Original Article

Screening and identification of *p,p′*-DDT degrading soil isolates

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Abstract

DDT is an organochlorine pesticide that can persist in the environment resulting in environmental problem with chronic effects on human and animal health. The determination of *p,p′*-DDT in soil samples from 23 agricultural areas in Songkhla Province found DDT residue in the ranges of 0.17-9.84 ng/g soil. After repeated culturing in mineral salts-yeast extract medium (MSYM) with an addition of 25 ppm *p,p′*-DDT (DDT$_{25}$), 167 morphologically different bacterial strains were isolated. Out of 167 isolates, only 5 strains showed *p,p′*-DDT degrading ability as indicated by clear zone around the colony when grown on nutrient agar supplemented up to 100 ppm *p,p′*-DDT. These 5 isolates include SB1A01, SB2A02, SB1A10, SB1A12 and SB1B05. Growth of these isolates in MSYM+DDT$_{25}$ after 10 days indicated reductions of *p,p′*-DDT by 30.5, 20.3, 37.4, 30.4 and 32.2%, respectively. Based on the morphological characteristics and 16S rDNA analysis, isolate SB1A10 which showed the highest degradation ability was found to be 99% identical (1360/1362) to *Staphylococcus haemolyticus*.

Keywords: biodegradation, *p,p′*-DDT, soil isolates, *Staphylococcus*

1. Introduction

1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane (*p,p′*-DDT), an organochlorine compound used as a pesticide in agricultural activities throughout the world. Although DDT functioned as a highly effective and efficient pesticide, it is also very recalcitrant and appeared to undergo slow degradation in the environment. Thus, DDT was banned in most countries for agricultural usages since 1970s but continued to be used for mosquito controlling of malaria in many developing countries. High level of DDT have been detected in human adipose tissues, blood plasma, liver, brain, placenta and even in breast milk (Jaga, 2000)

For several decades since its introduction as pesticide, DDT was extensively used in both agricultural and public health programs in Thailand. In 1983, use of DDT as pesticide in Thailand was banned due to the apparent environmental and human health effects. However, widespread contamination of DDT in soil and sediments had already

occurred. Senglai (1997) reported that 38 percent of water samples and 73 percent of sediment samples collected from various areas in Songkhla Province (Muang District and Hatyai District) and Pattalung Province (Muang District and Khoa Chaison District) were contaminated with *o,p′*-DDT and *p,p′*-DDT, respectively. La-Ongsiriwong and Mudmarn (1999) collected and analyzed water samples from Songkhla Lake for their organochlorine pesticide residues level. DDT and hexachlorocyclohexane (HCH) were found to be the two major contaminants, with DDT concentrations at 23, 143 and 47 ng/l in the inner, middle and outer lake regions, and HCH concentration at 9.5 ng/g sediment, respectively.

Microorganisms play an important role in organochlorine pesticides degradation. They possess several enzymes able to degrade pesticides, i.e., reductive dechlorination, dehydrochlorination, oxidation and isomerization of the parent molecules (Nawab *et al.*, 2003). Microbial degradations of organochlorine pesticides have been observed under both aerobic and anaerobic conditions. Aerobic degradation of DDT by several type of bacteria have been reported such as *Alcaligenes eutrophus* A5, *Serratia Marcescens* DT-1P, *Micrococcus varians*, *Lactobacillus plantarum*, and

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Pseudomonas sp. (Nadeau *et al.*, 1994; Abou-Arab, 2002; Bidlan and Manonmani, 2002; Chandrappa and Harichandra, 2004). The mechanisms involved in aerobic biodegradation of DDT by *Pseudomonas* sp. and *Alcaligenes eutrophus* A5 were shown to occur via a *meta*-cleavage pathway yielding 4-chlorobenzoic acid (Nadeau *et al.*, 1994; Chandrappa and Harichandra, 2004). Under anaerobic condition, DDT is converted to dichlorodiphenyldichloroethane (DDD) by the reductive dechlorination reaction (Häggblom and Valo,1992). Beside bacterial degradation, mineralization of DDT has been reported in white rot fungi, *Phanerochaete chrysosporium* (Fernando *et al.*, 1989).

