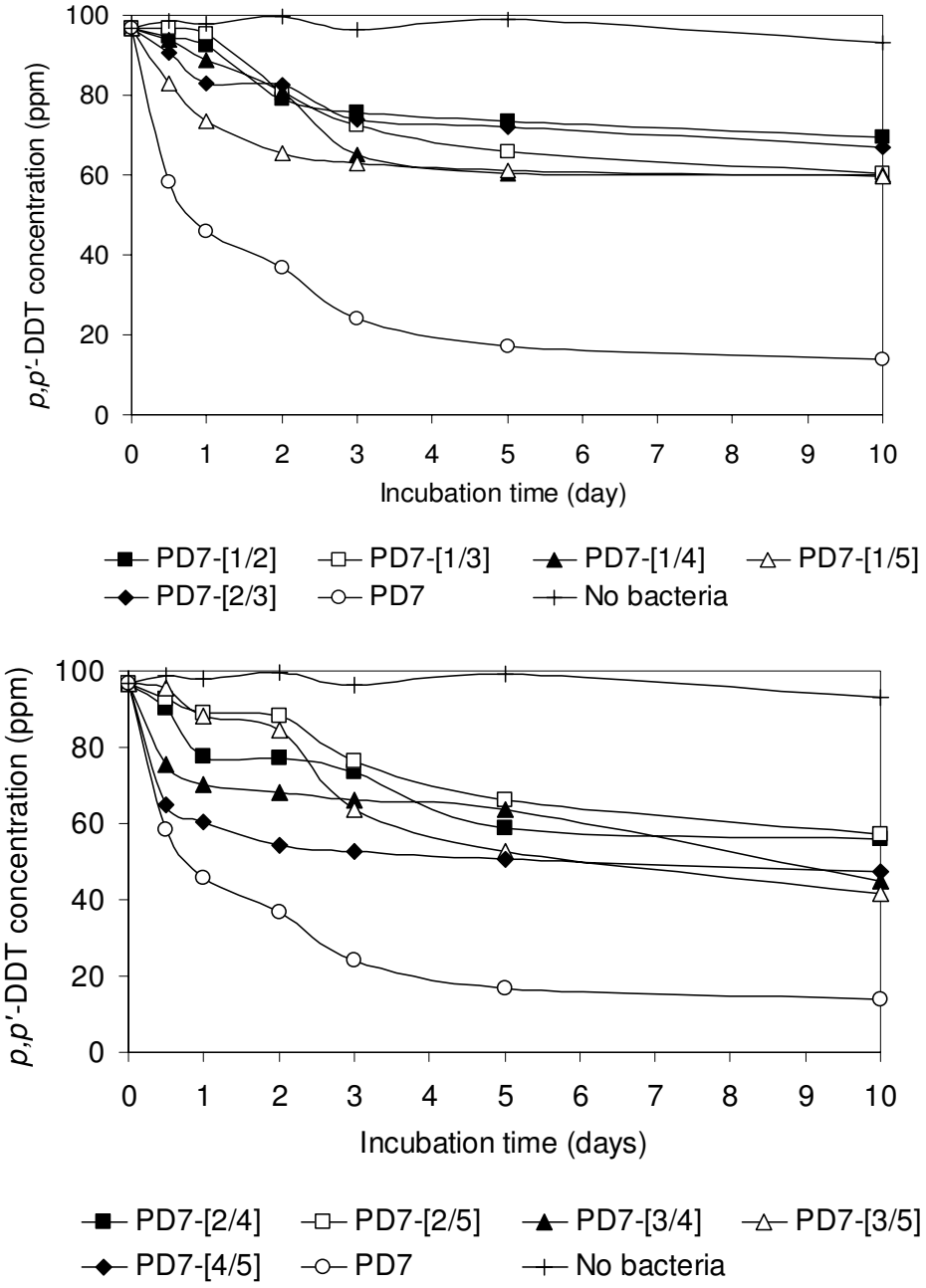


Figure 23. *p,p'*-DDT transformation profiles of mixed two bacterial cultures grown in MSYM+DDT_{100} at room temperature, 150 rpm for 10 days.

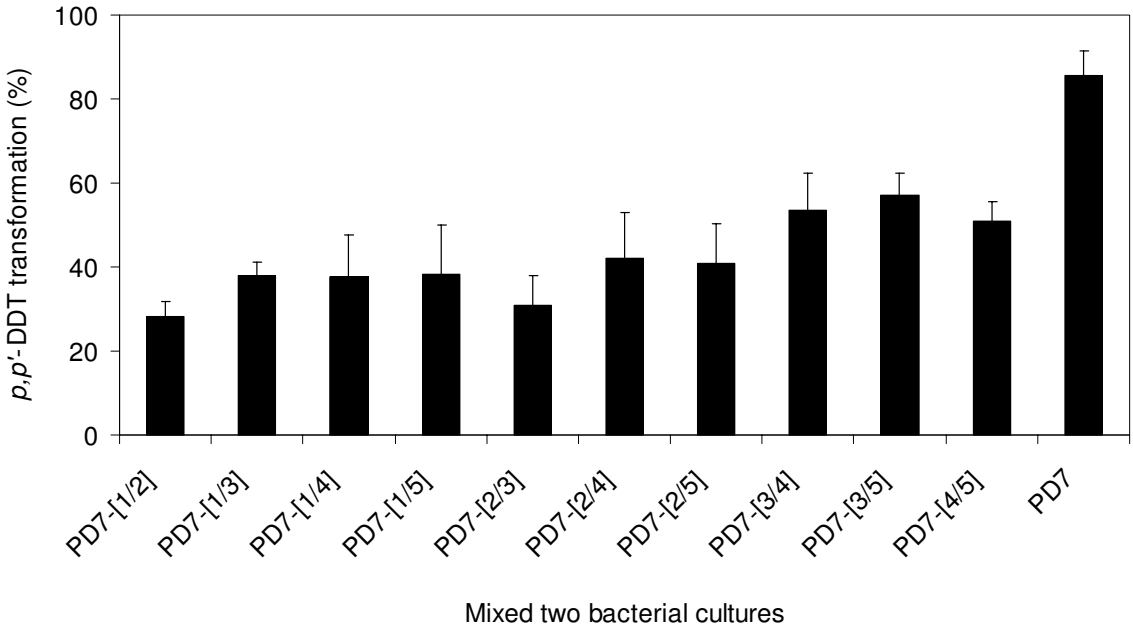


Figure 24. *p,p′*-DDT transformation levels by mixed two bacterial cultures after 10 days of incubation in MSYM+DDT_{100} at room temperature and 150 rpm. Bars represent the standard deviation from three determinations. (Negative control = No bacteria; Level of *p,p′*-DDT transformation = 3.94%)

The data suggests that bacterial isolates PD7-1, PD7-2 and PD7-3 do not play a significant role of *p,p′*-DDT transformation within the mixed two bacterial cultures in comparison with those of PD7-4 and PD7-5. These were in concerted with the result of single isolates study which reported that bacterial isolates PD7-1, PD7-2 and PD7-3 exhibited lower *p,p′*-DDT transformation levels in comparison with that of PD7-4 and PD7-5. In the cases of bacterial isolate PD7-1, which displayed insignificant role in *p,p′*-DDT transformation, it did show evident of a role in the co-metabolism process as seen by high cellular mass generated when the isolate was present in single and mixed cultures (Fig. 19 and 22).

According to the above results, it can be concluded that mixed two bacterial cultures are insignificant to the optimization of the transformation rate and level. Although, transformation products of *p,p′*-DDT such as DDE may be further metabolized as seen by the continuous growth of some mixed two bacterial cultures

when no significant *p,p′*-DDT transformation was observed. Hence, it is necessary to further investigate the details related to the role of these bacterial isolates in *p,p′*-DDT transformation pathway. In many instances during *p,p′*-DDT analysis, there were appearance of DDD, which is the anaerobic metabolite product during the transformation of *p,p′*-DDT, shown in the GC-ECD chromatogram. These may occur due to the impurities of the chemical compound and/or the minor anaerobic metabolism product when the bacteria could not access enough oxygen from the agitation (shaking) process during cultivation.

5. Study of *p,p′*-DDT transformation by mixed three bacterial cultures

Prior to the cultivation, bacterial isolates were grown as starter culture in MYSM+DDT_{20} until turbidity was observed (OD_{600} = 0.5). Starter culture of single isolate with the same cell density (OD_{600} value of approximately 0.5) in the ratio of 1:1:1 (v/v/v) were combined to give the final mixed bacterial culture inoculum concentrations that was approximately equivalent to that of single isolates and bacterial consortium inoculum concentrations. This mixed bacterial cultures was then cultivated in MYSM+DDT_{100} at room temperature, 150 rpm for up to 10 days.

Maximum growth of all mixed three bacterial cultures were shown to be higher than those of single isolates and mixed two bacterial cultures. Growth appeared to have rapid rate with no lag phase and reached stationary phase within 1 - 3 days of cultivation. Mixed three bacterial cultures PD7-[1/2/4] and PD7-[2/4/5] showed maximum growth at day 1 of incubation with 157.5 and 151.7 μg/ml of total cell protein, respectively. While mixed three bacterial cultures PD7-[1/2/5], PD7-[1/3/5], PD7-[1/3/4], PD7-[3/4/5], PD7-[2/3/4] and PD7-[2/3/5] had maximum growth at day 2 of incubation with total cell protein ranging between 121.5 to 198.1 μg/ml. Mixed three bacterial cultures PD7-[1/2/3] and PD7-[1/4/5] reached maximum growth on day 3 of incubation with 140.0 and 106.1 μg/ml of total cell protein, respectively (Fig. 25).

The transformation profiles of *p,p′*-DDT by mixed three bacterial cultures generally showed high rate of transformation from the initial incubation time up to days 3 and then began to be transformed at a slower rate afterward (Fig. 26).

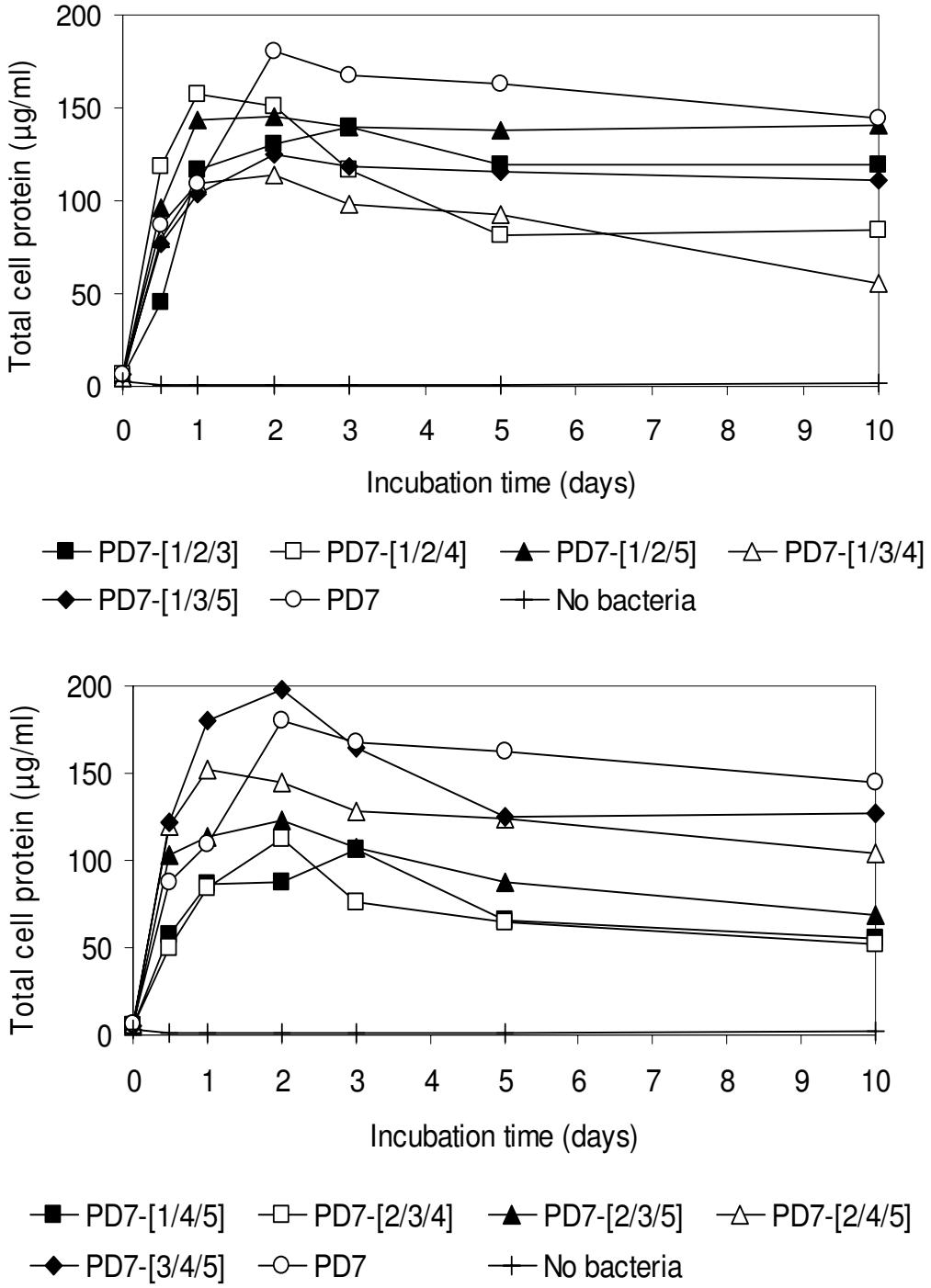


Figure 25. Bacterial growth profiles of mixed three bacterial cultures grown in MSYM+DDT_{100} at room temperature, 150 rpm for 10 days.

