Effects of bacterium *Ralstonia pickettii* addition on DDT biodegradation by *Daedalea dickinsii*

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Abstract
DDT (4,4-Dichlorodiphenyltrichloroethane) is one of the persistent organic pollutants that was extensively used in the 1940s as insecticide to control malaria. Even though DDT was banned since 1970, DDT and its metabolites are still present ubiquitously in the environment and have negative effects on human health. In this study, the ability of brown-rot fungus (BRF) Daedalea dickinsii to degrade DDT with the addition of Ralstonia pickettii was investigated. R. pickettii was added into 10 mL of D. dickinsii culture at 1, 3, 5, 7 and 10 ml (1 mL ≈ 1.44 x 10^{13} bacteria cells). DDT recovery was analyzed by HPLC. The addition of 10 mL R. pickettii to D. dickinsii culture yielded the highest DDT degradation of about 68.62%. This result was higher than DDT degradation by D. dickinsii only (about 53.61%) and by 10 mL R. pickettii only 19.84%. This result indicated that the addition of 10 mL R. pickettii can enhance DDT degradation by D. dickinsii.

Keywords: Biodegradation, *Daedalea dickinsii*, *Ralstonia pickettii*, DDT.

Introduction
In the past few decades, large quantities of persistent organic pollutants have been released into the environment for agricultural and public health purposes. Some pollutants are persistent and have ecotoxic properties[1]. DDT (4,4-Dichlorodiphenyltrichloroethane) is one of the persistent organic pollutants that was extensively used in the 1940s as insecticide to control malaria. DDT was the first synthetic insecticide to be developed and has been used worldwide since the 1940s. DDT was extensively used as an organochlorine insecticide for agricultural crops and control of vector-borne disease such as malaria. The use of DDT as insecticide continued for 25 to 30 years after World War II in most countries[2].

Technical grade of DDT that was used as a pesticide was composed of 14 chemical compounds (DDTs), with 65-80% of the active compound as *p,p'*-DDT, the other components were distributed as follows: 15 to 21% of *o,p'*-DDT, up to 4% *p,p'*-DDD and up to 1.5% 1-(*p*-chlorophenyl)-2,2,2-trichloro ethanol[3]. The most persistent compounds of DDTs are *p,p'*-DDT and *o,p'*-DDT[4]. DDT was also used in antifouling paint on fishing ships and industry for its transformation into dicofol, a miticide product[5].

Although DDT was banned since 1970, DDT and its metabolites are still present ubiquitously in the environment and have negative effects on human health. The presence of DDT in environment is a great concern due to its persistent and toxic biological effects. Some studies reported that DDT's half-life is between 4-30 years in soil, 1-4 years in sediment and 26-56 days in water[1]. DDTs pollution in both soil and sediment can reach up to 1600 mg/kg for some highly contaminated environments[6]. Due to its physicochemical properties and specially its persistence related with its half-life, DDT has been linked to several health and social problem that are due to its accumulation and biomagnification properties in the environment and living organisms[1].

Long-term exposure to small amounts of DDT (20-50 mg kg^{-1} of body weight every day) in animal studies can affect the liver, reproductive organs and the adrenal gland. DDT exposure poses a serious risk to human health; exposure is often accompanied by many harmful effects including neurological and immunodeficiency effects[7,8]. Toxic effects on humans together with long-lasting effects on wildlife made the removal of DDT from environment an environmental priority.

Some DDT remediation processes have been studied including physicochemical and biological process. Some physicochemical processes such as electrolysis, reductive dechlorination using metals and hydrodechlorination were already reported[9-12]. Although chemical and physical treatments are more rapid than biological treatments, they are more energy-intensive and often more expensive than bioremediation[13]. Among the several methods, biodegradation is relatively low-cost, more secure and efficient.