In this study, indigenous soil bacteria from agricultural area were isolated by selective enrichment screening with *p,p'*-DDT as a sole carbon source. Bacterial isolates able to grow in the presence of *p,p'*-DDT were obtained and screened for their degradation ability. The *p,p'*-DDT degrading isolates were then identified by their morphological and 16S rDNA characteristics. The isolation of indigenous soil bacteria with the ability to degrade organochlorine pesticide such as *p,p'*-DDT has provided us with alternative tool in the remediation of recalcitrance from the environment. However, further investigations have to be performed on the diversity of organochlorine pesticide degrading community and the degradation role/relationship of microorganisms within, the optimal degradation conditions, and field testing. These will allow for better understanding of the biodegradation mechanism of organochlorine pesticide and will eventually lead to the most efficient bioremediation process.

2. Materials and Methods

2.1 Culture media

Mineral salt-yeast extract medium (MSYM) used for screening of soil bacteria consisted of (per 1 liter of distilled water) 4 g of Na_2NO_3, 0.5 g Na_2HPO_4, 1.5 g $KHPO_4$, 0.01 g $CaCO_3$, 0.005 g $FeCl_3$ and 0.2 g $MgSO_4$, 0.01% yeast extract (modified from Nadeau *et al.*, 1994). Nutrient agar (NA) used for primary screening of *p,p'*-DDT-degrading bacteria consisted of (per 1 liter of distilled water) 5 g peptone, 3 g beef extract and 15 g agar. All media were sterilized by autoclaving at 121°C for 15 min. *p,p'*-DDT (AR grade, 98%; Aldrich Chemical Company, Inc.) was filter-sterilized and added to both media at the concentration of 25, 50 and 100 ppm.

2.2 Collection of soil samples

Soil samples were collected from 2 agricultural areas, Bangleang Subdistrict and Tungwang Subdistrict, in Songkhla Province with history of continued farming activities for more than 30 years. Surface soil from 0-15 cm were collected, placed in plastic bags, transported on ice to the laboratory and stored at 4°C until analysis. Soil samples were air-dried and sieved through a 10 mm mesh prior to *p,p'*-DDT analysis

and bacterial screening.

2.3 Extraction and quantitative determination of pesticides in soil samples

Extraction of *p,p'*-DDT was performed according to the method described by Senglai (1997). Ten grams of soil sample were vigorously shaken with 20 ml acetone for 1 hr. The organic phase were decanted into 250 ml flask and soil slurry was mixed with acetone:hexane (1:1) mixture. Samples were again vigorously shaken for 1 hr. The organic phase was combined and further extracted with 20 ml hexane and 50 ml distilled water in a 250 ml separatory funnel. Water phase was discarded and anhydrous Na_2SO_3 was added to remove remaining moisture. Solvents were evaporated with rotary evaporator and the extract was dissolved with hexane to a volume of 1 ml. Extract was then cleaned in a 5 g florisil column and eluted with 40 ml dichloromethane. Purified extract was analyzed for *p,p'*-DDT concentration by Gas chromatography-electron capture detector (GC-ECD) with the following conditions: HP-35 capillary column; detector and injector temperatures of 320°C and 250°C, respectively; oven temperature programmed at: 150°C for 1 min, 20°C/min to 250°C 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 splitless injection technique.

2.4 Screening and isolation of *p,p'*-DDT degrading bacteria

Ten grams of soil sample were added to 100 ml MSYM and enriched with an addition of 25 ppm *p,p'*-DDT (DDT$_{25}$). Samples were incubated on rotary shaker (150 rpm) at 30°C for 7 days and then transferred to a fresh medium and incubated at the same conditions, after which, the cultures were regularly transferred every 3-4 days or until increased turbidity were evidenced. After 3-4 times of repeated subculturing, 0.1 ml culture broth was pipetted and spreaded on MSYM+DDT$_{25}$ agar. Single colonies were selected and streaked on nutrient agar (NA) supplemented with 25, 50 and 100 ppm *p,p'*-DDT. Cultures were incubated at 30°C for 3 days. *p,p'*-DDT degrading isolates were selected from isolates which developed clear zone surrounding their colonies when grown on NA supplemented with 25, 50 and 100 ppm *p,p'*-DDT.