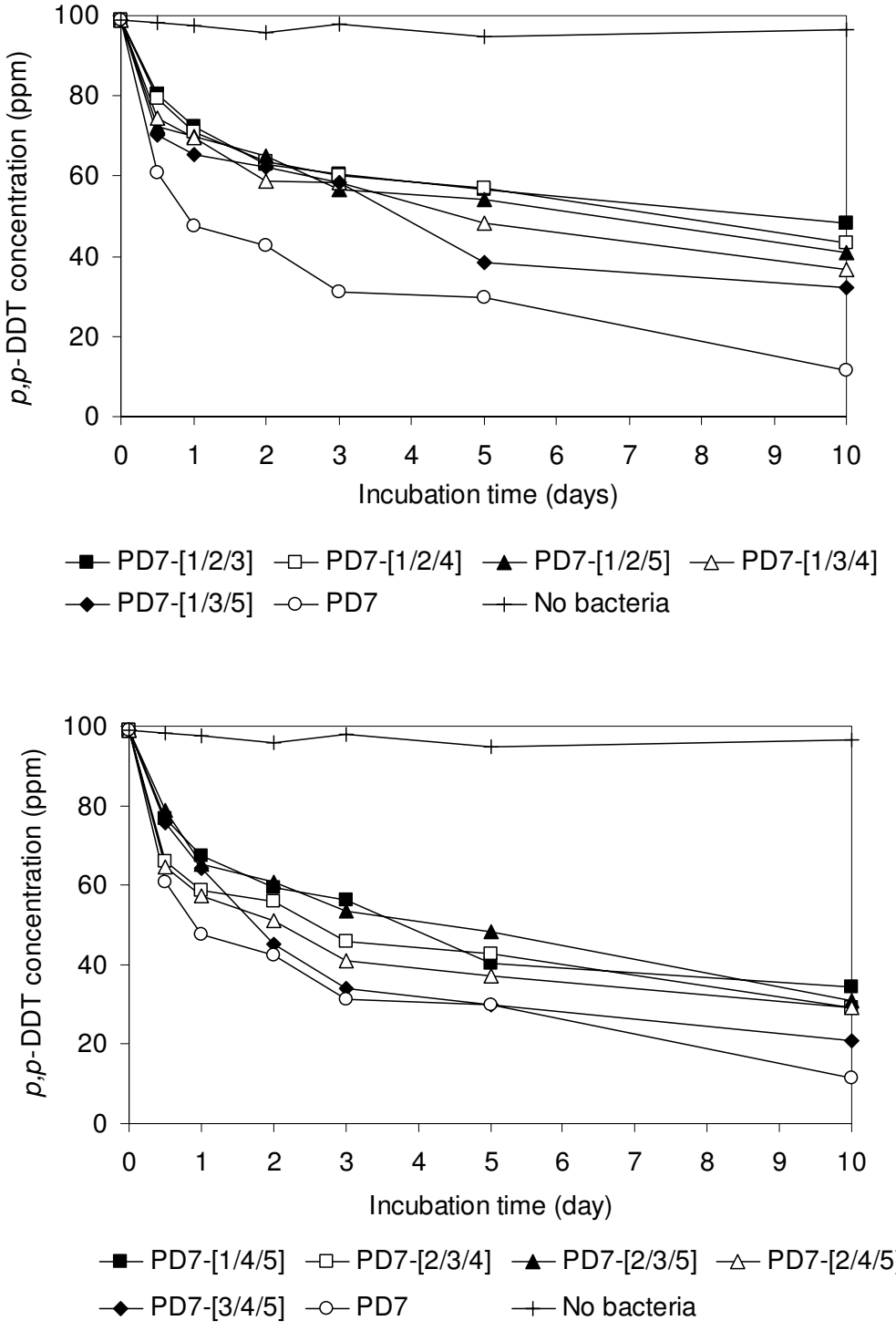


Figure 26. *p,p'*-DDT transformation profiles of mixed three bacterial cultures grown in MSYM+DDT_{100} at room temperature, 150 rpm for 10 days.

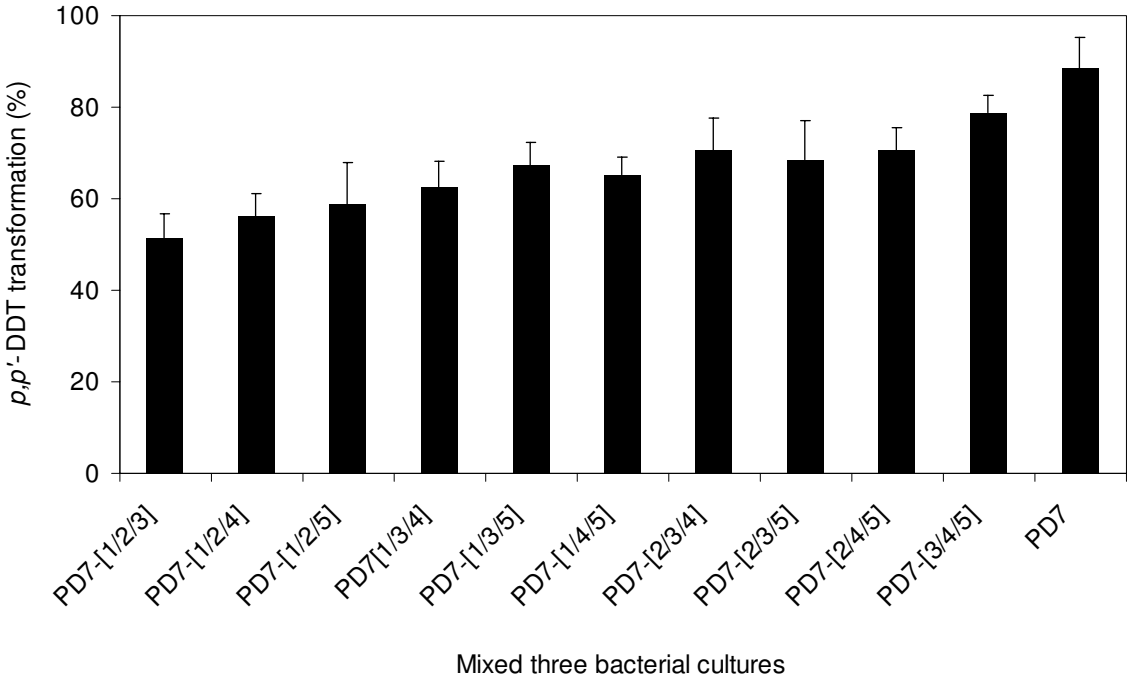


Figure 27. *p,p′*-DDT transformation levels by mixed three bacterial cultures after 10 days of incubation in MSYM+DDT_{100} at room temperature and 150 rpm. Bars represent the standard deviation from three determinations. (Negative control = No bacteria; Level of *p,p′*-DDT transformation = 2.54%)

Interestingly, mixed three bacterial culture PD7-[3/4/5] exhibited the highest *p,p′*-DDT transformation level and displayed relatively similar *p,p′*-DDT transformation profile with that of bacterial consortium PD7 during the first 5 days of cultivation. However, bacterial consortium PD7 continued to transform *p,p′*-DDT at a higher rate during days 5 to 10, thus resulting in an overall higher transformation level (Fig. 26). These transformation results were in agreement with the growth profile data, which indicated that mixed three bacterial cultures PD7-[3/4/5] had higher maximum growth of 198.1 μg/ml total cell protein at day 2 of incubation, whereas the maximum growth of bacterial consortium PD7 was only 180.7 μg/ml. However, the growth of PD7-[3/4/5] significantly declined during stationary phase to become less than that of PD7 (Fig. 25). Longer exponential phase of growth shown by bacterial consortium PD7 in comparison with that of mixed bacterial cultures PD7-[3/4/5]

suggested that PD7 has a more efficient co-metabolic process of *p,p′*-DDT transformation products as well as the metabolism of the initial *p,p′*-DDT compound.

In general, mixed three bacterial cultures exhibited higher *p,p′*-DDT transformation levels in comparison with those of single and mixed two bacterial isolates (Fig. 21, 24 and 27). The transformation levels ranged between 51.4 - 78.7% of initial 100 ppm *p,p′*-DDT after 10 days of incubation. The highest *p,p′*-DDT transformation level was achieved by mixed three bacterial cultures PD7-[3/4/5] at 78.7% in comparison to 88.4% by bacterial consortium PD7 (Fig. 27). However, mixed three bacterial cultures PD7-[1/2/3], PD7-[1/2/4] and PD7-[1/2/5] showed no significant increase on transformation level when compared to that of single isolates (transformation levels of 27.8 - 50.63%) (Fig. 21 and 27). These data suggest that bacterial isolates PD7-1 and PD7-2 had less significant roles in the overall *p,p′*-DDT transformation, especially when both were included in mixed three bacterial cultures. Antagonistic interaction may be the reason for this occurrence as was reported in a study involving mixed culture of hydrocarboclastic strains (*Corynebacterium* sp. and *Sphingomonas* sp.) isolated from contaminated marine sediment which were able to degrade *eicosane* (C_{20}) and phenanthrene (Syakti *et al.*, 2004). Nevertheless, mixed three bacterial cultures PD7-[1/3/4], PD7-[1/3/5], PD7-[1/4/5], PD7-[2/3/4], PD7-[2/3/5], PD7-[2/4/5] and PD7-[3/4/5] all significantly increased *p,p′*-DDT transformation level (more than 62.7% transformation) in comparison with that of single isolates.

From the results obtained, mixed three bacterial cultures was demonstrated to increase *p,p′*-DDT transformation in comparison with that of single isolates. Thereby, supporting the report that the involvement of numerous bacteria in the transformation process of contaminant may potentially increases the efficiency with complex transformation regulatory pathway (Neilson, 1995). Although the growth and *p,p′*-DDT transformation profiles of each mixed three bacterial cultures followed the same trend, the levels were different from one another. These differences may be attributed to the distinct bacterial composition within the mixed three bacterial communities. Hence, besides understanding the transformation ability of each isolate within the community, it is also important to determine the appropriate bacterial composition.

6. Study of *p,p′*-DDT transformation by mixed four bacterial cultures

Mixed four bacterial cultures were constructed by excluding one bacterial isolate, either PD7-1 PD7-2 PD7-3 PD7-4 or PD7-5, from the mixed cultures. Prior to the cultivation, bacterial isolates were grown as starter culture in MYSM+DDT_{20} until turbidity was observed (OD_{600} = 0.5). Starter culture of single isolate with the same cell density (OD_{600} value of approximately 0.5) in the ratio of 1:1:1:1 (v/v/v/v) were combined to give the final mixed bacterial culture inoculum concentrations that was approximately equivalent to that of single isolates and bacterial consortium inoculum concentrations. This mixed bacterial cultures was then cultivated in MYSM+DDT_{100} at room temperature, 150 rpm for up to 10 days.

Growth and *p,p′*-DDT transformation profiles of mixed four bacterial cultures showed close resemblance to those of mixed two and three bacterial cultures. Growth occurred from the start of inoculation without any lag phase, reached stationary phase in 2 - 3 days, and steadily declined until the end of the experiment (days 10) (Fig. 28). Maximum growths were between 135.7 - 183.8 μg/ml total cell proteins.

Transformation of *p,p′*-DDT by mixed four bacterial cultures did not displayed lag phase and can be observed from the start of cultivation. Transformation occurred rapidly until day 1 and gradually slowed down until the end of the experiment (days 10) (Fig. 29). Similar to the results shown by mixed three bacterial cultures, mixed four bacterial cultures also displayed significant increase in *p,p′*-DDT transformation levels in comparison with that of single bacterial isolates. The transformation levels ranged from 56.8 to 78.5% after 10 days of incubation. The highest *p,p′*-DDT transformation level was achieved by mixed four bacterial cultures PD7-[2/3/4/5] or by excluding PD7-1 at 78.5%. The lowest transformation level was obtained from mixed four bacterial cultures PD7-[1/2/3/4] or by excluding PD7-5 at 56.8%. Whereas, bacterial consortium PD7 achieved *p,p′*-DDT transformation levels of 90.07% (Fig. 30). It is interesting to note that *p,p′*-DDT concentration in all studies with mixed bacterial cultures seem to continuously decrease until days 10. Thus, if these trend hold through, *p,p′*-DDT concentration should further reduce below the amount achieved in these studies and *p,p′*-DDT transformation level may potentially be higher than seen within 10 days of cultivation.

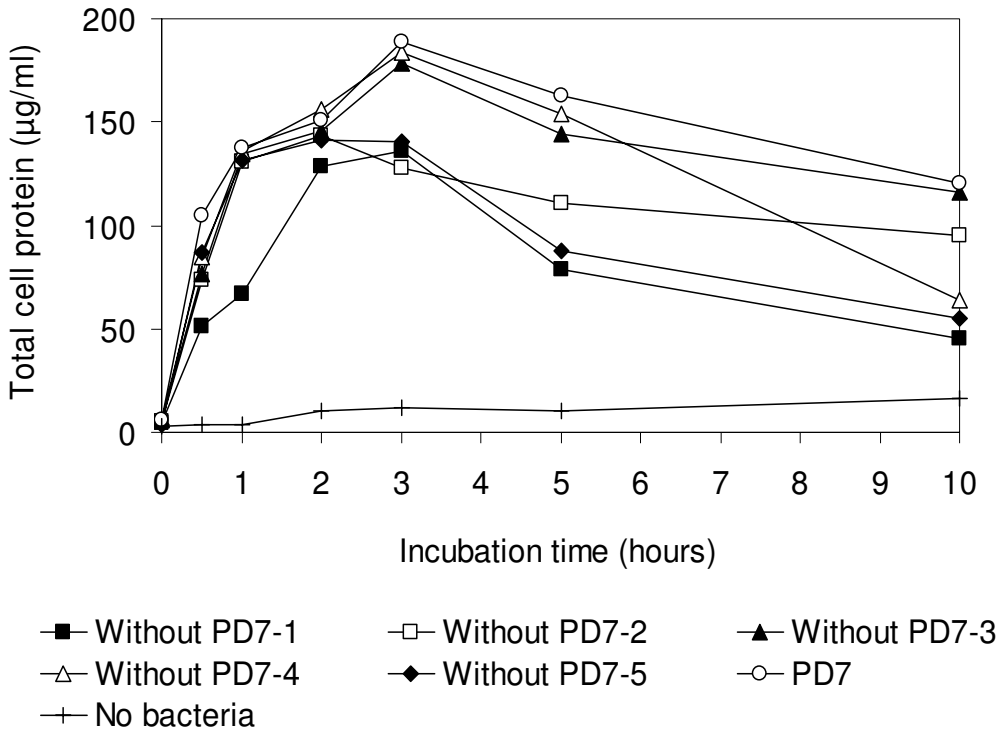


Figure 28. Bacterial growth profile of mixed four bacterial cultures grown in MSYM+DDT_{100} at room temperature, 150 rpm for 10 days.