Purnomo et al[14-17] reported that *Daedalea dickinsii*, one kind of brown rot fungus (BRF), has the ability to degrade DDT via Fenton reaction. *D. dickinsii* can degrade DDT by about 47% in potato dextrose broth (PDB) after a 14-day incubation period. However, the amount of DDT degradation by *D. dickinsii* is still low and has a long incubation time. Therefore, the process needs to be improved by modifying the culture so as to enhance the ability of *D. dickinsii* to degrade DDT.

Modified methods of enhancing bioremediation have been developed. Mixed cultures of fungi and bacteria have the most potential because of their ability to degrade the complex mixture of organic pollutants in a contaminated area[18]. Some studies on bioremediation using mixed cultures of fungi and bacteria had reported that bacteria addition can increase the degradative ability of fungi.

Recently, Purnomo et al[19] reported that the addition of biosurfactant-producing bacteria, *Bacillus subtilis* and *Pseudomonas aeruginosa*, enhances DDT biodegradation by *Pleurotus ostreatus*. Furthermore, *B. subtilis* addition also enhances DDT degradation by *Phlebia brevispora*[20]. On the other hand, Sariwati et al[21] reported that *B. subtilis* addition also enhances DDT biodegradation by brown-rot fungus *Fomitopsis pinicola*.

Another study also reported that the removal of phenantrene from soil can be enhanced from 20% to 73% by the addition of *Ralstonia picketii* and *P. aeruginosa* to *Penicillium* sp. culture as co-cultures[22]. However, DDT biodegradation by *D. dickinsii* with *R. pickettii* addition has not been reported yet. In a previous study, *R. pickettii* was reported to have the capability on remediation of some organic pollutants such as chlorobenzene, petroleum and BTEX[23-26].

Besides, *R. pickettii* also has the ability to produce biosurfactant[27]. Biosurfactants can increase the solubility of DDT by optimizing DDT degradation process[28]. Therefore, the effects of *R. pickettii* on DDT biodegradation by *D. dickinsii* were investigated in the present study.

Material and Methods

Chemicals: DDT was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). *n*-hexane and acetone were purchased from Anhui Fulltime Specialized Solvent and Reagent Co. Ltd. (Anhui, China). Methanol, dimethylsulfoxide (DMSO) and sodium sulphate anhydrous were purchased from Merck Millipore (Darmstadt, German).

Culture Condition: *Daedalea dickinsii* NBRC 31163 (NITE Biological Resource Center, NBRC; Chiba, Japan) was used in this study. This fungus was maintained as cultures on 9 cm diameter of potato dextrose agar plates (PDA; Merck, Darmstadt, German). This fungus had been incubated at 30°C. *D. dickinsii* mycelia (1 cm diameter) was inoculated into 10 ml PDB medium (Difco). Cultures were pre-incubated at 30°C for 7 days[14,15].

Bacteria cultures *Ralstonia pickettii* NBRC 102503 (NITE Biological Resource Center, NBRC; Chiba, Japan) were maintained on nutrient agar (NA; Merck, Darmstadt, German), which was incubated at 37°C. The colony was inoculated into 60 mL nutrient broth (NB; Merck, Darmstadt, German). Cultures were pre-incubated at 37°C for 30 hours with shaker condition 180 rpm [19-21,29,30].

DDT Biodegradation by *D. dickinsii*: Pre-incubated cultures of *D. dickinsii* were added with 10 mL PDB medium (final volume 20 mL) and 50 µl of 5 mM DDT in DMSO was added to each inoculated flask. Each flask was flushed with oxygen and sealed with a glass stopper and sealing tape to prevent the DDT volatilization. The cultures were incubated statically for 7 days at 30°C. As a control, the cultures were terminated by autoclave (121°C, 15min) after pre-incubation. The experiments were performed in duplicate[14,15].

DDT Biodegradation by *R. pickettii*: After pre-incubation for 30 hours, 1, 3, 5, 7 and 10 mL *R. pickettii* (1 mL ≈ 1.44 x 10^{13} bacteria cells) were inoculated into PDB medium (final volume 20 mL) and 50 µl of 5 mM DDT in DMSO was added to each inoculated flask. Each flask was flushed with oxygen and sealed with a glass stopper and sealing tape to prevent the DDT volatilization. The cultures were incubated statically for 7 days at 30°C. As a control, the cultures were terminated by autoclave (121°C, 15 min) after pre-incubation. The experiments were performed in duplicate[19-21].