2.5 Biodegradation of *p,p'*-DDT by bacterial isolates

Selected bacterial isolates were grown in MSYM+DDT$_{25}$ and incubated on rotary shaker at 150 rpm, 30°C for 10 days. Samples were collected every 24 hrs for the determination of *p,p'*-DDT concentration and cell growth via total protein measurement by Lowry's method (Stoscheck, 1990).

To determine *p,p'*-DDT concentration, 2 ml culture

broth was extracted with 4 ml ethylacetate (repeated 3 times) and evaporated under vacuum condition until dryness. The extract was redissolved with hexane to a volume of 2 ml and analyzed with GC-ECD.

2.6 Characterization of bacterial isolates

1) Morphological studies

Bacterial isolates were grown in MSYM+DDT$_{25}$ and incubated at 30°C until either turbidity or colony was observed. Gram's stain and cell morphology was investigated under microscope (1000x magnification).

2) 16S rDNA analysis

Genomic DNA was extracted by Boiling method (modified from Yamada *et al.*, 2002). One ml of cell culture grown in nutrient broth (NB) at 30°C for 18-24 hrs was centrifuged at 10,000 rpm for 10 min then washed 2 times with TE-buffer (pH 7.8). Washed cell was resuspended with 0.3 ml TE-buffer (pH 7.8) then boiled at 100°C for 10 min, followed immediately by cooling at 0°C for 5 min. This boiling/cooling step was repeated 3 times.

16S rDNA from the cell extract obtained with the above procedure was amplified by PCR using 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1492R (5'-ACGGCTACCTTGTTACGACTT-3') primers. The reaction composition consisted of 0.2 mM each dNTP, 0.2 μM of each primer, 5 μl 10x PCR buffer, 10 μl cell extract, 2.5 units *Taq* DNA polymerase and sterile deionized water to a final volume of 50 μl. Following a hot start (94°C for 3 min), 25 cycles of amplification were used (94°C for 1 min, 50°C for 45 sec, 72°C for 2 min) followed by a final single extension of 72°C for 10 min. The PCR products were separated by electrophoresis on a 1% agarose gel and visualized under UV light after staining with ethidium bromide.

The amplified PCR product was purified using QIA quick PCR Purification Kit (QIAGEN, Inc.) according to the manufacturer's instruction. DNA sequencing was performed by Macrogen, Inc. (South Korea) and 16S rDNA sequences were BLAST searched against GenBank database (http://www.ncbi.nlm.nih.gov/). Phylogenetic tree was constructed using Treeview software (Page, 1996).

3. Results and Discussions

3.1 Quantitative determination of pesticides in soil samples

Soil samples from a total of 23 sites in 2 agricultural areas were collected, of which 18 were analyzed and found to be contaminated with *p,p'*-DDT in the ranges of 0.17-9.84 ng/g soil (Table 1). Most sites (13 sites) contained *p,p'*-DDT in the range below 1 ng/g soil, with 3 sites containing between 1-2 ng/g soil and 2 sites at a level higher than 6 ng/g soil. The

areas under investigation have a history of pesticides usage for more than 30 years and, although, DDT has been banned since the 1980s it is still being used by the public health sector for the control of malaria. Thus, its existence in the environment is not a rare occurrence. DDT generally persists in the environment with a half-life between 15 to 20 years depending on soil and compound compositions (Nawab *et al.*, 2003).