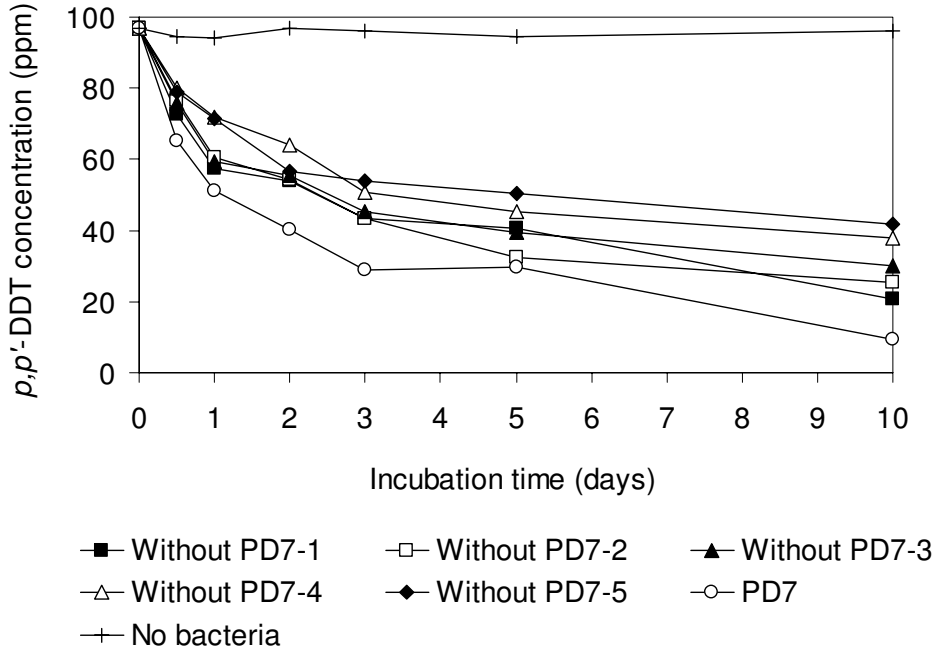


Figure 29. *p,p'*-DDT transformation profile of mixed four bacterial cultures grown in MSYM+DDT_{100} at room temperature, 150 rpm for 10 days.

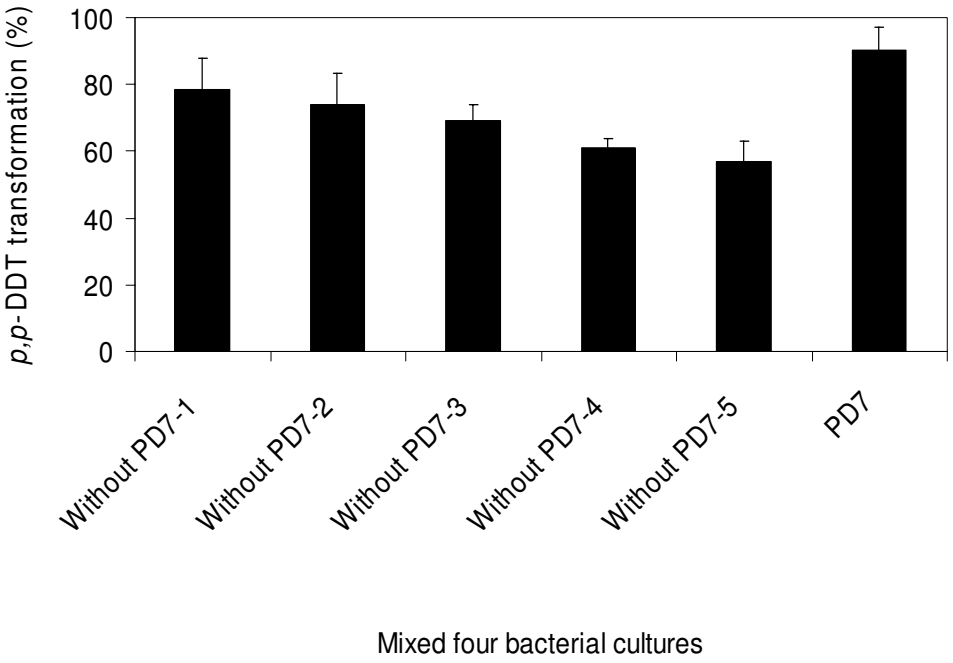


Figure 30. *p,p′*-DDT transformation levels by mixed four bacterial cultures after 10 days of incubation in MSYM+DDT_{100} at room temperature (30±2°C) and 150 rpm. Bars represent the standard deviation from three determinations. (Negative control = No bacteria; Level of *p,p′*-DDT transformation = 0.90%)

The exclusion of bacterial isolates PD7-1 or PD7-2 from the mixed four bacterial cultures resulted in high *p,p′*-DDT transformation levels of 78.5 and 74.13%, which was comparable to that from the mixed three bacterial cultures PD7-[3/4/5] (78.75%) (Fig. 27 and 30). Bacterial isolates PD7-1, PD7-2 and PD7-3 exhibited low *p,p′*-DDT transformation ability of not more than 40% when cultivated alone in comparison with those by PD7-4 and PD7-5 (49.6 and 50.63%, respectively) (Fig. 33). Although exclusion of bacterial isolates PD7-1, PD7-2 and PD7-3 did not significantly decrease the overall *p,p′*-DDT transformation by the mixed cultures, but removing either isolates PD7-4 or PD7-5 resulted in the decrease of transformation levels to 60.89 and 56.79%, respectively (Fig. 30). Besides having the highest *p,p′*-DDT transformation level, growth of mixed culture without PD7-1 reached the lowest total cell protein when compared to other mixed four bacterial cultures. As suggested

in the earlier part of this work, bacterial isolate PD7-1 may played an important part in the co-metabolism process within the *p,p′*-DDT transformation community. In co-metabolism, the bacterial isolate prefers to utilize the metabolized product for growth instead of the initial substrate. Therefore, mixed bacterial cultures without isolate PD7-1 will continue to transform *p,p′*-DDT to a high degree without the addition of growth or increase in the total cell protein contributed by isolate PD7-1.

7. Study of *p,p′*-DDT transformation by full mixed bacterial cultures

Mixed five bacterial culture study was constructed by mixing isolates PD7-1, PD7-2, PD7-3, PD7-4 and PD7-5 starter culture in the ratio of 1:1:1:1:1 (v/v) and incubated in MYSM+DDT_{100} at room temperature, 150 rpm for up to 10 days. Prior to cultivation, each bacterial isolate was grown as starter culture in MYSM+DDT_{20} until turbidity was observed (OD_{600} = 0.5). Starter culture of single isolate with the same cell density (OD_{600} value of approximately 0.5) in the above ratio of 1:1:1:1:1 (v/v) were combined to give the final mixed bacterial culture inoculum concentrations that was approximately equivalent to that of single isolates and bacterial consortium inoculum concentrations.

Full mixed five bacterial culture (PD7-[1/2/3/4/5]) showed high level of *p,p′*-DDT transformation at 81.57% of the initial 100 ppm, while the transformation level by bacterial consortium PD7 was at 90.07%. Comparison of growth and *p,p′*-DDT transformation profiles of full mixed five bacterial culture (PD7-[1/2/3/4/5]) and bacterial consortium PD7 revealed similar features (Fig. 31). Both mixed bacterial cultures showed rapid growth rate from the start of cultivation, slowed down after day 1, reached maximum growth on days 3 and gradually declined afterward. These growth profiles were in association with *p,p′*-DDT transformation profiles as they showed high rate of *p,p′*-DDT transformation within first day of incubation and had slower transformation rate afterward (Fig. 31). The similar trends in both growth and *p,p′*-DDT transformation suggested that the bacterial number and composition within consortium PD7 and full mixed cultures of PD7-[1/2/3/4/5] were approximately the same. To investigate this hypothesis, viable cell count of each bacterial isolate, PD7-1, PD7-2, PD7-3, PD7-4 and PD7-5, was determined from consortium PD7 during *p,p′*-DDT transformation study (Fig. 32).

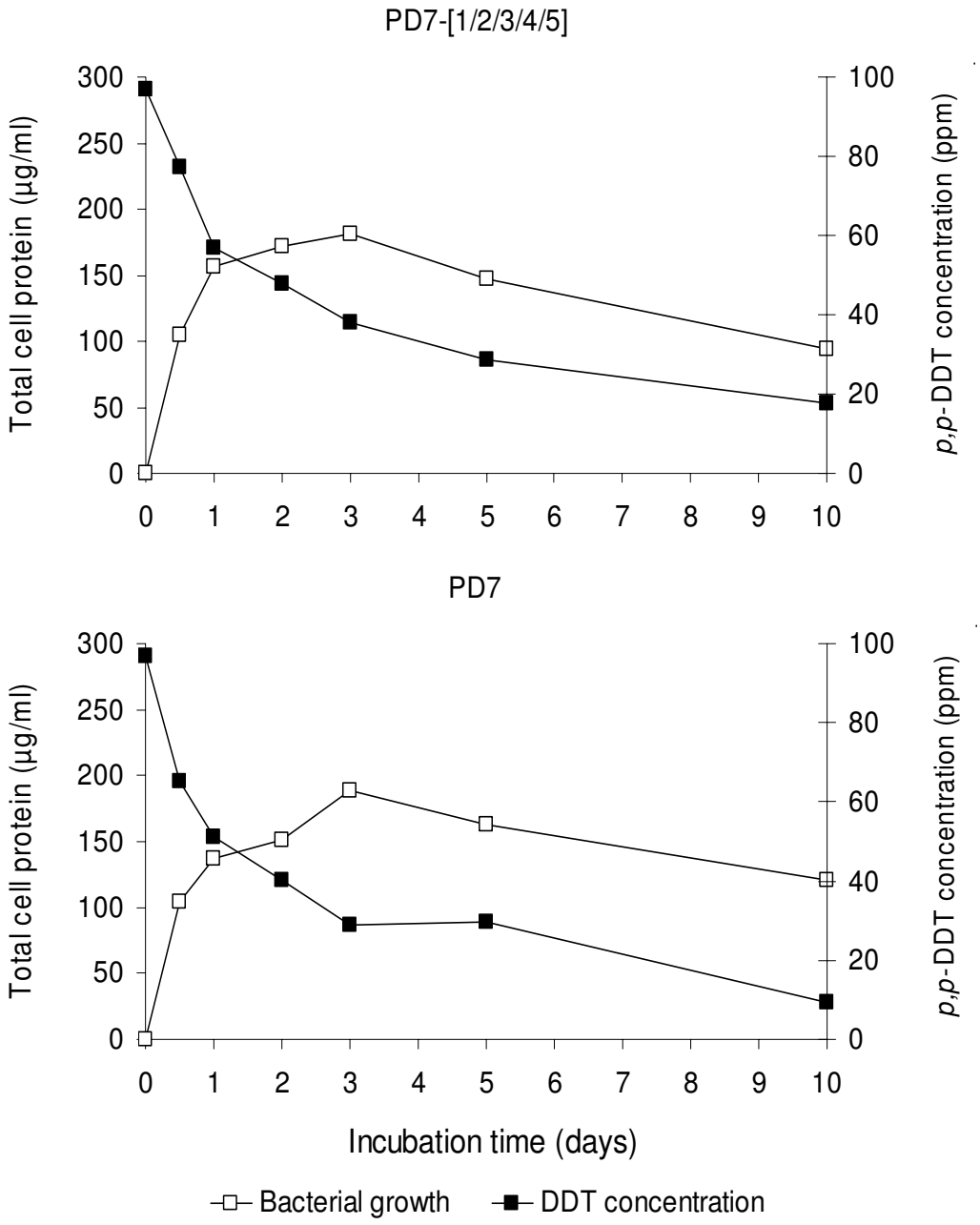


Figure 31. Comparison of bacterial growth and *p,p'*-DDT transformation profile of PD7-[1/2/3/4/5] and PD7 grown in MSYM+DDT_{100} at room temperature, 150 rpm for 10 days.

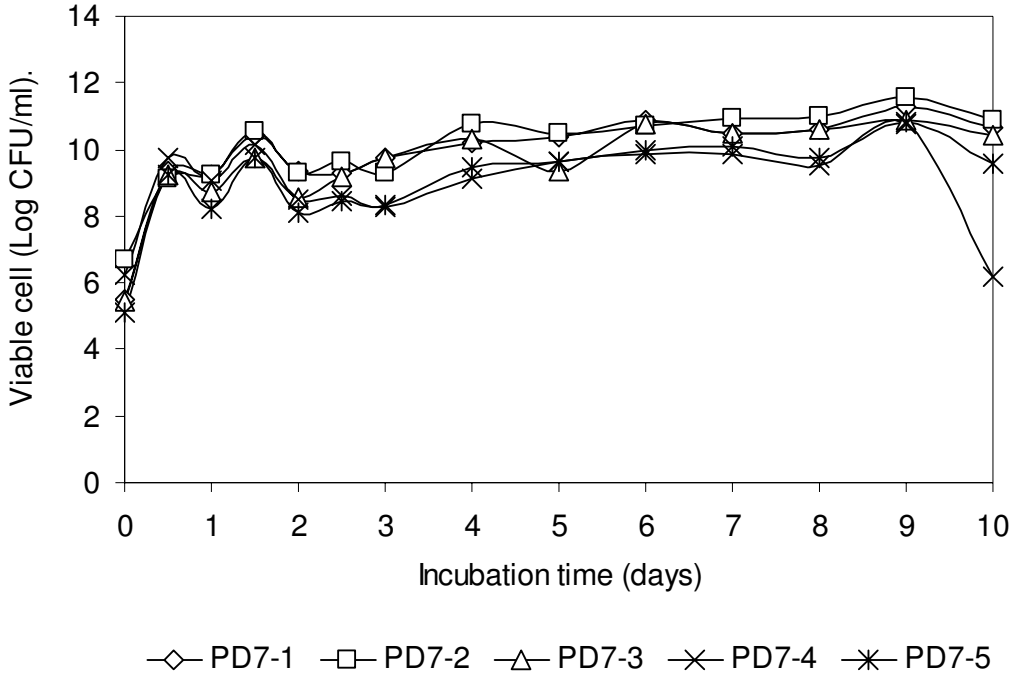


Figure 32. Growth profiles of bacterial isolates from consortium PD7, i.e. isolates PD7-1, PD7-2, PD7-3, PD7-4 and PD7-5, grown in MSYM+DDT_{100} at room temperature, 150 rpm for 10 days.

