The Effect of *Pseudomonas aeruginosa* Addition on 1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) Biodegradation by Brown-rot Fungus *Fomitopsis pinicola*

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ABSTRACT

Effect of addition of Pseudomonas aeruginosa *on 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) biodegradation by* Fomitopsis pinicola *had been investigated.* P. aeruginosa *was added into* F. pinicola *culture at 1, 3, 5, 7 and 10 mL (1 mL ≈ 1.53 x 10^9* P. aeruginosa *bacteria cells/mL culture). The addition of 10 mL of* P. aeruginosa *showed the highest DDT biodegradation approximately 68% during 7 days incubation in Potato Dextrose Broth (PDB) medium, which was higher than biodegradation of DDT by* F. pinicola *only (42%) at the same incubation time. 1,1-Dichloro-2,2-bis(4-chlorophenyl)ethane (DDD), 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene (DDE) and 1-chloro-2,2-bis(4-chlorophenyl)ethylene (DDMU) were detected as metabolites from DDT biodegradation by mixed cultures of* F. pinicola *and* P. aeruginosa*.*

Keywords: *biodegradation; DDT;* Fomitopsis pinicola*;* Pseudomonas aeruginosa

ABSTRAK

Pengaruh penambahan Pseudomonas aeruginosa *terhadap biodegradasi 1,1,1-trikloro-2,2-bis(4-klorofenil)etana (DDT) oleh* Fomitopsis pinicola *telah diteliti.* P. aeruginosa *ditambahkan ke dalam kultur* F. pinicola *masing-masing sebesar 1, 3, 5, 7 dan 10 mL (1 mL ≈ 1,53 x 10^9 sel bakteri* P. aeruginosa*/mL kultur). Biodegradasi DDT tertinggi ditunjukkan pada penambahan bakteri 10 mL dengan jumlah degradasi sebesar 68% dengan waktu inkubasi 7 hari, lebih tinggi dibandingkan dengan biodegradasi DDT yang hanya menggunakan jamur* F. pinicola *(42%). 1,1-Dikloro-2,2-bis(4-klorofenil)etana (DDD), 1,1-dikloro-2,2-bis(4-klorofenil)etilena (DDE) dan 1-kloro-2,2-bis(4-klorofenil)etilena (DDMU) terdeteksi sebagai metabolit dari biodegradasi DDT menggunakan campuran kultur* F. pinicola *dan* P. aeruginosa*.*

Kata Kunci: *biodegradasi; DDT;* Fomitopsis pinicola*;* Pseudomonas aeruginosa

INTRODUCTION

The use of pesticides is ubiquitous in modern agriculture and important to increase crop yield and reduce post-harvest losses [1]. 1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) is one of the most extensively used organochlorine pesticides, which has been widely used as an insecticide to control mosquito-borne malaria and thypus [2].The presence of chlorine atom in conjunction with their low solubility and tendency to partition preferentially into the lipophilic phase makes them highly toxic to the higher organism [2]. Due to their great persistence and toxicity, the US Environmental Protection Agency has classified DDT as priority pollutants [3]. Its residues are lipophilic which tend to accumulate in fatty tissues of the ingesting organism along the food chain, resulting in a problem such as breast cancer and reproductive faults even in humans [3]. Therefore, it is necessary to develop a remediation method to clean up the residues of DDT.

Most of the research works on biodegradation of DDT have been carried out using individual fungal and bacterial culture by mineralization under aerobic and anaerobic condition [4-5]. Even though bacteria play dominant roles in bioremediation, fungi are likely than bacterial intracellular enzyme to initiate an attack on high molecular weight pollutants in the soil because of their ability to diffuse in the soil and release extracellular enzymes [6]. However, owing to recalcitrant structures of the pesticides, only specific fungi have been reported to be capable of degrading them [1]. *Fomitopsis pinicola* could degrade DDT up to

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84% during 28 days incubation in Potato Dextrose Broth (PDB) [7], which it consumed long incubation time. Besides, the study of DDT biodegradation by *P. aeruginosa* that isolated from microflora of soil and water in the agricultural area shows that the biodegradation of DDT produced DDD, DDE, alkane and cycloalkene as metabolites [8]. In addition, the *P. aeruginosa* could also transform DDT into DDE, DDMU and DDOH in broth medium [9]. However, there appears to be no report on the combination of *F. pinicola* and *P. aeruginosa* to degrade DDT.