In our study, the collected soil samples were mainly composed of loose sediment of rock particles and sand, with small amount of clay and red sediments (a characteristic of iron oxide and aluminum hydroxide) (Table 1). Sandy soil has a greater chance of pesticides leaching out into the groundwater due to high infiltration capacities. DDT has an adsorption partition coefficient (K_d) of 243,000 compared to only 10 and 29 for aldicarb and carbofuran. Therefore, DDT adheres strongly to soil particles and organic contents in the environment hindering its bioavailability for degradation by indigenous microorganisms and other means of degradation processes. The levels of DDT observed in our study show resemblance to those determined by Nahn *et al.* (1999) which examined sediments along the coast of North Vietnam and found level of DDT contamination in the ranges of 6.2-10.4 ng/g soil. These levels are relatively lower than those observed in agricultural fields in Alabama, USA, where the ranges of residual DDT were 24.6-30.5 ng/g soil (Harner *et al.*, 1999).

### 3.2 Screening and isolation of *p,p'*-DDT degrading bacteria

The bacterial population in soil samples collected for screening was in the ranges of 10^4 - 10^8 CFU/g soil (Table 1). The amounts of bacteria in soil also depend on factors such as soil properties, soil nutrient levels and soil types (Himaman, 1998). Sites with low bacteria population (10^4 - 10^6 CFU/ml) may be the result of high level of accessible DDT in the area. As previously reported, growth of *Bacillus* sp. and metha-nogenic bacteria were inhibited by increasing the concentration of γ-HCH and endosulfan, respectively (Zargar and Johri, 1995; Bharati *et al.*, 1999).

After successive enrichment and culturing in MSYM+DDT$_{25}$, a total of 167 bacterial strains capable of growing in the presence of 25 ppm *p,p'*-DDT were obtained (data not shown). All 167 isolates were screened for potential DDT-degrading bacteria by plating on nutrient agar with the addition of 25, 50 and 100 ppm *p,p'*-DDT. The results showed 5 strains with clear zone surrounding their colonies at *p,p'*-DDT concentration up to 100 ppm (Figure 1). These isolates included SB1A01, SB02A02, SB1A10, SB1A12 and SB1B05. As observed from the screening results, a diverse group of bacteria both grams negative and positive show the ability to tolerate and some to degrade DDT. According to other studies, similar results were obtained showing both gram negative, gram positive bacteria and even fungi with DDT-degrading capabilities, such as *Alcaligenes*

Table 1. Soil characteristic, amount of *p,p'*-DDT residue, and total amount of soil bacteria grown in the presence of *p,p'*-DDT

Area	*p,p'*-DDT concentration (ng/g soil)	Amount of bacteria grown in the presence of *p,p'*-DDT(CFU/g soil)	Soil texture
Bangleang Subdistrict			
Area 1 Broccoli Field	ND	1.29×10^7	Loam
Area 2 Broccoli Field	ND	4.83×10^7	Loam
Area 3 Sediment from irrigation ditch	ND	4.50×10^7	Clay
Area 4 Broccoli Field	ND	8.62×10^7	Loam
Area 5 Sediment from Bangleang River	ND	4.30×10^7	Silty clay
Area 6 Cabbage Field	0.19	1.23×10^5	Loam
Area 7 Broccoli Field	0.80	5.41×10^6	Loam
Area 8 Broccoli Field	0.52	1.45×10^6	Loam
Area 9 Sediment from irrigation ditch	1.81	1.24×10^5	Silty clay
Area 10 Water Spinach Field	0.34	4.21×10^5	Loam
Area 11 Broccoli Field	0.84	1.52×10^6	Loam
Area 12 Chilli Field	6.27	3.20×10^5	Loam
Area 13 Yu Choy Field	0.95	5.25×10^4	Loam
Area 14 Chinese Parsley Field	0.24	2.51×10^5	Loam
Area 15 Broccoli Field	9.84	8.41×10^6	Laterite
Area 16 Chinese Kale/Broccoli Field	0.62	3.47×10^6	Loam
Area 17 Lettuce Field	0.79	4.21×10^6	Laterite
Tungwang Subdistrict			
Area 18 Watermelon Field	0.17	1.20×10^6	Sandy loam
Area 19 Watermelon Field	0.18	5.20×10^8	Sandy loam
Area 20 Watermelon Field	0.57	4.70×10^6	Sandy loam
Area 21 Watermelon Field	0.18	2.30×10^8	Sandy loam
Area 22 Watermelon Field	1.12	5.40×10^6	Sandy loam
Area 23 Watermelon Field	1.27	3.90×10^6	Sandy loam