Time dependent viable cell count profile of each bacterial isolate showed similarity in number and trend, which suggested the composition of each isolates within the full mixed bacterial cultures PD7-[1/2/3/4/5] and consortium PD7 were approximately the same (Fig. 32). Hence, the growth and transformation profiles/levels of both PD7-[1/2/3/4/5] and PD7 were not significantly different (P<0.05).

Summary of all the results from the studies of bacterial consortium PD7, single and mixed bacterial cultures indicated significant increases in *p,p′*-DDT transformation levels as the numbers of isolate increase, e.g. in mixed three, four and five (full mixed) bacterial cultures (Fig. 33). Besides the importance of bacterial isolate numbers within the transformation community, the isolates composition are of significant. In the case of *p,p′*-DDT transformation, mixed bacterial cultures of three isolates PD7-[3/4/5] or four isolates without PD7-1 (PD7-[2/3/4/5]) with isolate ratio of approximately 1:1 (v/v) are comparable to that of bacterial consortium PD7.

Furthermore, bacterial isolate PD7-1 displayed evidence of significant role in co-metabolic process of when grown in the present of 100 ppm *p,p'*-DDT since the involvement of PD7-1 in the mixed cultures may help increase cell growth with no apparent increase of *p,p'*-DDT transformation rates.

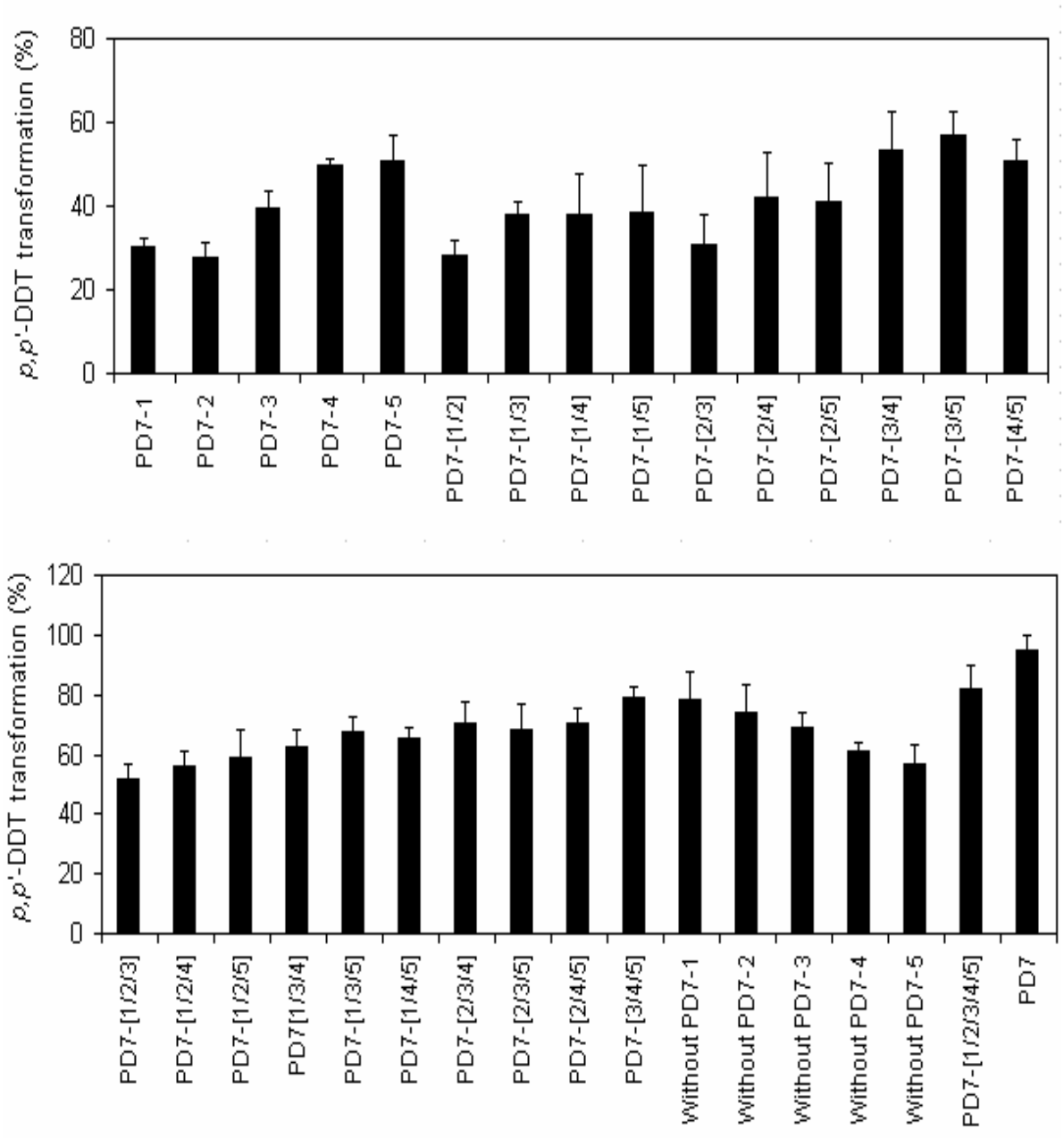


Figure 33. Comparison of *p,p'*-DDT transformation levels (%) between bacterial consortium PD7, single and mixed bacterial cultures.

8. Identification of bacterial isolates by biochemical characteristics and 16S rRNA sequences analysis

Based on their colony/cell morphologies and biochemical properties (Table 8), all 53 bacterial isolates were identified to be from the genus *Moraxella*, *Proteus*, *Klebseilla*, *Neisseria*, *Pseudomonas*, *Proteus*, *Enterobacter and Branhamella* (Table 9). There have been previous report that various bacterial species had the ability to transform and degrade DDT such as *Alcaligenes euthrophus* (Nadeau *et al.*, 1994), *Pseudomonas* sp. (Cruz *et al.*, 1999; Kamanavalli and Ninnekar, 2004; Huerta *et al*., 2007), *Aeromonas* sp., *Enterobacter* sp., *Arthrobacter* sp., *Dermabacter* sp., *Lactobacillus* sp. (Huerta *et al.*, 2007), *Pseudomonas fluorescens* (SantaCruz *et al.,* 2005).

For the five bacterial isolates from consortium PD7, i.e. PD7-1, PD7-2, PD7-3, PD7-4 and PD7-5 further molecular identifications were performed by 16S rRNA sequences analysis. Full length sequences from each isolate (more than 1300 bases) were BLAST to GenBank database with the returned result between 95 - 99% identities. Based on 16S rRNA sequences analysis data, all five bacterial isolates were identified to be closely related to *Curtobacterium citreum* (PD7-1), *Rhodococcus pyridinivorans* (PD7-2), *Niabella* sp. (PD7-3), *Bacillus anthracis* (PD7-4) and *Shinella zoogloeoides* (PD7-5) (Table 10). DNA sequence analyses identified most of the bacterial isolates from consortium PD7 to be Gram-positive bacteria, whereas, earlier Gram staining identified them as Gram-negative bacteria from the genus *Pseudomonas* and *Moraxella* (Table 8, 9 and 10). The differences in conventional identification based on colony/cell morphologies and biochemical properties from those based on molecular identification may be attributed to several reasons.

Gram staining had been criticized on its limitation of not being able to differentiate Gram-variable bacteria, where age of the culture and synthesized mucoid (lipid/wax) substance can interfere the results of both Gram-staining and biochemical tests. In this study, 13 bacterial isolates from various consortia were observed to excrete extracellular mucoid-liked substance (Table 7). Thus, Gram staining has limited usage in environmental study, where samples usually require long cultivation period and mucoid substances are sometimes produced in certain conditions (Spiegelman *et al.*, 2005).

Biochemical analyses composed of numerous tests, e.g. indole test, motility, H_2S production, methyl red test, urease test, citrate utilization test, acid production test from glucose and lactose fermentation, and nitrate reduction test. Visual-based identification method such as biochemical properties analyses is the conventional method, which requires a qualitative observation. Limitation associated with this conventional method includes low sensitivity, inability to detect unculturable bacteria and unknown species, very slow turnaround time, and poor reproducibility (Waters *et al.*, 2006). Thus, the disadvantage of colony/cell morphology observations and biochemical tests may represented a serious problem during the experiment since the error in one-step of identification may influence all set of subsequent identification results. However, morphological and biochemical characteristics are still reliable for preliminary identification due to their simple procedure, required low cost of identification, can be rapidly performed and suitable for applying to multiple bacterial samples at one time.

On the other hand, molecular biological-based identification methods comprised a broad range of techniques that are based on the analysis and differentiation of microbial DNA such 16S rRNA sequences analysis, which was used for further identification in this study, are more precise and possesses several distinct advantages over conventional identification methods. Unlike most other commonly used methods, which require the production of secondary materials via the manipulation of microbial growth, molecular biological-based methods recover and test of their source materials (DNA) directly from the microbial cells themselves. This eliminates both the time required for growth and the biases associated with cultured growth, which was unavoidably selective. The recovered nucleic acid was specifically amplified using polymerase chain reaction (PCR) and subsequently sequenced. Molecular methods are highly sensitive and allow for a high degree of specificity, which, coupled with the ability to separate similar but distinct DNA molecules, means that a great deal of information can be obtained from even very complex microbial communities (Spiegelman *et al.*, 2005).

Table 8. Morphological and biochemical properties of *p,p'*-DDT transforming bacteria from DTT contaminated soil.

Morphological and Biochemical characteristics	Isolates								
	PD1-1	PD1-2	PD1-3	PD1-4	PD1-5	PD1-6	PD2-1	PD2-2	PD2-3
Gram staining	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Cell shape	rod	cocci	cocci	cocci	rod	cocci	rod	cocci	rod
Indole test	-	-	-	-	-	-	-	-	-
Motility	+	-	+	-	-	-	-	+	
H_2S production	+	+	+	-	-	-	+	-	-
Methyl red test	-	-	-	-	-	-	-	-	-
Urea	+	+	-	+	-	-	+	-	-
Citrate utilization	-	-	+	-	-	+	-	-	-
Acid Production test:									
- Glucose	+	+	+	+	-	+	+	-	-
- Lactose	-	-	-	-	+	-	-	-	-
Nitrate reduction	-	-	+	-	-	+	+	+	+

Table 8. (Continued)

Morphological and Biochemical characteristics	Isolates								
	PD2-4	PD3-1	PD3-2	PD3-3	PD3-4	PD3-5	PD4-1	PD4-2	PD4-3
Gram staining	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Cell shape	rod	cocci	rod	cocci	rod	rod	cocci	rod	rod
Indole test	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+		-	+	-
H_2S production	-	-	-	+	-	+	-	-	-
Methyl red test	-	-	-	+	+	-	-	-	-
Urea	-	-	-	+	+	+	+	-	+
Citrate utilization	-	-	-	+	-	+	+	-	+
Acid Production test:									
- Glucose	-	-	-	-	-	+	-	-	-
- Lactose	-	-	-	+	+	-	+	-	-
Nitrate reduction	-	-	-	+	+	+	-	-	+

Table 8. (Continued)

Morphological and Biochemical characteristics	Isolates								
	PD4-4	PD5-1	PD5-2	PD5-3	PD5-4	PD5-5	PD5-6	PD6-1	PD6-2
Gram staining	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Cell shape	rod	cocci	cocci	cocci	cocci	cocci	cocci	rod	rod
Indole test	-	-	-	+	+	-	-	-	+
Motility	+		+	+	+	-	-	+	+
H_2S production	-	-	-	-	-	-	+	-	-
Methyl red test	-	-	-	-	-	-	-	-	-
Urea	+	-	-	-	-	+	+	+	-
Citrate utilization	-	-	-	-	-	+	-	-	-
Acid Production test:									
- Glucose	-	-	-	+	+	-	-	-	+
- Lactose	-	-	-	-	+	-	-	-	-
Nitrate reduction	+	+	+	-	-	+	+	-	-

Table 8. (Continued)

Morphological and Biochemical characteristics	Isolates								
	PD6-3	PD6-4	PD6-5	PD6-6	PD7-1	PD7-2	PD7-3	PD7-4	PD7-5
Gram staining	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Cell shape	rod	cocci	cocci	rod	rod	rod	cocci	rod	rod
Indole test	-	-	-	-	-	-	+	-	-
Motility	+	-	-	+	+	+	+	+	-
H_2S production	-	+	+	-	-	+	+	-	+
Methyl red test	-	-	-	+	-	-	-	+	
Urea	+	+	+	-	+	-	-	-	+
Citrate utilization	-	-	-	-	-	-	-	-	+
Acid Production test:									
- Glucose	-	-	-	+	-	-	-	-	-
- Lactose	-	-	-	+	-	-	+	+	-
Nitrate reduction	-	+	+	+	+	+	-	+	+

Table 8. (Continued)

Morphological and Biochemical characteristics	Isolates								
	PD8-1	PD8-2	PD8-3	PD8-4	PD9-1	PD9-2	PD9-3	PD9-4	PD9-5
Gram staining	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Cell shape	cocci	cocci	rod	rod	cocci	rod	cocci	cocci	rod
Indole test	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	-	-	+	-
H_2S production	-	-	-	-	+	-	+	+	-
Methyl red test	-	-	+	+	+	-	-	+	-
Urea	-	-	+	-	-	-	+	+	-
Citrate utilization	-	-	-	-	-	-	-	-	-
Acid Production test:									
- Glucose	-	-	+	+	+	-	-	-	-
- Lactose	-	-	-	+	-	-	-	-	-
Nitrate reduction	-	-	-	+	-	-	-	-	-

Table 8. (Continued)

Morphological and Biochemical characteristics	Isolates							
	PD10-1	PD10-2	PD11-1	PD11-2	PD11-3	PD12-1	PD12-2	PD12-3
Gram staining	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Cell shape	rod	rod	cocci	cocci	rod	rod	rod	rod
Indole test	-	-	-	-	-	+	+	-
Motility	+	-	-	+	-	+	-	+
H_2S production	-	+	+	-	-	-	-	-
Methyl red test	-	-	-	+	-	+	+	-
Urea	-	-	+	+	-	+	+	+
Citrate utilization	-	-	-	-	-	-	+	+
Acid Production test:								
- Glucose	-	-	-	+	-	-	+	-
- Lactose	-	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	+	+	+	+

Table 9. Identification of isolated bacteria based on morphological features and biochemical properties.