Biodegradation of DDT may be more efficient using mixed fungal bacterial cultures rather than the individual strain alone. Fungal hyphae may function as transport vectors for bacteria, thereby facilitating a more effective spreading of degrader organism [10]. In the case of degradation by mixed cultures that bacteria and fungi grew together, the accumulation of biomass and the capabilities of their enzymes can work synergistically to degrade pollutants [11]. It is probable that bacteria utilize intermediate decomposition products released by fungi. The fungus metabolizes the initial step of oxidation liberating to the polar mineral medium extracellular intermediary metabolites for degradation and mineralization by bacteria [12]. The membrane of bacterial cells is hydrophobic that increases the difficulty of biodegradation for decreasing the availability of hydrocarbon for uptake by bacterial cells [13]. *P. aeruginosa* can be produced extracellularly or as part of the cell membrane biosurfactant rhamnolipid. Biosurfactants would decrease the surface and interfacial tensions in the culture medium thereby increasing the aqueous dispersion of organic level compounds at the molecular level [14]. With the aim to address the important knowledge gap illustrated above, in this study, biodegradation of DDT using mixed cultures was investigated. The biodegradation of DDT was conducted using incubation period for 7 days, which is shorter than the previous reports [7,16]. The efficiency of DDT biodegradation by *P. aeruginosa* addition on *F. pinicola* was then compared to the culture with only fungus or bacteria and mixed cultures with different concentration of bacteria.

EXPERIMENTAL SECTION

Materials

Chemical materials

1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane(DDT) and pyrene were purchased from Tokyo Chemical Industry Co, *n*-hexane and acetone were purchased from Anhui Fulltime Specialized Solvent & Reagent Co., Ltd (Anhui, China). Methanol, dimethylsulfoxide (DMSO), and sodium sulfate anhydrous were purchased from Merck Millipore (Darmstadt, Germany).

Cultures and medium

Culture *P. aeruginosa* bacteria NBRC 3080 (NITE Biological Resources Center, NBRC; Chiba, Japan) were maintained on nutrient agar (NA; Merck, Darmstadt, Germany) plate that had been incubated at 37 °C. The colony was then inoculated into 60 mL nutrient broth (NB; Merck, Darmstadt, Germany). Cultures were pre-incubated at 37 °C for 21 h with a shaker at a speed of 180 rpm. Colony dry weight at a various concentration of 1, 3, 5, 7 and 10 mL (1 mL ≈ 1.53 x 10^9 *P. aeruginosa* bacteria cells/mL) was shown in Table 1.

Culture fungus *F. pinicola* NBRC 8705 was maintained as cultures on Potato Dextrose Agar (PDA; Merck Darmstadt, Germany). This fungus had been incubated at 30 °C. The mycelia (1 cm diameter) was inoculated into 10 mL Potato Dextrose Broth (PDB; Merck Darmstadt, Germany) then pre-incubated at 30 °C for 7 days). Mycelial dry weight of *F. pinicola* was shown in Table 1.

Instrumentation

The HPLC was conducted with a Shimadzu LC-20AT pump and a Shimadzu SPD-M20A diode array detector fitted with an Inertsil ODS-3 column (150 mm) with an inner diameter of 4.6 mm (GL Science, Japan). The samples were eluted with 82% methanol in 0.1% trifluoro acetic acid aqueous solution at a flow rate of 1 mL min^{-1}.

The GC/MS was performed on a 7890A GC system (Agilent Technologies, USA) linked to a 5975C VL MSD Triple-Axis Detector (Agilent Technologies, USA) with a 30-m Agilent 19091S-433 column (Agilent Technologies, USA). The oven temperature was programmed at 100 °C for 3 min, followed by a linear increase to 200 °C at 20 °C min^{-1} and held for 3 min and increased again to 250 °C at 10 °C min^{-1}. The injector temperature was set at 100 °C. The injection was splintedless approximately 1 µL.