ND = not determined
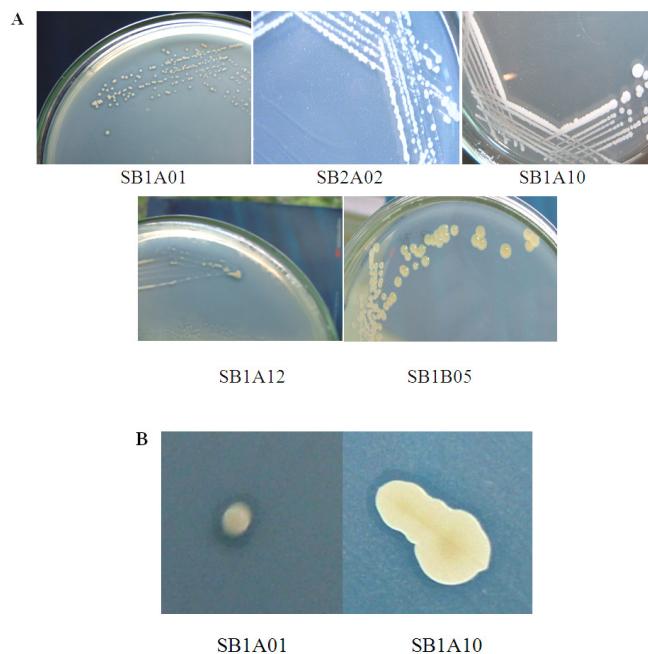
Figure 1. (A) Bacterial colonies grown on nutrient agar (NA) with an addition of 100 ppm *p,p'*-DDT. (B) Clear zone surrounding the colony of strains SB1A01 and SB1A10.

eutrophus A5, *Serratia Marcescens* DT-1P, and *Pseudomonas* sp., (Nadeau *et al.*, 1994; Bidlan and Manonmani, 2002; Chandrappa and Harichandra, 2004), *Micrococcus varians* and *Lactobacillus plantarum* (Abou-Arab, 2002), and *Phanerocheaete chrysosporium* (Fernando *et al.*, 1989), respectively.