Consortium	Isolate	Genus
PD1	1	*Proteus*
	2	*Neisseria*
	3	*Neisseria*
	4	*Neisseria*
	5	*Enterobacter*
	6	*Neisseria*
PD2	1	*Proteus*
	2	*Branhamella*
	3	*Pseudomonas*
	4	*Pseudomonas*
PD3	1	*Moraxella*
	2	*Pseudomonas*
	3	*Branhamella*
	4	*Klebseilla*
	5	*Proteus*
PD4	1	*Moraxella*
	2	*Pseudomonas*
	3	*Pseudomonas*
	4	*Pseudomonas*
PD5	1	*Branhamella*
	2	*Branhamella*
	3	*Neisseria*
	4	*Neisseria*
	5	*Branhamella*
	6	*Branhamella*

Table 9. (Continued)

Consortium	Isolate	Genus
PD6	1	*Pseudomonas*
	2	*Proteus*
	3	*Pseudomonas*
	4	*Branhamella*
	5	*Branhamella*
	6	*Klebseilla*
PD7	1	*Pseudomonas*
	2	*Pseudomonas*
	3	*Moraxella*
	4	*Pseudomonas*
	5	*Pseudomonas*
PD8	1	*Moraxella*
	2	*Moraxella*
	3	*Proteus*
	4	*Klebseilla*
PD9	1	*Neisseria*
	2	*Pseudomonas*
	3	*Moraxella*
	4	*Moraxella*
	5	*Pseudomonas*
PD10	1	*Pseudomonas*
	2	*Pseudomonas*
PD11	1	*Moraxella*
	2	*Neisseria*
	3	*Pseudomonas*
PD12	1	*Pseudomonas*
	2	*Proteus*
	3	*Pseudomonas*

Table 10. 16S rRNA gene sequences analysis of *p,p'*-DDT transforming bacterial isolates from consortium PD7.

Isolates	Predicted genus/specie	Identity	Sequences similarity
PD7-1	*Curtobacterium citreum*	99%	1482/1483
PD7-2	*Rhodococcus pyridinivorans*	99%	1461/1463
PD7-3	*Niabella* sp.	95%	1325/1390
PD7-4	*Bacillus anthracis*	99%	1449/1451
PD7-5	*Shinella zoogloeoides*	98%	1426/1446

16S rRNA sequences analysis of bacterial isolate PD7-1 to GenBank database revealed 99% identity to *Curtobacterium citreum* (Table 10; Fig. 34). *Curtobacterium* was the current name of the genus *Brevibacterium*. It is a Gram-positive rod-shaped, obligated aerobic soil bacterium with no report on human infection. This strain has never been reported to have transformation or degradation ability to any contaminant (Faddin, 1999; Fang *et al.*, 2007). Based on the morphological and biochemical properties, bacterial isolate PD7-1 was previously identified to be from the genus *Pseudomonas*. The differences between morphological/biochemical-based and molecular-based identification results may be due to the similarity between both genus and long cultivation time.

Curtobacterium is a non-spore forming bacterium with optimum growth at 20-37^{o}C (or 20-30^{o}C for some strain). It also displays a negative result in glucose fermentation test (Faddin, 1999). These morphological and biochemical characteristics are similar with those of *Pseudomonas* obtained in the previous observation. The cell of *Curtobacterium* does not contain mycolic acid (Faddin, 1999), the presence of which may increase resistant to chemical damage and dehydration, and prevent the effective activity of hydrophobic antibiotics. In addition, mycolic acid allows the bacterium to grow readily inside macrophages. This is the possible reason that PD7-1 exhibited low transformation result in comparison to that of other bacterial isolates from consortium PD7.

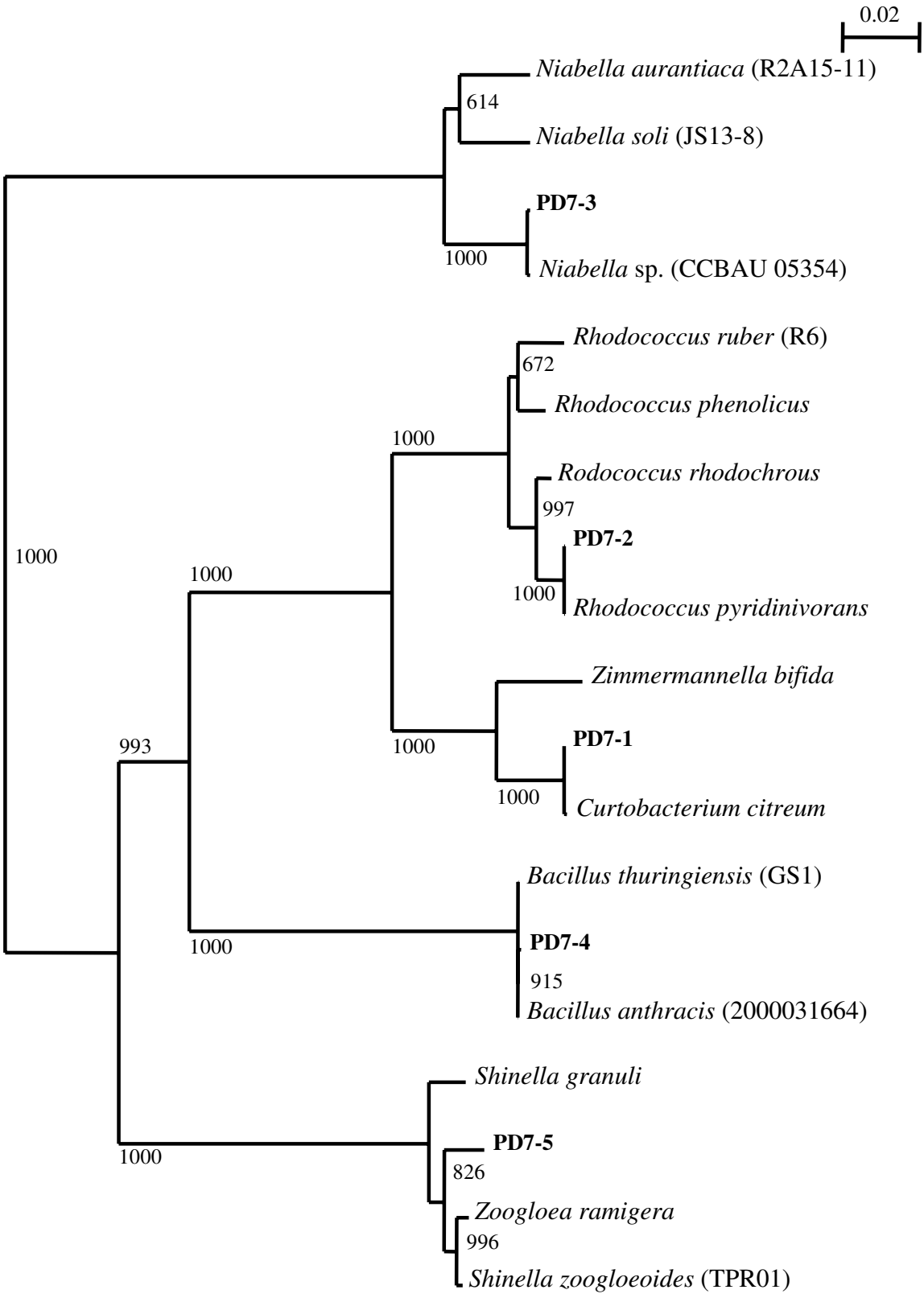


Figure 34. Phylogenetic analysis of the 16S rRNA sequence analyses of *p,p'*-DDT transforming bacterial isolates PD7-1, PD7-2, PD73, PD7-4 and PD7-5.

Bacterial isolate PD7-2 showed 99% identity to *Rhodococcus pyridinivorans* with sequences similarity of 1461/1463 bases (Table 10; Fig. 34). *Rhodococcus pyridinivorans* is a Gram-positive bacterium, usually found in soil, water and some eukaryotic cells (Faddin, 1999; Yoon *et al.*, 2000). In the previous identification analysis by morphological and biochemical features, bacterial isolate PD7-2 was also determined to be from the genus *Pseudomonas*. This difference in identification may be the result of close similarity in many biochemical features such as non-spore forming, negative results in glucose fermentation and urease test, and positive result in nitrate reduction test. *Rhodococcus pyridinivorans* has also been shown to have the ability of pyridine degradation (Yoon *et al*., 2000). Pyridine is an aromatic heterocyclic organic compound with the chemical formula C_5H_5N. It is used as a precursor to agrochemicals and pharmaceuticals, and is also an important solvent and reagent. It is structurally related to benzene, wherein one -CH group in the aromatic six-membered ring is replaced by a nitrogen atom (Fig. 35). Thus, being the possible reason of *Rhodococcus* ability for DDT transformation. Furthermore, *Rhodococcus pyridinivorans* was also known to contain mycolic acid in its cell, thus making it more resistant to chemical damage from the exposure of toxic compound.

After BLAST search through GenBank database, bacterial isolate PD7-3 showed 95% identity to the genus *Niabella* with sequence similarity of 1325/1390 bases (Table 10; Fig. 34). A 95% identity on sequence similarity, especially on a full length such as in this instance suggests only a slight possibility of the bacterial isolate being in the genus *Niabella*. However, *Niabella* is a known non-motile, short rod, aerobic Gram-negative bacterium found in soil samples (Yeon *et al.*, 2008). There is very little information available on this newly discovered bacterial strain.

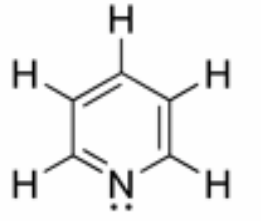


Figure 35. The structure of pyridine.
Source : http://en.wikipedia.org

Bacterial isolate PD7-4 received 99% identity to *Bacillus anthracis* from a sequence similarity of 1449/1451 bases. *Bacillus anthracis* is a Gram-positive spore forming bacterium, which is widely known as the cause of anthrax disease. The sources of *Bacillus anthracis* are soil, animal carcasses and feces (including sheep, goats, cattle, bison, horses, and deer), and animal products (e.g., hides and wool). Previous identification by morphological and biochemical properties placed isolate PD7-4 in the genus *Pseudomonas*. Spore forming and positive nitrate reduction are the similar features between genus *Bacillus* and *Pseudomonas*. Sporulation by *Bacillus* allows the strain to survive in the extreme conditions such as chemical exposure or high temperature (35 - 65^{o}C) (Faddin, 1999). The other possible reason to its high *p,p′*-DDT transformation ability in comparison with that of other bacterial isolates may be attributed to its ability to grow in aerobic and facultative anaerobic condition. Thus, this isolate could transform *p,p′*-DDT via either aerobic or anaerobic pathways. As observed by Beunink and Rehm (1988), synchronous interaction between anaerobic and aerobic transformation of *p,p′*-DDT by immobilized mixed culture system could improve transformation process. Although DDT is metabolized through co-metabolic pathways by many facultative and obligated anaerobic microorganisms under suitable environmental conditions, no organism is known which utilizes DDT as a sole carbon and energy sources. Nevertheless, complete mineralization of the insecticide by sequential cooperation of microbial populations through co-metabolic reactions is possible (Focht and Alexander 1970; Pfaender and Alexander 1972).

From this study, the bacterial isolate with the highest *p,p′*-DDT transformation, PD7-5, was identified to be 98% similar in sequences (1426/1446 bases) to *Shinella zoogloeoides.* This strain has been found in activated sludge and reported to be able to degrade pyridine (Sun *et al.*, 2008). The ability of *Shinella zoogloeoides* in degrading pyridine, which is also an aromatic-based compound, may be the reason for its ability to transform *p,p′*-DDT as well. There is little information related to this bacterial strain, thus it was of the interest that *Shinella zoogloeoides* can transformed up to 50.63 % from the initial 100 ppm of *p,p′*-DDT in the cultivation medium.

CHAPTER IV

CONCLUSIONS

Twelve bacterial consortia with 53 distinct bacterial isolates were obtained from 12 soil samples collected from various agricultural areas in Songkhla Province, Thailand, through selective enrichment and acclimatization processes in mineral salt yeast extract medium supplemented with 100 ppm of *p,p′*-DDT (MSYM+DDT_{100}). All twelve bacterial consortia were shown to have the ability of 37.03 to 100% transformation from initial 100 ppm of *p,p′*-DDT within 10 days of incubation. Bacterial consortium PD7 was chosen for further study since it exhibited high *p,p′*-DDT transformation ability (94.72%) and generate high total cell protein (193.83 μg/ml). Mixed two bacterial cultures exhibited no significant *p,p′*-DDT transformation increase (28.19 to 57.08%) in comparison with those of single bacterial isolates ($P<0.05$). Nevertheless, mixed three bacterial cultures of PD7-[1/3/4], PD7-[1/3/5], PD7-[1/4/5], PD7-[2/3/4], PD7-[2/3/5], PD7-[2/4/5] and PD7-[3/4/5] all have significantly increased *p,p′*-DDT transformation levels in comparison with that of single isolates ($P<0.05$). The exception was for PD7-[1/2/3], PD7-[1/2/4] and PD7-[1/2/5] which showed no significant increase in transformation levels in comparison with that of the single isolates. Mixed four and five bacterial culture all exhibited significant increase of *p,p′*-DDT transformation levels in comparison with that of single isolates ($P<0.05$). Data from this study suggests that bacterial isolates PD7-1, PD7-2 and PD7-3 contributed less significant role on the overall transformation process compared with that offered by PD7-4 and PD7-5 when they work as either single culture or community of mixed bacterial culture study. Bacterial isolate PD7-1 appeared to utilize *p,p′*-DDT and it metabolites in a co-metabolic mode of activity. Based on 16S rRNA sequences analysis, all five bacterial isolates were identified to be *Curtobacterium citreum* (PD7-1), *Rhodococcus pyridinivorans* (PD7-2), *Niabella* sp. (PD7-3), *Bacillus anthracis* (PD7-4) and *Shinella zoogloeoides* (PD7-5). It will be interesting to further evaluate the applicable potential of these *p,p′*-DDT

transforming community in natural environmental such as contaminated agricultural area or water reservoir.