Procedure

Biodegradation of DDT by P. aeruginosa

After pre-incubation for 21 h, a various concentration of bacteria cultures at 1, 3, 5, 7 and 10 mL (1 mL ≈ 1.53 x 10^9 bacteria cells/mL culture) was inoculated to PDB culture (final volume 20 mL). Each inoculated flask was added 50 µL of 5 mM DDT in DMSO (final concentration of 0.25 µmol). The headspace of each flask was flushed with oxygen and

Table 1. Biomass of *F. pinicola* and *P. aeruginosa*

Amount of *P. aeruginosa* (mL)	Biomass (mg)	
	P. aeruginosa	Mixed cultures
0	-	38.05 ± 0.002 [a]
1	6.15 ± 1.00	39.40 ± 2.00
3	6.65 ± 1.50	39.60 ± 2.00
5	5.85 ± 1.50	45.00 ± 2.00
7	5.95 ± 1.00	45.60 ± 2.00
10	6.70 ± 2.00	42.90 ± 2.00

[a]*F. pinicola only*

then sealed with a glass stopper. The sealing tape is used to prevent the volatilization of the substrate. The cultures were incubated statically for 7 days at 30 °C. As a control, the cultures were killed by autoclave (121 °C, 20 min) after pre-incubation. The experiments were performed in duplicate [15].

Biodegradation of DDT by F. pinicola

After pre-incubation for 7 days, 10 mL of PDB medium was added into cultures (final volume of 20 mL) and 50 µL of 5 mM DDT in DMSO was added to each *F. pinicola* inoculated flask (final concentration 0.25 µmol). The headspace of each flask was flushed with oxygen and then sealed with a glass stopper and sealing tape is used to prevent the volatilization of DDT. The cultures were incubated statically for 7 days at 30 °C. As a control, the cultures were killed by autoclave (121 °C, 20 min) after pre-incubation. The experiments were performed in duplicate [7,16-17].

Biodegradation of DDT by co-cultures of F. pinicola and P. aeruginosa

After pre-incubation of fungus for 7 days and bacteria 21 h, a various concentration of bacteria of 1, 3, 5, 7 and 10 mL (1 mL ≈ 1.53 x 10^9 bacteria cells/mL culture) was separately added into *F. pinicola* cultures and PDB medium was then added into final volume of 20 mL. The biomass of mixed cultures was shown in Table 1. Each of mixed inoculated flasks was then added 50 µL of DDT 5 mM in DMSO (final concentration 0.25 µmol). To prevent the volatilization of substrate, the flask was sealed with a glass stopper and sealing tape after the headspace of each flask was flushed with oxygen. The cultures were incubated statically for 7 days at 30 °C. As a control, the cultures were terminated by autoclave (121 °C, 20 min) after pre-incubation. The experiments were performed in duplicate. The ratio of optimization was calculated by amount of degradation by mixed cultures per total amounts of degradation by fungus and bacteria.

Recovery of DDT

The samples were added 50 µL of pyrene 5 mM in DMSO (final concentration of 0.25 µmol) as internal standard and homogenized with 20 mL of methanol and then washed with 5 mL of acetone and centrifuged at 3000 rpm for 10 min. The biomass and supernatant were separated. The supernatant was filtrated with Whatman filter paper 41 (GE Healthcare life Science, UK). The filtrates were evaporated at 64 °C and extracted with 200 mL of *n*-hexane and the organic fractions were collected and dried over anhydrous sodium sulfate. The extracts were evaporated at 68 °C and concentrated to dryness under reduced pressure. The concentrate was diluted with methanol then analyzed using high-performance liquid chromatography (HPLC; Jasco, Japan) to quantify the amount of DDT. DDT and its metabolites were identified on the basis of the retention time and absorption maximum at specific wavelengths in comparison with authentic standard. For quantitative analysis, the peak area of DDT and its metabolites were compared to the peak area of pyrene [18-22]. To identify the other metabolites that could not be detected by HPLC, samples were further diluted with *n*-hexane and then analyzed by GC/MS.