3.3 Biodegradation studies of *p,p'*-DDT by bacterial isolates

Each of the 5 potential *p,p'*-DDT degrading isolates was inoculated into MSYM+DDT$_{25}$ medium at 10% inoculum, after which growth profile and *p,p'*-DDT level were followed during the 240 hours of cultivation. The selected bacterial isolates were found to grow and degrade DDT within 72-96 hours (Figure 2A-E). Isolate SB1A12 gave the highest growth followed by SB1A10, SB1B05, SB1A01 and SB2A02, respectively. All isolates could grow better in MSYM with an addition of *p,p'*-DDT than without *p,p'*-DDT. Results indicated that these isolates could used *p,p'*-DDT as a sole carbon and energy sources. Sahu *et al.* (1993) also observed that *Pseudomonas* sp. isolated from soil grew better in mineral salt media with the addition of α, β, and γ-HCH than without these compounds.
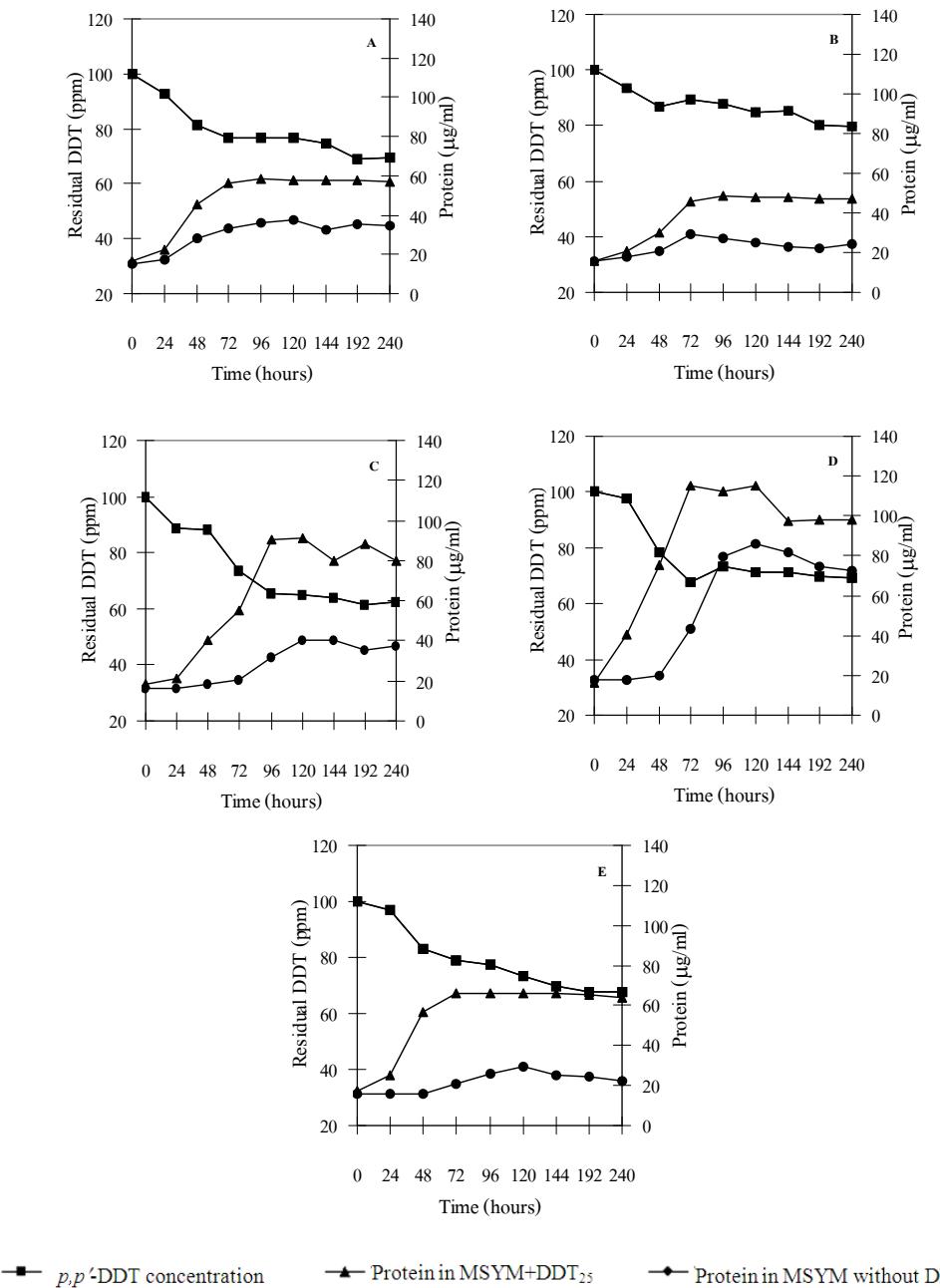
Figure 2. Growth and degradation of *p,p'*-DDT by bacterial isolates grown in MSYM with the addition of 25 ppm *p,p*-DDT after 240 hrs of cultivation. (A = SB1A01; B = SB2A02; C = SB1A10; D = SB1A12; E = SB1B05)