Suggestion for further study:

1. Bacterial identification based on colony and cell morphologies are reliable for preliminary screening, which are necessary to help reduce the unwarranted study of identical isolates. However, for environmental samples which require extended cultivation time, precaution has to be taken as to not miss identify the Gram-variable isolates. Otherwise, subsequent biochemical analyses will be misleading and may contradict molecular-based identification results. Further study should be performed on the identification of bacterial isolates from all *p,p′*-DDT transforming consortia. This will provide information on the biodiversity of *p,p′*-DDT transforming soil bacteria.
2. *p,p′*-DDT biotransformation study should include detail analysis of the metabolites to understand which catabolic pathway the compound followed. GC-ECD analysis can precisely determined the concentration of even trace amount of halogenated compound such as *p,p′*-DDT and its metabolites. However, applying GC-ECD has a limitation since the analysis requires standard to determine the amount of each compound. Thus, a known *p,p′*-DDT transformation metabolites has to be determined other analytical instrument such as GC-MS. In addition, tracer study using isotopic compound can be used to follow the transformation pathway.
3. The data obtained from this study were laboratory proven data. Thus, it is important to investigate the reliability of these bacterial *p,p′*-DDT transformation abilities in the direct application for environment remediation. These subsequent studies may utilize the information from related studies for conservation and remediation of the natural environment.

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APPENDICES

APPENDIX 1

Culture media

1. Mineral salt-yeast extract medium (MSYM)

NH_4NO_3	0.25	g
K_2HPO_4	0.675	g
$Ca(NO_3)_2$	0.1	g
$MgSO_4.7H_2O$	0.2	g
Yeast extract	0.01	g
Agar	15	g
Distilled water	1	liter

APPENDIX 2

Gram staining and biochemical test

1. Gram staining

Reagent and equipment

1. microscope
2. glass slide
3. crystal violet
4. safranin
5. iodine solution
6. 95% alcohol

Method

1. Inoculate isolated bacteria in nutrient broth for 18-24 hr. Transfer a loop of the liquid culture to the surface of a clean glass slide, and spread over a small area.
2. A loopful of a pure culture is smeared on a slide and allowed to air dry.
3. The cells were fixed to the slide by heating through a flame.
4. Crystal violet (a basic dye) was then added by covering the heat-fixed cells with a prepared solution. Allow to stain for approximately 1 minute.
5. The slide was briefly rinsed with water. The heat-fixed cells should look purple at this stage.
6. Iodine (Gram's iodine) solution (1% iodine, 2% potassium iodide in water) was added for 1 minute. This step was to fix the dye, making it more difficult to decolorize and reducing some of the variability of the test (briefly rinse with water).
7. The sample was decolorized by applying 95% ethanol to ensure that all the color was rinsed. In this step was to wash away unbound crystal violet, leaving Gram-positive organisms stained purple with Gram-negative organisms colorless (rinse with water to stop decolorization)

8. The slide was rinsed with a counterstain (safranin or carbol fuchsin) which stains all cells red. The counterstain stains both gram-negative and gram-positive cells. However, the purple gram-positive color was not altered by the presence of the counter-stain, it's effect is only seen in the previously colorless gram-negative cells which now appear pink/red.
9. Gently blotted and allowed the slide to dryness.
10. All slides of bacteria were observed for the color and shape under the oil immersion lens (100x magnification).

Observation

1. Gram positive bacteria : bacteria will appear to be crystal violet purple colored.
2. Gram negative bacteria : bacteria will appear to be red safranin colored.

2. Indole test

Reagent and equipment

1. Tryptone broth
2. Kovac's reagent

Method

1. Inoculate 5 ml of isolated bacteria in tryptone broth for 24-48 hr.
2. Five ml of Kovac's reagent was added in the culture.

Observation

Positive : a red-violet color in the surface of broth.

Negative : no color appear, if the broth appear orange color that show variable result due to tryptophan being oxidized to skatole (methyl indole)

3. Motility test

Reagent and equipment

1. motility test medium
2. needle

Method

1. Isolated bacterial was stabbed into motility test medium.
2. Incubated at room temperature for 24-48 hr.

Observation

Positive : bacteria migrate away from line of inoculation

Negative : bacteria will grow in line of inoculation

4. Hydrogen sulfide (H_2S) production test

Reagent and equipment

1. Triple sugar iron agar (TSI)
2. Needle

Method

1. Isolated bacteria was streaked on the surface of agar and stabbed into the bottom of slant.
2. Incubated at room temperature for 5 days.

Observation

Positive :
1. Yellow is appeared at the bottom of slant while the color of slant surface is not changed that show the bacteria can use only glucose.
2. Yellow also appeared on the slant surface that show the bacteria can use lactose or sucrose
3. If the gas is produced the agar will raise up from the bottom. If the sulfur is reduced and hydrogen sulfide is produced, it will form a visible black of ferric sulfide (FeS) in the tube

5. Methyl red test

Reagent and equipment

1. MR-VP broth
2. methyl red

Method

1. Isolated bacteria was inoculated in 5 ml MR-VP broth for 5 days
2. Five or six drops of methyl red were added into the broth.

Observation

Positive : red

Negative : orange or yellow

6. Urease test

Reagent and equipment

1. Urea agar
2. loop

Method

1. Isolated bacterial was streaked on urea agar slant.
2. Incubated for 24-48 hr.

Observation

Positive : pink color

Negative : no color change

7. Citrate Utilization Test

Reagent and equipment

1. Simmons' citrate agar
2. needle

Method

1. Isolated bacterial was spotted Simmons' citrate agar slant.
2. Incubated for 24-48 hr (maximum 7 days).

Observation

Positive : Agar color will change from green to blue due to bacterial growth.

Negative : Agar color will not change.

8. Acid production from Carbohydrate utilization

Reagent and equipment

1. Fermentation carbohydrate medium
2. Gas tubes

Method

1. The bacteria were cultured in fermentation carbohydrate medium and incubated at room temperature for 18-24 hr.
2. The samples were observed in color changing and gas production.

Observation

Positive : 1. The color of medium turned yellow but there is no bubble in gas tube showing that bacteria can ferment carbohydrate to produce acid.

2. Medium color changed to yellow with bubble in gas tube showing that bacteria can ferment carbohydrate to produce acid and gas.

Negative: The color of medium will not changed

9. Nitrate reduction test

Reagent and equipment

1. Nitrate broth
2. sulfinilic acid
3. ∝ -naphthylamine
4. Zinc

Method

1. Isolated bacteria was inoculated in nitrate broth medium.
2. Incubated at room temperature for 24-48 hr.
3. Each of sulfanilic acid and ∝ -naphthylamine (10-15 drops) was added to the medium . the broth will turn a deep red within 5 minutes at this step, if the bacterium produced nitrate reductase.
4. If no color change is observed, then the result is inconclusive. Add a small amount of zinc to the broth.

Observation

Positive : colorless.

Negative : turn to be red

10. Medium composition for biochemical tests

(each formula was dissolved in 1 liter of distilled water)

Nutrient agar

Beef extract	3.0	g.
Peptone	5.0	g.
Agar	15.0	g.

Motility test medium

Tryptone	10.0	g.
Sodium chloride	5.0	g.
Agar	5.0	g.

Nitrate broth

Beef extract	3.0	g.
Peptone	5.0	g.
Potassium nitrate	1.0	g.

Tryptone broth

Tryptone	10	g.

MR-VP medium

Buffered Peptone	7.0	g.
Dipotassium phosphate	5.0	g.
Dextrose	5.0	g.

Final pH 6.9 at 25 degree Celsius

Simmons' citrate agar

$MnSO_4$	0.2	g.
$(NH_4)H_2PO_4$	1.0	g.
K_2HPO_4	1.0	g.
NaCl	5.0	g.
Sodium citrate	2.0	g.
Bromthymol blue	0.08	g.
Agar	15.0	g.

Urea agar

Pancreatic Digest of Gelatin	1.0	g.
Dextrose	1.0	g.
NaCl	5.0	g.

$NaPO_4$	2.0	g.
Urea	20.0	g.
Agar	15.0	g.
Phenol red	12.0	mg.
Final pH 6.8 ±0.2 at 25°C		

Triple sugar iron agar (TSI)

Pancreatic Digest of Casein	15.0	g.
Yeast Extract	3.0	g.
Beef Extract	3.0	g.
Proteose peptone	5.0	g.
Sodium chloride	5.0	g
Lactose	10.0	g.
Sucrose	10.0	g.
Dextrose	1.0	g.
Ferrous sulfate	0.2	g.
Sodium Thiosulfate	0.3	g.
Phenol Red	24.0	mg.
Agar	12.0	g

3% Hydrogen peroxide

H_2O_2	3.0	g.
Distilled water	100.0	ml.

Methyl red solution

Methyl red	0.8	g.
Ethanol 95%	300.0	ml.
Distilled water	200.0	ml.

Nitrate test solution

Sulfanilic acid	0.8	g.
5 N acetic acid	100.0	ml.

Fermentation Carbohydrate medium (pH 6.8-7.0)

Beef extract	3.0	g.
Peptone	5.0	g.
Sugar (lactose or glucose)	10.0	g.
Bromthymol blue 1.6%	4.0	g.

APPENDIX 3

Determination of total cell protein by modified Lowry's method

1. Lowry's reagent

Solution A - 20 g Na_2CO_3 in 260 ml H_2O
- 0.4 g $CuSO_4.5H_2O$ in 20 ml H_2O
- 0.2 g sodium potassium tartrate in 20 ml H_2O
- Those solutions are then mixed together

Solution B - 10 g of sodium dodecyl sulfate (SDS) in 100 ml H_2O

Solution C - NaOH 1 N solution

Note: Lowry's reagent is prepared by mixing solution A, B and C in the ratio of 3:1:1.

2. Folin-Ciocalteu reagent

Folin-Ciocalteu reagent is freshly prepared before analysis by diluting with distilled water in the ratio of 10:1 (0.2 N)

3. Standard curve of BSA protein

[BSA] (μg/ml)	OD_{750}					Average
0	0.135	0.129	0.138	0.128	0.13	0.132 ± 0.004
10	0.147	0.152	0.147	0.143	0.157	0.1492 ± 0.005
20	0.173	0.174	0.17	0.179	0.169	0.173 ± 0.004
40	0.215	0.22	0.225	0.21	0.216	0.2172 ± 0.006
60	0.26	0.262	0.268	0.257	0.258	0.261 ± 0.004
80	0.285	0.302	0.287	0.303	0.289	0.2932 ± 0.009
100	0.322	0.345	0.328	0.33	0.341	0.3332 ± 0.010

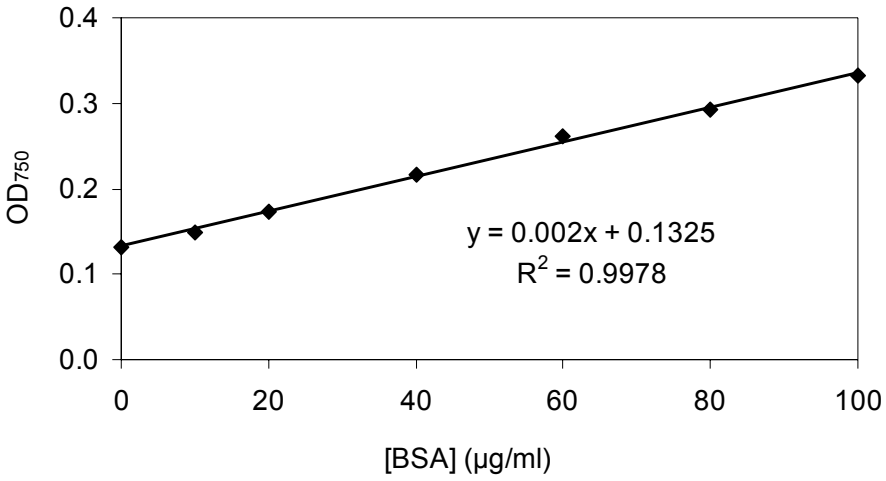

Figure 36. Standard curve of BSA for protein determination.

4. Microplate reader instrument

Figure 37. Microplate reader Zenyth 200rt

APPENDIX 4

Determination of *p,p'*-DDT concentration by GC-ECD

1. Standard curve

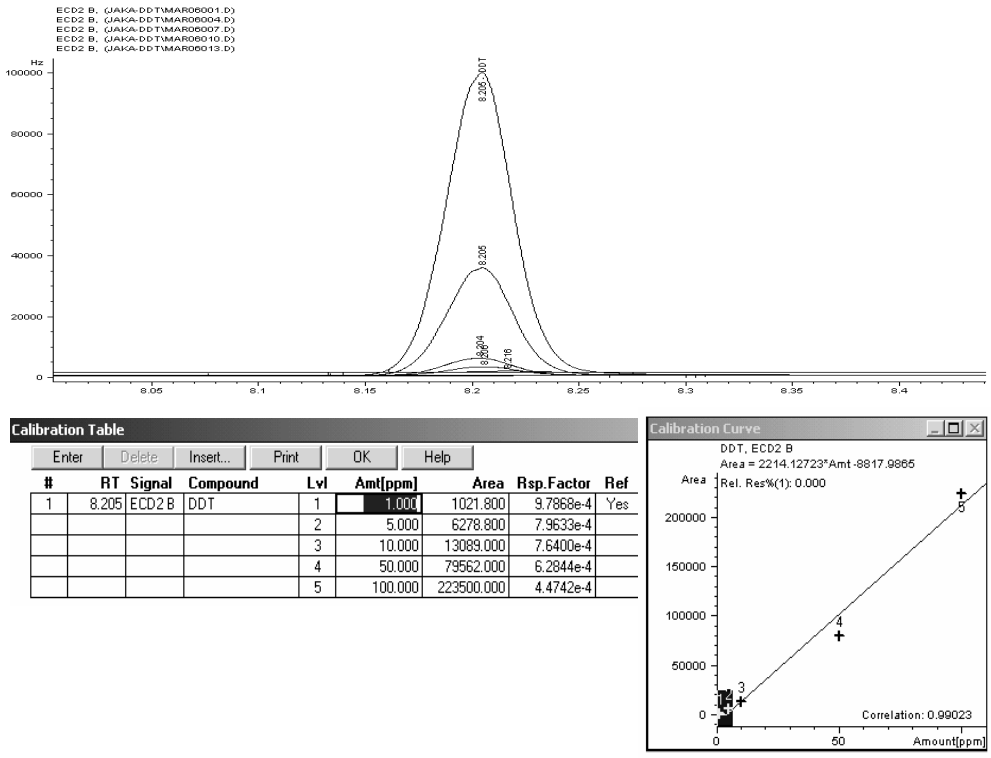

Figure 38. Standard curve of *p,p'*-DDT determination by GC-ECD.