RESULT AND DISCUSSION

Biodegradation of DDT

Microbes are potentially useful for the biotransformation of DDT. Bacteria and fungi metabolize DDT via reductive dechlorination. Ligninolytic fungi and the chlorophenyl-degrading bacteria carried out ring cleavage of DDT under aerobic conditions [20]. The syntropic activity of fungi and bacteria has been found to be very efficient in degrading both natural and synthetic organic substances, which the degradation rate is highly influenced by microbial density. Besides, diversity the association is general more efficiently degrades complex recalcitrant molecules like lignin and hemicellulose, which are first enzymatically attacked by fungi to form product that become the substrates for bacterial metabolism [20].

In this study, biodegradation of DDT was carried out in PDB medium. PDB medium was the most suitable medium for the proliferation of brown-rot fungi compared to the other liquid media such as low nitrogen (LN) and high nitrogen (HN) media [7]. After 7 days of incubation period, *F. pinicola* degraded DDT by approximately 42%. This result was higher than previous reports [7,16] due to higher amount of PDB medium. It indicated that the amount of medium affected the result of DDT biodegradation. DDT biodegradation by *F. pinicola* might be depended of enzyme because *F. pinicola* does not produce extracellular hydroxyl radical via Fenton reaction [7,16]. Enzymatic key reactions of aerobic biodegradations are

oxidation catalyzed by oxygenases and peroxidases [23]. *F. pinicola* has shown oxidoreductase activities as superoxide dismutase and catalase [24]. Besides, *F. pinicola* produced P450 monooxygenase and peroxidase [25]. However, the key enzyme of DDT degradation by *F. pinicola* is needed for further investigation.

Percentage of biodegradation of DDT by bacteria *P. aeruginosa* was shown in Table 2. The bacteria degraded DDT by approximately 75, 82, 86, 90 and 66% at 1, 3, 5, 7 and 10 mL of bacteria in PDB medium, respectively. The results indicated that the higher concentration of *P. aeruginosa*, the higher percentage of biodegradation of DDT was obtained, where the highest percentage of biodegradation of DDT was approximately 90% at 7 mL of bacteria. The results indicated that *P. aeruginosa* is potential bacteria for biodegrading DDT. However, in excess concentration of *P. aeruginosa* (10 mL) resulted a decreasing in DDT biodegradation (66%). It indicated the competition of bacteria for surviving rather than to degrade DDT. Some secondary metabolites might be produced by bacteria in stationary phase under abundant population of bacteria, which might be toxic for others in order to survive.

The correlation of DDT biodegradation by particular fungus *F. pinicola* and bacteria *P. aeruginosa* and the mixed cultures was shown in Fig. 1, while the total biomass was shown in Table. 1. The addition of *P. aeruginosa* into culture of *F. pinicola* had an influence on DDT biodegradation which tend to increase the DDT biodegradation comparing to culture *F. pinicola* without the addition of *P. aeruginosa*. The addition of 10 mL of *P. aeruginosa* in cultures of *F. pinicola* gave the highest degradation of about 67%. *P. aeruginosa* produced rhamnolipid biosurfactant form that can improve the solubility of DDT for degradation [26-27]. Beside producing biosurfactant, this bacterium was also able to metabolize DDT [8-9]. The ability of particular bacteria to degrade DDT was not equivalent with its mixed cultures. It indicated that synergistic between fungus *F. pinicola* and bacteria *P. aeruginosa* might be occurred on DDT biodegradation. A single bacterium did not possess enzymatically capability to degrade all or even most of organic compounds. Mixed microbial communities have the most powerful biodegradative potential because the genetic information of more than one organism is necessary to degrade the complex mixture [28]. Particularly, 7 mL of *P. aeruginosa* showed the highest DDT biodegradation, however, its mixed cultures with *F. pinicola* at 10 mL showed the highest DDT biodegradation (Fig. 1). It indicated that the amount of addition of bacteria affected the synergistic relationship between *F. pinicola* and *P. aeruginosa*. Based on these results, mixed cultures *F. pinicola* with 10 mL of *P. aeruginosa* showed the best mixed cultures that have the highest biodegradation of DDT (Fig. 1). Thus, it was selected for further experiment on identification of metabolites.