The highest and lowest *p,p'*-DDT reduction was achieved by isolate SB1A10 and SB02A02 at 37.4 and 20.3%, respectively. Other isolates, namely SB1B05, SB1A01 and SB1A12, showed slightly lower degradation ability at 32.2, 30.5 and 30.4% DDT reduction, respectively (Figure 3). The maximum *p,p'*-DDT reduction by isolate SB1A10 of only 37.4% is probably the result of utilizing non-optimal culturing and degradation conditions during the courses of the biodegradation studies. As reported by Bidlan and Manonmani (2002), an inoculum of *Serratia marcescens* DT-1P grown in glucose-yeast extract medium then transferred to basal mineral medium (M4) with 5 ppm DDT showed only

50% reduction of DDT by 48 hours. However, pre-exposing the inoculum with 10 ppm DDT in glucose-yeast extract medium for 72 hours prior to cultivation resulted in nearly 90% DDT reduction. Furthermore, optimization of inoculum size, the addition of co-substrates such as glycerol, peptone and tryptone, and adjustment of pH to 7.0-7.5 led to complete degradation of up to 15 ppm DDT. These findings suggest that optimization studies will be required to obtain maximum degradation of *p,p'*-DDT by the microorganism under investigation. Another interesting aspect is the ability of bacterial consortium to mineralize or achieve higher degradation of persistent xenobiotics than that of single
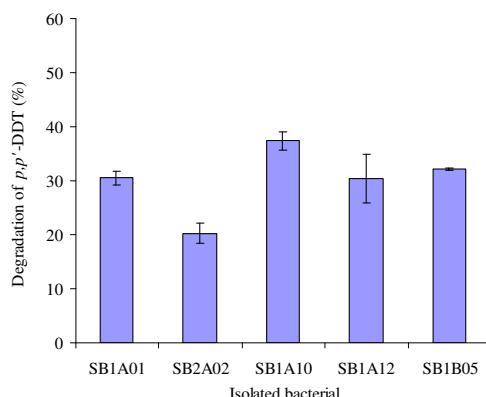
Figure 3. Comparison of *p,p′*-DDT degradation by the bacterial isolates.

bacterium. It has been reported that bacterial consortium could completely degraded 5, 10, 15 and 20 ppm *p,p′*-DDT within 48, 72, 96 and 120 hours, respectively (Bidlan and Manonmani, 2002). Moreover, mixed culture of *Pseudomonas* sp., *Agrobacterium radiobacter* and *Flavobacterium gleum* were shown to degrade pentachlorophenol by 80% compared to 10, 30 and 50% degradation by individual *Pseudomonas* sp., *Agrobacterium radiobacter* and *Flavo-*

bacterium gleum, respectively (Yu and Ward, 1996). Thus besides optimization studies of the degradation conditions, further comparison studies on the degradation by single and mixed cultures need to be addressed.

3.4 Characterization and identification of *p,p′*-DDT degrading bacteria

Morphological characteristics of all 5 *p,p′*-DDT degrading bacteria were determined by observation of colony morphology, Gram's staining and cell morphology under a microscope at 1000x magnification. All 5 isolates were non-motile or slightly motile aerobic bacteria. Three isolates (SB1A01, SB1A10 and SB1B05) were Gram-positive coccoid bacteria. Two isolates (SB2A02 and SB1A12) were Gram-negative rod bacteria.

Since isolate SB1A10 appeared to have the highest degradation ability at 37.4% and sufficient growth/ or degradation rates within 96 hours, it was selected for further optimization studies and identification by 16S rDNA analysis. The results obtained indicated that this isolate has 99% identity (1360/1362) with *Staphylococcus haemolyticus* (Figure 4).