DDT Biodegradation by mixed culture of *D. dickinsii* and *R. pickettii*: 1, 3, 5, 7 and 10 mL pre-incubated *R. pickettii* (1 mL ≈ 1.44 x 10^{13} bacteria cells) was added separately into pre-incubated *D. dickinsii* cultures and PDB medium was added up to the final volume 20 mL. DDT (50 µl of 5 mM) in DMSO was added to each inoculated flask. Each flask was flushed with oxygen and sealed with a glass stopper and sealing tape to prevent the DDT volatilization. The cultures were incubated statically for 7 days at 30°C. As a control, the cultures were terminated by autoclave (121°C, 15min) after pre-incubation. The experiments were performed in duplicate[19-21].

DDT Recovery: After the incubation process, 50 µL of pyrene 5 mM in DMSO (final concentration, 0.25 µmol) was added into each flask as internal standard. The cultures were homogenized with 20 mL of methanol and then washed with 5 mL acetone. The residual biomass was removed by centrifugation at 3000 rpm for 10 min. The supernatant was filtered with Whatmann Filter Paper 41 (GE Healthcare Life Science, UK) and the filtrate was evaporated at 64°C and extracted with 200 mL *n*-hexane. The organic fraction was 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 by high-performance liquid chromatography (HPLC) to quantify the amount of substrate.

HPLC was conducted with a Shimadzu LC-20AT pump and a Shimadzu SPD-M20A diode array detector fitted with an Inertsil ODS-4 column (250 mm) with an inner diameter of 4.6 mm (GL Science, Tokyo). The samples were eluted with 82% methanol in a 0.1% trifluoroacetic acid aqueous solution at a flow rate of 1 ml/min[31-35].

Results and Discussion

In this study, the ability of *D. dickinsii* to biodegrade DDT with *R. pickettii* addition was investigated. *D. dickinsii* is one of the brown-rot fungi capable of being a bioremediation agent. *D. dickinsii* has already been reported to have the ability to degrade some pollutants. Rizqi et al[36] reported that *D. dickinsii* can transform methylene blue dye up to 54% in a 14-day incubation period. Mahmood et al[37] reported that *D. dickinsii* can decolorize some dyes such as disperse violet, yellow brown, red W4BS and yellow SRLP. Kim et al[38] also reported that *D. dickinsii* can also remove copper. In previous studies, *D. dickinsii* has been reported which can degrade DDT (final concentaration 0,25 µM DDT/Flask) about 47% in potato dexrose broth (PDB) for 14 days incubation[14-17]. However, DDT degradation by *D. dickinsii* was still low and needs long incubation time. So, in this study the abilities of *D. dickinsii* to degrade DDT with addition of *R. pickettii* were investigated.

In this study, *D. dickinsii* degraded DDT by 53.61% during the 7-day incubation period in PDB medium (table 1), which was different from a previous report (47 % in 14 days)[14]. This may be because the total volume of *D. dickinsii* culture (20 mL) used was larger than those used in previous study (10 mL)[14]. The ability of *D. dickinsii* to degrade DDT is dependent on its ability to produce extracellular hydroxyl radical via Fenton reaction and enzymatic process in their methabolism[14-17].

The production of ·OH is a universal characteristic of wood degradation by brown rot fungi which a part of their wood-degrading system[39]. *D. dickinsii* can also produce some enzymes that are responsible for degradation of organic, inorganic and aromatic compounds present in plant biomass on their metabolism to get their energy[37,40].