Analysis of Metabolites and Proposed Degradation Pathway

The analysis of metabolites of DDT biodegradation was performed using GCMS. The internal standard peaks appeared in control and treatment samples at m/z 202, which identified as pyrene (Fig. 2). The other peaks of metabolites were showed in Fig. 2 that had retention time at 13.3; 13.6; 12.8 and 11.3 min. The peak at a retention time of 13.3 min had m/z 235, which identified as DDD. The peak at retention time 13.6 min had m/z 235, which identified as DDT. The peak at retention time of 12.8 min had m/z 246, which identified as DDE. The peak at a retention time of 11.3 min had m/z 212, which identified as DDMU. *P. aeruginosa* metabolized DDT into DDD and DDMU, whereas *F. pinicola* produced DDD and DDE (data not shown). In previous report, DDD was the only metabolite detected from DDT biodegradation by *F. pinicola*, which indicated as main as well as end metabolite from DDT biodegradation during 28 days incubation [7,16]. In this study, biodegradation occurred during 7 days incubation, which converted DDT into DDE and DDD. The results indicated that initial transformation of DDT might be

Table 2. Biodegradation of DDT by *P. aeruginosa* in PDB medium during 7-days incubation period

Bacteria Amount (mL)	% DDT Biodegradation (%)
1	75.17 ± 2.06
3	82.13 ± 2.34
5	86.15 ± 0.51
7	90.20 ± 0.16
10	65.98 ± 0.87

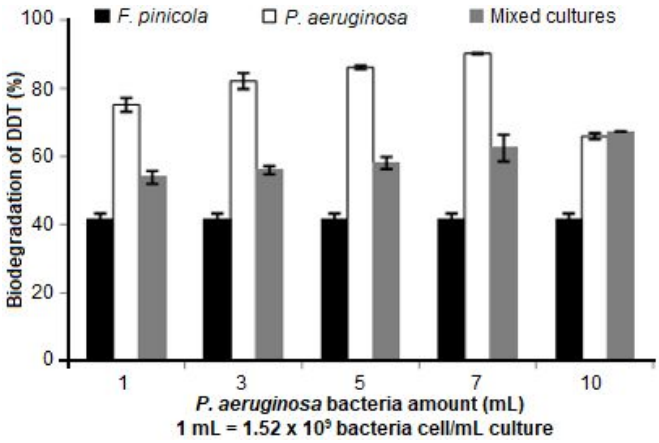

Fig 1. Biodegradation of DDT by *F. pinicola* with the addition of *P. aeruginosa* at various amounts

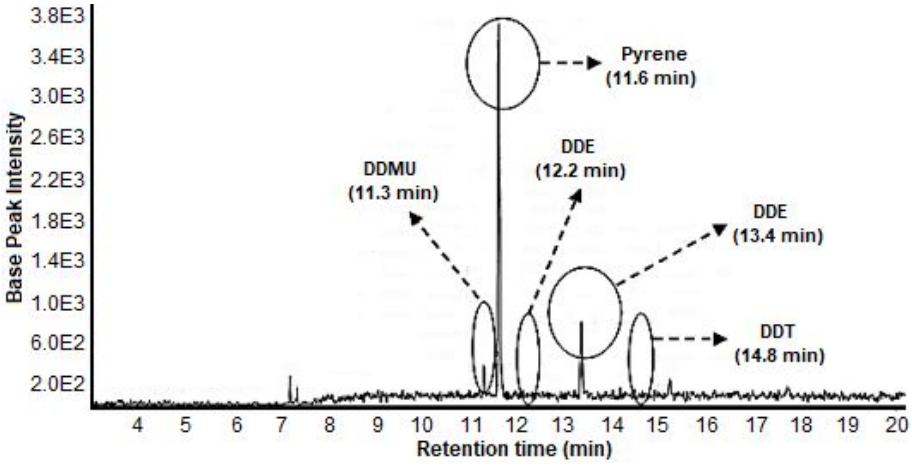

Fig 2. Chromatogram of DDT biodegradation by mixed cultures of *F. pinicola* and 10 mL of *P. aeruginosa* in 7 days incubation periods