2. *p,p'*-DDT chromatogram

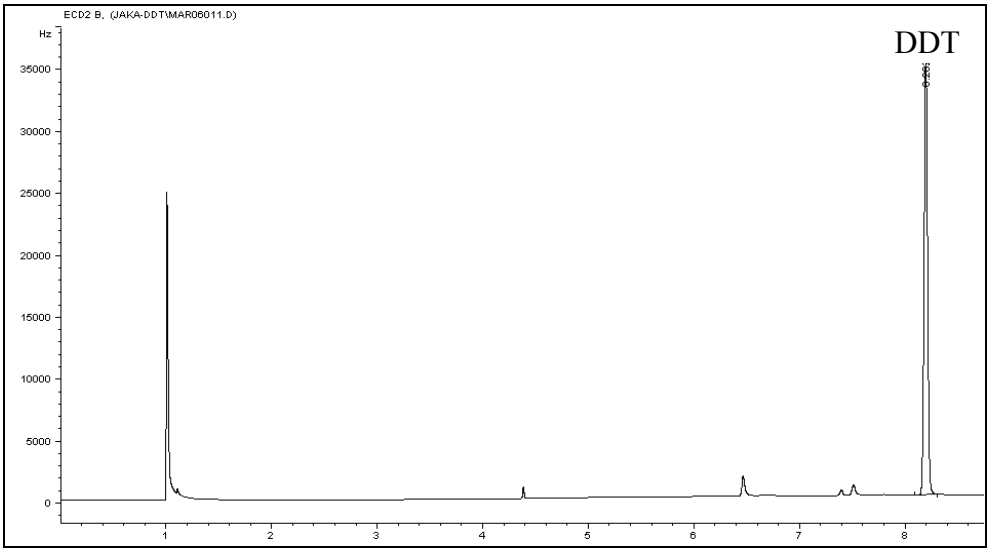

Figure 39. GC-ECD chromatogram of *p,p'*-DDT.

3. GC-ECD instrument

Figure 40. GC-ECD Hewlett-Packard model 6890.

APPENDIX 5

p,p'-DDT concentration, degradation level and total cell protein

Table 11. *p,p'*-DDT concentration during cultivation with bacterial consortium in MSYM+DDT_{100} medium at room temperature and 150 rpm.

DDT concentration (ppm)	Cultivation time (days)						
	0	0.5	1	2	3	5	10
PD1	101.65	97.53	88.65	88.97	80.74	86.84	64.05
PD2	101.65	96.65	92.55	94.13	92.47	51.15	50.08
PD3	101.65	100.00	84.41	60.40	59.00	53.72	45.28
PD4	101.65	85.46	79.15	78.19	73.16	53.66	34.21
PD5	101.65	96.47	69.49	67.53	68.96	48.48	36.57
PD6	101.65	68.68	60.47	60.93	47.73	52.67	29.01
PD7	101.65	72.95	66.29	59.43	59.67	33.70	5.31
PD8	101.65	102.26	97.46	89.05	87.23	63.80	57.43
PD9	101.65	96.67	88.44	88.71	84.05	72.78	53.41
PD10	101.65	93.33	89.63	92.26	83.79	84.74	42.13
PD11	101.65	99.89	101.64	83.22	86.62	82.68	0.00
PD12	101.65	96.20	95.52	93.38	77.88	58.87	61.65
No bacteria	101.65	96.70	96.82	98.48	99.72	93.60	98.08

Table 12. *p,p′*-DDT transformation level after 10 days of incubation by individual bacterial consortium.

p,p′-DDT transformation level	Consortium												No bacteria
	PD1	PD2	PD3	PD4	PD5	PD6	PD7	PD8	PD9	PD10	PD11	PD12	
transformation (%)	37.03	50.70	55.33	66.34	63.96	71.55	94.72	43.52	47.39	58.63	100	39.40	3.47
Standard deviation	3.58	5.57	13.03	1.86	7.57	8.44	5.02	3.99	9.39	5.78	0	6.95	4.64

Table 13. Total cell protein of bacterial consortium during cultivation in MSYM+DDT_{100} medium at room temperature and 150 rpm.

Total cell protein (μg/ml)	Cultivation time (days)													
	0	0.5	1	1.5	2	2.5	3	4	5	6	7	8	9	10
PD1	7.67	59.67	66.67	69.50	69.00	71.00	82.17	79.00	78.17	73.50	76.17	74.83	75.50	80.67
PD2	7.06	62.00	75.17	77.67	106.33	148.17	174.83	176.17	161.17	148.17	144.33	132.50	136.33	126.83
PD3	6.97	50.32	55.40	64.00	76.11	120.32	155.23	189.44	153.82	112.95	94.70	102.07	108.91	108.74
PD4	5.90	51.83	61.17	61.50	64.50	67.00	60.33	55.83	44.33	34.33	36.17	39.00	45.17	36.33
PD5	7.03	28.21	46.98	54.35	53.12	47.51	35.93	53.30	69.79	55.05	67.51	221.02	301.54	161.02
PD6	6.27	36.11	51.19	60.49	62.42	45.23	44.35	47.51	62.77	61.54	69.26	202.95	201.02	109.61
PD7	6.41	67.00	70.67	79.00	88.50	112.00	178.17	193.83	161.17	123.17	106.00	113.17	119.17	119.00
PD8	5.97	43.51	61.76	56.05	56.84	56.21	51.43	61.76	69.70	77.48	84.46	94.30	103.51	89.06
PD9	7.23	15.32	34.71	38.05	41.38	44.56	57.44	51.53	50.47	41.98	36.68	38.95	37.44	38.65
PD10	6.17	13.20	19.11	16.98	17.29	16.98	28.80	60.17	57.89	38.80	43.50	32.29	33.05	17.89
PD11	5.87	16.52	69.68	76.85	75.35	71.02	65.85	66.35	65.18	68.68	52.68	48.18	46.85	42.35
PD12	7.16	10.85	15.18	20.52	52.85	57.35	65.02	52.52	49.18	53.18	53.52	58.85	59.68	58.02
No bacteria	3.27	3.33	6.17	15.83	18.83	19.17	14.33	20.50	22.83	34.50	37.00	38.50	39.83	31.33

Table 14. *p,p′*-DDT concentration during cultivation with single bacterial culture in MSYM+DDT_{100} medium at room temperature and 150 rpm.

DDT concentration (ppm)	Cultivation time (days)						
	0	0.5	1	2	3	5	10
PD7-1	99.41	94.61	92.31	79.05	75.62	73.36	69.49
PD7-2	99.41	94.16	93.54	77.00	72.17	70.61	71.79
PD7-3	99.41	96.70	95.39	81.14	72.40	65.88	60.18
PD7-4	99.41	98.24	98.62	80.61	68.40	57.02	50.09
PD7-5	99.41	97.03	93.67	88.33	71.94	58.59	49.15
PD7	99.41	97.65	90.93	76.83	59.32	43.18	9.73
No bacteria	99.41	97.47	97.52	92.11	94.11	94.90	96.62

Table 15. *p,p′*-DDT transformation level after 10 days of incubation by single bacterial culture.

p,p′-DDT transformation level	Single culture						
	PD7-1	PD7-2	PD7-3	PD7-4	PD7-5	PD7	No bacteria
Transformation (%)	30.12	27.80	39.40	49.60	50.63	90.23	2.81
Standard deviation	2.17	3.44	4.19	1.85	6.28	2.36	3.04

Table 16. Total cell protein of single culture during cultivation in MSYM+DDT_{100} medium at room temperature and 150 rpm.

Total cell protein (μg/ml)	Cultivation time (days)						
	0	0.5	1	2	3	5	10
PD7-1	5.20	8.04	20.32	24.88	40.14	61.89	56.46
PD7-2	5.60	28.56	31.89	44.88	38.21	29.44	17.33
PD7-3	4.70	11.19	21.02	39.79	45.93	55.40	42.60
PD7-4	5.04	25.75	30.14	31.89	28.39	28.74	23.30
PD7-5	4.78	18.74	20.49	27.16	55.05	79.96	103.82
PD7	5.60	20.84	34.35	79.31	138.96	149.88	97.68
No bacteria	2.93	1.37	2.07	3.82	5.93	4.18	7.33

Table 17. *p,p'*-DDT concentration during cultivation with mixed two bacterial cultures in MSYM+DDT_{100} medium at room temperature and 150 rpm.

DDT concentration (ppm)	Cultivation time (days)						
	0	0.5	1	2	3	5	10
PD7-[1/2]	96.79	94.61	92.31	79.05	75.62	73.36	69.49
PD7-[1/3]	96.79	96.70	95.39	81.14	72.40	65.88	60.18
PD7-[1/4]	96.79	93.91	88.62	80.61	65.07	60.35	60.09
PD7-[1/5]	96.79	82.81	73.42	65.34	62.96	60.93	59.63
PD7-[2/3]	96.79	90.42	83.07	82.46	73.89	72.07	67.03
PD7-[2/4]	96.79	90.05	77.49	77.04	73.39	58.77	56.09
PD7-[2/5]	96.79	92.49	89.11	88.31	76.53	66.13	57.05
PD7-[3/4]	96.79	75.61	70.33	68.31	66.20	63.50	44.92
PD7-[3/5]	96.79	95.33	88.14	84.67	63.62	52.78	41.59
PD7-[4/5]	96.79	65.03	60.34	54.48	52.55	50.75	47.48
PD7	96.79	58.28	45.64	36.78	23.92	16.93	13.84
No bacteria	96.79	98.65	97.92	99.71	96.53	98.99	93.02

Table 18. *p,p′*-DDT transformation level after 10 days of incubation by mixed two bacterial cultures.

p,p′-DDT transformation level	Mixed two bacterial cultures											No bacteria
	PD7-[1/2]	PD7-[1/3]	PD7-[1/4]	PD7-[1/5]	PD7-[2/3]	PD7-[2/4]	PD7-[2/5]	PD7-[3/4]	PD7-[3/5]	PD7-[4/5]	PD7	
Transformation (%)	28.19	37.81	37.77	38.22	30.79	42.19	40.92	53.55	57.08	50.89	85.66	3.94
Standard deviation	3.65	3.27	9.95	11.67	7.03	10.77	9.47	8.87	5.28	4.75	5.70	5.80

Table 19. Total cell protein of mixed two bacterial cultures during cultivation in MSYM+DDT_{100} medium at room temperature and 150 rpm.

Total cell protein (μg/ml)	Cultivation time (days)						
	0	0.5	1	2	3	5	10
PD7-[1/2]	4.48	9.44	29.09	59.96	72.42	110.49	134.70
PD7-[1/3]	5.53	8.56	18.74	26.46	44.00	72.07	97.16
PD7-[1/4]	6.20	30.67	28.56	25.05	25.75	21.54	11.54
PD7-[1/5]	5.20	25.23	27.16	36.98	69.79	96.81	121.19
PD7-[2/3]	4.98	28.74	30.84	40.49	35.40	33.12	24.35
PD7-[2/4]	5.32	30.67	35.05	26.98	23.12	22.07	13.12
PD7-[2/5]	4.90	21.19	31.72	30.32	40.14	66.98	87.51
PD7-[3/4]	5.08	40.14	45.05	47.68	46.60	35.05	20.67
PD7-[3/5]	6.09	32.25	35.05	51.89	52.95	62.95	25.05
PD7-[4/5]	5.54	28.04	41.37	32.07	20.84	17.51	7.33
PD7	5.80	133.93	141.46	142.44	185.96	156.51	104.48
No bacteria	2.10	1.72	2.95	5.05	4.35	4.00	4.88

Table 20. *p,p′*-DDT concentration during cultivation with mixed three bacterial cultures in MSYM+DDT_{100} medium at room temperature and 150 rpm.

p,p′-DDT concentration (ppm)	Cultivation time (days)						
	0	0.5	1	2	3	5	10
PD7-[1/2/3]	98.96	80.34	72.43	63.05	60.38	56.69	48.10
PD7-[1/2/4]	98.96	79.50	71.10	63.77	60.04	57.08	43.52
PD7-[1/2/5]	98.96	72.45	70.05	64.93	56.48	54.26	40.77
PD7-[1/3/4]	98.96	74.43	69.68	58.73	58.47	48.27	36.83
PD7-[1/3/5]	98.96	70.44	65.29	62.29	58.41	38.41	32.33
PD7-[1/4/5]	98.96	76.69	67.50	59.54	56.15	40.44	34.33
PD7-[2/3/4]	98.96	65.81	58.79	55.98	45.75	42.64	29.09
PD7-[2/3/5]	98.96	78.65	65.38	60.77	53.35	48.25	31.01
PD7-[2/4/5]	98.96	64.68	57.39	51.00	41.14	36.99	29.11
PD7-[3/4/5]	98.96	75.70	64.27	45.21	34.18	29.96	20.99
PD7	98.96	60.77	47.65	42.52	31.20	29.87	11.60
No bacteria	98.96	98.28	97.43	95.93	97.98	94.93	96.46

Table 21. *p,p′*-DDT transformation level after 10 days of incubation by mixed three bacterial cultures.

p,p′-DDT transformation level	Mixed three bacterial cultures											No bacteria
	PD7-[1/2/3]	PD7-[1/2/4]	PD7-[1/2/5]	PD7 [1/3/4]	PD7-[1/3/5]	PD7-[1/4/5]	PD7-[2/3/4]	PD7-[2/3/5]	PD7-[2/4 /5]	PD7-[3/4/5]	PD7	
Transformation (%)	51.40	56.04	58.68	62.71	67.24	65.26	70.62	68.47	70.47	78.75	88.43	2.54
Standard deviation	5.35	5.20	9.39	5.62	5.17	3.84	6.97	8.44	5.09	4.00	6.85	4.86

Table 22. Total cell protein of mixed three bacterial cultures during cultivation in MSYM+DDT_{100} medium at room temperature and 150 rpm.