DDT degradation by *R. pickettii* only was also investigated. In this study, DDT degradation by *R. pickettii* was carried out with the difference initial cell density at 1, 3, 5, 7 and 10 mL (1 mL ≈ 1.44×10^{13} bacteria cells). The results of degradation of DDT by *R. pickettii* alone are shown in table 1. DDT was degraded by 8.62%, 12.07%, 15.64%, 30.87% and 19.84% by *R. pickettii* at the addition of 1, 3, 5, 7 and 10 mL of bacterial culture, respectively, within 7 days of incubation in PDB medium (table 1). These results indicated that at a higher volume of *R. pickettii*, a higher rate of DDT degradation was obtained (table 1).

The highest of DDT degradation was obtained at volume of 7 mL of *R. pickettii* of approximately 31%. This indicated that *R. pickettii* has a greater potential to degrade DDT. However, excess *R. pickettii* (10 mL) resulted in a decrease in DDT degradation amount (20%) due to competition for survival among the bacteria rather than promotion of DDT degradation. The bacteria may produce some secondary metabolites in an abundant population of bacteria in the stationery phase that may be toxic for others in order to survive.

R. pickettii is one of the aerobic bacteria found in water and soil[41]. *R. pickettii* also has ability to survive and thrive in low nutrient condition[42]. On the other hand, *R. pickettii* has been shown to have biodegradative abilities for some organic pollutants[23]. Zhang et al[24] reported that *R. pickettii* is able to degrade chlorobenzene as the sole source of carbon and energy. Plaza et al[25] also reported that *R. pickettii* also has the ability to produce biosurfactants and degrade petroleum hydrocarbons. In addition, *R. pickettii* has also been reported to greatly degrade benzene, toluene, ethyl benzene and xylenes[26].

The ability of *R. pickettii* to degrade organoaromatic pollutants is based on their ability to grow and use organoaromatic compounds as sole sources of carbon and energy under both aerobic and oxygen-limited denitrifying conditions[43,44]. The initial step of organoaromatics metabolism by *R. picketti* involves the hydroxylation of the organoaromatic compounds, which is then further hydroxylated to catechol, prior to ring cleavage by meta-fission dioxygenase[45].

The results of biodegradation of DDT by co-cultures of *D. dickinsii* and *R. pickettii* are shown in table 1. After 7 days of incubation, 53.98%, 62.29%, 52.21%, 60.93% and 68.62% of DDT was degraded by co-cultures of *D. dickinsii* with the addition of *R. pickettii* at 1, 3, 5, 7 and 10 mL respectively. Based on table 1, the addition of 10 mL of *R. pickettii* into *D. dickinsii* culture enhanced the DDT degradation ability by approximately 68.62%. The excess amount of bacteria might produce more biosurfactant to enhance the process of DDT degradation that was effective in the degradation process by the mixed cultures. The results indicated that the amount of addition of *R. pickettii* affected the synergistic relationship on DDT degradation by *D. dickinsii*.

Bioremediation of organic pollutants by mixed cultures of fungi with the addition of bacteria has been previously reported. Chavez-Gomez et al[22] reported that the addition of *R. pickettii* and *P. aeruginosa* to *Penicillium* sp. culture as co-cultures can enhance the soil removal of phenantrene from 20% to 73% and 63% respectively. Purnomo et al[19] reported that *B. subtilis* and *P. aeruginosa* addition increased DDT biodegradation by *P. ostreatus*. Sariwati et al[21] also reported that *B. subtilis* addition increased DDT biodegradation by *F. pinicola*.

The enhanced ability to degrade DDT can be correlated to biosurfactant produced by *R. pickettii*. *R. pickettii* is one of the bacteria which can produce biosurfactant. Plaza et al[25,27] reported that *R. pickettii* can produce biosurfactants which enhances their ability to degrade petroleum hydrocarbon. In another study, Plociniczak et al[28] reported that biosurfactant can increase the solubility of DDT thereby optimizing the DDT degradation process. Because DDT is a hydrophobic compound, the biosurfactant that is produced from addition

of *R. pickettii* can increase the solubility of DDT thereby optimizing the DDT degradation process.