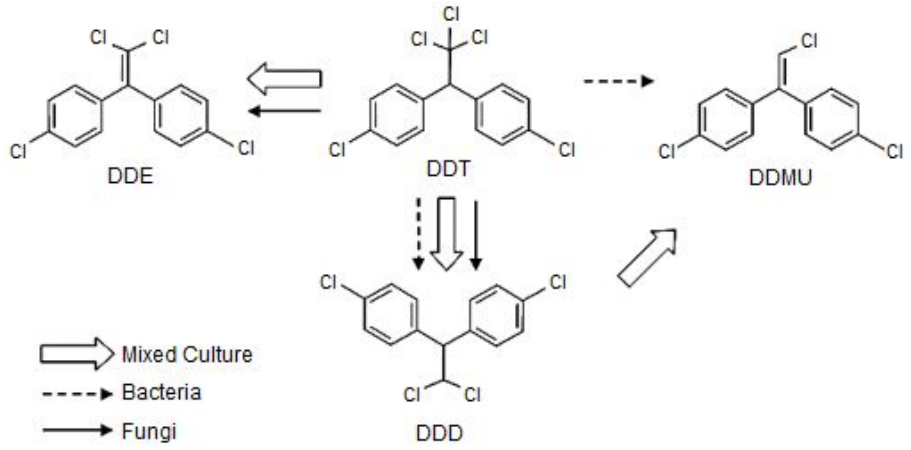

Fig 3. Proposed degradation pathway

Table 3. LD_{50} of DDT, DDD, DDE, DDMU

Subtrates	LD_{50} (mg/kg)	References
DDT	113.0 (in rat oral)	Hodgson et al. [33]
DDD	3400.0 (in rat oral)	Hodgson et al. [33]
DDE	880.9 (in the mouse oral)	An et al. [34]
DDMU	2700.0 (in the mouse oral)	An et al. [34]

converted to DDE and DDD, and then followed by the transformation DDE into DDD as the end product during longer incubation.

In general, the bacteria can degrade DDT aerobically or anaerobically, which produce DDE and DDD, respectively [5]. Biodegradation of DDT by the *P. aeruginosa* isolated from microflora of soil and water in agricultural area produced DDD, DDE, alkane and cycloalkene as metabolites [8]. Besides, *P. aeruginosa* could transform DDT into DDE, DDMU and DDOH in broth medium [9]. *P. aeruginosa* has the dehalogenase hydrolytic [29], dehalogenases play roles in the detoxification of halogenated aromatics [30]. However, in this study, DDD and DDMU were detected as metabolites of DDT biodegradation by *P. aeruginosa,* which DDD was the main metabolite and DDMU was produced from reductive dechlorination of DDD.

The mixed cultures of *F. pinicola* and *P. aeruginosa* converted DDT to DDE, DDD and DDMU (Fig. 2). The present of DDE was resulted from biodegradation of DDT by *F. pinicola* as initial transformation, which then transformed to DDD. DDD might be produced by both of fungus and bacteria since this metabolite was obtained from DDT by *F. pinicola* as well as *P. aeruginosa*. DDMU might be produced from transformation of DDD by *P. aeruginosa* via dechlorination [31]. The DDT biodegradation pathway by mixed cultures *F. pinicola* and *P. aeruginosa* culture was proposed in Fig. 3. DDT is transformed into DDD by reductive dechlorination which involved single electron transfer, removal of a chlorine ion and transformation of an alkyl radical [31]. DDE is produced from DDT via hydrolytic dehalogenation includes replacement of chlorine atom with hydroxyl group [32]. All of identified metabolite

compounds (DDD, DDE, DDMU) still had complex structure, which also potentially hazard to the environment but less toxic than DDT as shown in Table 3 [33-34]. Then, further investigation is required to obtain simpler and harmless metabolic products such as CO_2, H_2O.

CONCLUSION

The addition of bacteria *P. aeruginosa* could increase the percentage of degraded DDT by *F. pinicola* during 7 days incubation period. The addition of 10 mL of *P. aeruginosa* to *F. pinicola* showed the highest DDT biodegradation (67%), where DDD, DDE and DDMU were detected as metabolites. The results indicated that the addition of *P. aeruginosa* enhanced the biodegradation of DDT by *F. pinicola*.

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