Total cell protein (μg/ml)	Cultivation time (days)						
	0	0.5	1	2	3	5	10
PD7-[1/2/3]	5.68	45.11	117.1	130.2	140	119.5	119.5
PD7-[1/2/4]	6.21	118.9	157.5	151.3	117	81.77	83.92
PD7-[1/2/5]	4.98	96.3	143.9	145.1	140	137.7	140.6
PD7-[1/3/4]	5.04	79.89	109.2	113.7	97.7	92.23	55.8
PD7-[1/3/5]	6.07	77.25	103.7	125.2	118.3	116.2	111.5
PD7-[1/4/5]	4.82	57.34	86.91	87.06	106.1	65.49	55.06
PD7-[2/3/4]	5.56	49.63	84.1	112.5	76.49	64.87	51.72
PD7-[2/3/5]	5.72	103.2	113.8	122.4	107.5	87.15	68.49
PD7-[2/4/5]	5.81	119.9	151.7	145	128.5	123.6	104.007
PD7-[3/4/5]	4.80	122.2	180.7	198.1	164.6	125.4	127.3
PD7	6.03	87.34	108.9	180.7	167.5	162.8	144.4
No bacteria	2.85	0.788	0.592	0.592	1.376	1.082	1.768

Table 23. *p,p′*-DDT concentration during cultivation with mixed four and five bacterial culture in MSYM+DDT_{100} medium at room temperature and 150 rpm.

p,p′-DDT concentration (ppm)	Cultivation time (days)						
	0	0.5	1	2	3	5	10
Without PD7-1	97.043	72.76	57.537	54	43.55	40.777	20.59
Without PD7-2	97.043	76.71	60.42	54.303	43.493	32.453	25.417
Without PD7-3	97.043	75.67	59.487	55.5	45.43	39.307	29.93
Without PD7-4	97.043	79.893	72.05	64.21	50.853	45.427	38.057
Without PD7-5	97.043	78.777	71.543	56.663	53.723	50.533	41.777
Mixed 5	97.043	77.45	56.683	47.803	37.947	28.497	17.637
PD7	97.043	65.1	51.1	40.144	28.983	29.717	9.3767
No bacteria	97.04	94.54	94.20	96.95	96.12	94.40	96.01

Table 24. *p,p′*-DDT transformation level after 10 days of incubation by mixed four and five bacterial cultures.

p,p′-DDT transformation level	Mixed four bacterial cultures					Mixed five bacterial cultures PD7-[1/2/3/4/5]	PD7	No bacteria
	Without PD7-1	Without PD7-2	Without PD7-3	Without PD7-4	Without PD7-5			
Transformation (%)	78.51	74.13	69.04	60.89	56.79	81.57	90.07	0.89
Standard deviation	9.09	9.05	4.92	3.03	6.09	8.35	7.10	4.57

Table 25. Total cell protein of mixed four and five bacterial culture during cultivation in MSYM+DDT_{100} medium at room temperature and 150 rpm.

Total cell protein (μg/ml)	Cultivation time (days)						
	0	0.5	1	2	3	5	10
Without PD7-1	4.34	51.63	66.58	128.3	135.7	78.49	45.15
Without PD7-2	5.05	73.53	130.6	143.2	127.9	110.6	95.34
Without PD7-3	5.75	76.77	134.6	145.7	178.8	143.9	115.9
Without PD7-4	6.14	84.96	135.7	156.2	183.8	153.7	64.3
Without PD7-5	3.98	87.25	131.8	141.3	140.3	87.72	55.34
Mixed 5	5.94	104.9	156.9	171.2	181.4	146.5	93.72
PD7	6.04	104.7	137.2	150.6	189.2	162.5	120.2
No bacteria	3.34	4.01	3.819	10.77	12.1	10.49	16.39

Table 26. Viable cell of isolate PD7-1, PD7-2, PD7-3, PD7-4 and PD7-5 grown as bacterial consortium in MSYM+DDT_{100} at room temperature, 150 rpm for 10 days.

Viable cell	Cultivation time (days)													
(Log CFU/ml)	0	0.5	1	1.5	2	2.5	3	4	5	6	7	8	9	10
PD7-1	5.49	9.33	9.22	10.43	9.35	9.28	9.75	10.18	10.35	10.91	10.49	10.59	11.27	10.66
PD7-2	6.66	9.13	9.25	10.52	9.29	9.61	9.31	10.77	10.51	10.71	10.95	11.01	11.56	10.88
PD7-3	5.46	9.23	8.70	9.75	8.55	9.17	9.72	10.33	9.38	10.77	10.46	10.62	10.88	10.41
PD7-4	6.26	9.75	9.06	10.15	8.53	8.59	8.25	9.13	9.65	9.85	9.84	9.54	10.77	6.16
PD7-5	5.10	9.25	8.24	9.75	8.09	8.45	8.35	9.47	9.66	9.97	10.12	9.76	10.84	9.60

APPENDIX 6

16S rRNA sequences of five bacterial isolates

1. Strain PD7-1

AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGAT
GATGCCCAGCTTGCTGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCC
TGACTCTGGGATAAGCGTTGGAAACGACGTCTAATACTGGATACGACTGCCGGCCGCATGGTC
TGGTGGTGGAAAGATTTTTTGGTTGGGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTA
ATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGA
GACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTG
ATGCAGCAACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTTAGTAGGGAAGA
AGCGAAAGTGACGGTACCTGCAGAAAAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGGTGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCG
TCTGCTGTGAAATCCCGAGGCTCAACCTCGGGCTTGCAGTGGGTACGGGCAGACTAGAGTGCG
GTAGGGGAGATTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGAT
GGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGCGCTAGATGTAGGGACCTTTCCACGG
TTTCTGTGTCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAAC
TCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCG
AAGAACCTTACCAAGGCTTGACATACACCGGAAACGGCCAGAGATGGTCGCCCCCTTGTGGTC
GGTGTACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
ACGAGCGCAACCCTCGTTCTATGTTGCCAGCGCGTTATGGCGGGGACTCATAGGAGACTGCCG
GGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTC
ACGCATGCTACAATGGCCGGTACAAAGGGCTGCGATACCGTAAGGTGGAGCGAATCCCAAAAA
GCCGGTCTCAGTTCGGATTGAGGTCTGCAACTCGACCTCATGAAGTCGGAGTCGCTAGTAATC
GCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCAT
GAAAGTCGGTAACACCCGAAGCCGGTGGCCTAACCCTTGTGGAAGGAGCCGTCGAAGGTGGGA
TCGGTGATTAGGACTAAGTCGTAACAAGGTAGCC

2. Strain PD7-2

AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGAT
GAAGCCCAGCTTGCTGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCT
GCACTCTGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATATGACCTCGGGATGCATGTCC
TGGGGTGGAAAGTTTTTCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAAT
GGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGA
CACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGAT
GCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCACCCATGACGAAG
CGCAAGTGACGGTAGTGGGAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATAC
GTAGGGTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTC
GTCTGTGAAATCCCGCAGCTCAACTGCGGGCTTGCAGGCGATACGGGCAGACTCGAGTACTGC
AGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGG
CGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGTAGCGAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCGCTAGGTGTGGGTTTCCTTCCACGGGA
TCCGTGCCGTAGCCAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTC
AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAA
GAACCTTACCTGGGTTTGACATGTACCGGACGACTGCAGAGATGTGGTTTCCCTTGTGGCCGG
TAGACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC
GAGCGCAACCCTTGTCCTGTGTTGCCAGCACGTGATGGTGGGGACTCGCAGGAGACTGCCGGG
GTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCAC
ACATGCTACAATGGTCGGTACAGAGGGCTGCGATACCGTGAGGTGGAGCGAATCCCTTAAAGC
CGGTCTCAGTTCGGATCGGGGTCTGCAACTCGACCCCGTGAAGTCGGAGTCGCTAGTAATCGC
AGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGA
AAGTCGGTAACACCCGAAGCCGGTGGCCTAACCCCTCGTGGGAGGGAGCCGTCGAAGGTGGGA
TCGGCGATTGGGACGAAGTCGTAACAAGGTAGCC

3. Strain PD7-3

AGAGTTTGATCCTGGCTCAGGATGAACGCTAGCGGCAGGCTTAATACATGCAAGTCGAGGGGC
AGCGCGGACTTCGGTCTGGCGGCGACCGGCAAACGGGTGCGGAACACGTACACAACCTTCCTT
TTAGTGGGGGATAGCCCAGAGAAATTTGGATTAATACCCCGTAAGATGTTAGTGAGGCATCTC
ACTGATATTATAGTGGCAACACGCTAGAAGACGGGTGTGCGTATGATTAGGTAGTTGGCGGGG
TAACGGCCCACCAAGCCTTCGATCATTAACTGGTGTGAGAGCACGACCAGTCACACGGGCACT
GAGACACGGGCCCGACTCCTACGGGAGGCAGCAGTAAGGAATATTGGTCAATGGAGGAAACTC
TGAACCAGCCATGCCGCGTGGAGGATGAAGGTCCTCTGGATTGTAAACTTCTTTTATATGGGA
CGAAAAAGGGACTTTCTAGTTCAACTGACGGTACCATATGAATAAGCACCGGCTAACTCCGTG
CCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTATCCGGATTCACTGGGTTTAAAGGGAGC
GTAGGTGGGTTGGTAAGTCAGAGGTGAAATCTCCGAGCTTAACTCGGAAACTGCCTTTGATAC
TATCAGTCTTGAATATTGTGGAGGTTAGCGGAATATGTCATGTAGCGGTGAAATGCTTAGATA
TGACATAGAACACCAATTGCGAAGGCAGCTGGCTACACATATATTGACACTGAGGCTCGAAAG
CGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACTATGGATACTCGACA
TACGCGATAAACAGTGTGTGTCTGAGCGAAAGCATTAAGTATCCCACCTGGGAAGTACGACCG
CAAGGTTGAAACTCAAAGGAATTGGCGGGGGTCCGCACAAGCGGTGGAGCATGTGGTTTAATT
CGATGATACGCGAGGAACCTTACCTGGGCTAGAATGCGAGTGCCGTACCGTGAAAGCGGTATT
TCTAGCAATAGACACAAAGCAAGGTGCTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCCCATCACTAGTTGCCATCAGGTAACGCTGGGAACTC
TAGTGAAACTGCCGTCGTAAGACGCGAGGAAGGAGGGGATGATGTCAAGTCATCATGGCCTTT
ATGCCCAGGGCTACACACGTGCTACAATGGGTAGGACAAAGAGTTGCAACACGGTGACGTGAA
GCTAATCTCAAAAACCTACTCTCAGTTCAGATCGTAGTCTGCAACTCGACTACGTGAAGCTGG
AATCGCTAGTAATCGTATATCAGCAATGATACGGTGAATACGTTCCCGGACCTTGCACACACC
GCCCGTCAAGCCATGAAAGCCGGGTGTACCTAAAGTCGGTAACCGTAAGGAGCTGCCTAGGGT
AAAACTGGTAATTGGGGCTAAGTCGTAACAAGGTAGCC

4. Strain PD7-4

AGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGC
GAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGT
AACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTT
GAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGT
CGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAG
AGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTA
GGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCT
TTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACC
TTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT
AGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGT
CTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTG
CAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACA
CCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAG
CAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAG
GGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCC
GCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTT
AATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGAT
AGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAA
GTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAA
TCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTG
CAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCA
ACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATAC
GTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCG
GTGGGGTAGCC

5. Strain PD7-5

AGAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAACGCC
CCGCAAGGGGAGTGGCAGACGGGTGAGTAACGCGTGGGAATCTACCCATCTCTACGGAATAAC
TCAGGGAAACTTGTGCTAATACCGTATACGCCCTTCGGGGGAAAGATTTATCGGAGATGGATG
AGCCCGCGTTGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGG
TCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCA
GTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCC
CTAGGGTTGTAAAGCTCTTTCACCGATGAAGATAATGACTGTAGTCGGAGAAGAAGCCCCGGC
TAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATTACTGGGCG
TAAAGCGCACGTAGGCGGGTATTTAAGTCAGGGGTGAAATCCCAGAGCTCAACTCTGGAACTG
CCTTTGATACTGGGTACCTAGAGTATGGAAGAGGTAAGTGGAATTCCGAGTGTAGAGGTGAAA
TTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTTACTGGTCCATTACTGACGCTGA
GGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGA
ATGTTAGCCGTCGGCATGCATGCATGTCGGTGGCGCAGCTAACGCATTAAACATTCCGCCTGG
GGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA
TGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCCCTTGACATCCCGATCGCGGTTAG
TGGAGACACTTTCCTTCAGTTAGGCTGGATCGGAGACAGGTGCTGCATGGCTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCTTAGTTGCCAGCAT
TCAGTTGGGCACTCTAAGGGGACTGCCGGTGATAAGCCGAGAGGAAGGTGGGGATGACGTCAA
GTCCTCATGGCCCTTACGGGCTGGGCTACACACGTGCTACAATGGTGGTGACAGTGGGCAGCG
AGACAGCGATGTCGAGCTAATCTCCAAAAGCCATCTCAGTTCGGATTGCACTCTGCAACTCGA
GTGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGG
CCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTTTTACCCGAAGGCGATGCGCTAACCG
CAAGGAGGCAGTCGACCACGGTAGGGTCAGCGACTGGGGTGAAGTCGTAACAAGGTAGCC

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List of Publication and Proceedings

Proceedings

Jaya, J. D., Maneerat, S. and Sobhon, V. 2008. Screening of *p,p'*-DDT Degrading Bacterial Consortium from Agricultural Soil in Songkhla Province, Thailand. The 20th Annual Meeting of the Thai Society for Biotechnology "Biotechnology for Global Care". October 14-17, 2008. Mahasarakham, Thailand.