Staphylococcus haemolyticus is a pathogenic bacte-
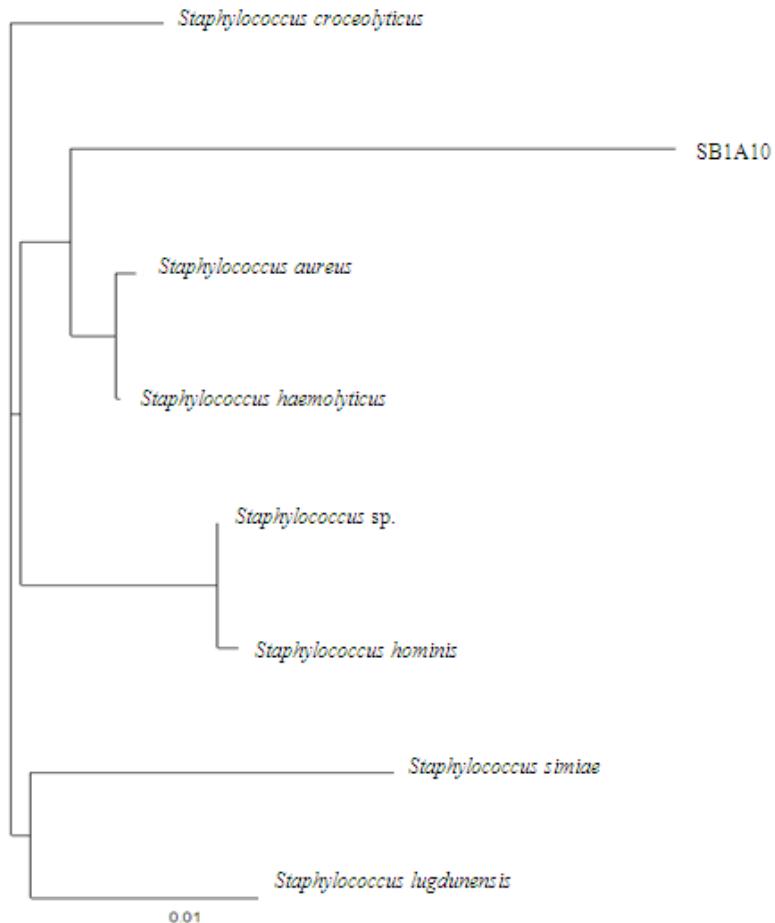
Figure 4. Phylogenetic analysis of *p,p′*-DDT degrading isolate SB1A10.

rium, which colonizes the skin and mucosal membranes of humans, and may also cause septicemia, peritonitis, otitis and urinary tract infections. This strain of *Staphylococcus* is also known to be highly resistant to antibiotics (Takeuchi *et al.*, 2005). Although there has never been report of *p,p′*-DDT degradation by *Staphylococcus haemolyticus*, it has been observed to degrade trichloroethane (TCE) in the presence of phenol as substrate (Fries *et al.*, 1997).

4. Conclusion

Investigation of *p,p′*-DDT residues in soil samples from agricultural areas in Songkhla Province revealed that 2 of the 18 sites contained *p,p′*-DDT higher than 6 ng/g soil, with the rest in a range below 2 ng/g soil. After repeated enrichment and culturing in MSYM+DDT$_{25}$ medium, 167 indigenous soil bacteria were isolated. Five isolates from these groups were shown to be capable of degrading *p,p′*-DDT at a concentration up to 100 ppm on nutrient agar. When grown in MSYM+DDT$_{25}$ medium (with *p,p′*-DDT as a sole carbon source), isolates SB1A01, SB2A02, SB1A10, SB1A12 and SB1B05 were shown to reduce *p,p′*-DDT after 10 days of cultivation by 30.5, 30.4, 37.4, 20.3 and 32.2%, respectively. Based on their morphological characteristics, 2 isolates (SB2A02 and SB1A12) were found to be Gram-negative rod aerobic bacteria and 3 isolates (SB1A01, SB1A10 and SB1B05) were Gram-positive coccoid aerobic bacteria. Additional identification of isolate SB1A10, which showed the highest degradation ability, by 16S rDNA analysis found it to have 99% identities (1360/1362) with *Staphylococcus haemolyticus*.

To maximize the efficiency of *p,p′*-DDT degradation by these isolates, optimization studies of the degradation conditions need to be performed. It will also be interesting to reveal the degradation relationship of each isolate in mixed culture and natural microbial community. Thus, a comparative study of *p,p′*-DDT degradation by single culture, mixed culture, and bacterial consortium will be investigated.

Acknowledgement

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