Metabolic products were also identified. DDD (1,1-dichloro-2,2-bis (4-chlorophenyl) ethane) and DDE (1,1-dichloro-2,2-bis (4-chlorophenyl) ethylene) were detected as metabolic products of DDT degradation by *D. dickinsii* only while DDD and DDMU (1-chloro-2,2-bis (4-chlorophenyl) ethylene) were produced by *R. pickettii* (7 mL) (data not shown). On the other hand, co-cultures of *D. dickinsii* and *R. pickettii* (10 mL) degraded DDT to DDD and DDMU (fig. 1). Since metabolites of degradation of DDT by *R. pickettii* only were similar as metabolites by co-cultures, it indicated that *R. pickettii* has dominant. DDD and DDE were degraded by approximately 39% and 47% respectively. However, no metabolic products were detected from these degradations suggesting that DDD and DDE were transformed into other metabolic products that could not be detected by HPLC.

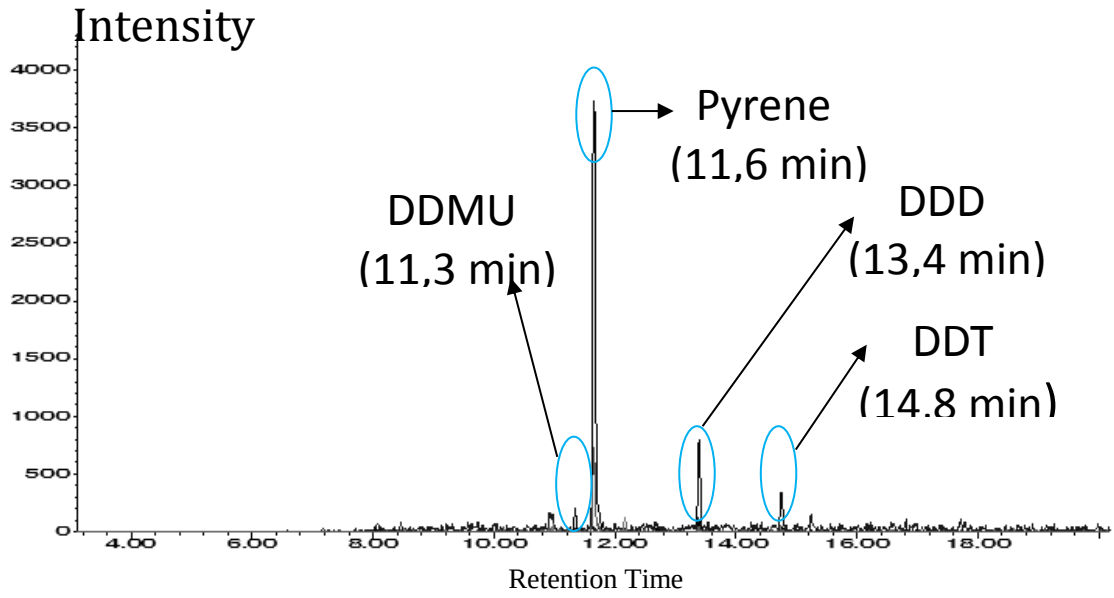

Figure 1: GCMS chromatogram of DDT degradation by co-cultures of *D. dickinsii* and *R. pickettii.*

Table 1
Degradation of DDT by *D. dickinsii* and *R. pickettii* as well as its co-cultures in PDB medium during the 7-day incubation period.

Volumes of *R. pickettii* (mL)	Degradation of DDT (%)	
	R. pickettii	Co-cultures
(*D. dickinsii only*)	53.61 ± 3.88	
1	8.62 ± 0.65	53.99 ± 1.41
3	12.08 ± 0.88	62.30 ± 0.54
5	15.64 ± 2.29	52.21 ± 0.95
7	30.87 ± 0.52	60.93 ± 0.04
10	19.84 ± 0.60	68.62 ± 1.80

Analyses were conducted by HPLC. Data are mean ± standard deviation (n = 2, triplicated).
1 mL of bacteria is equal to 1.44 x 10^{13} of bacterial cells/mL culture.

Further investigation for the identification of metabolic products was conducted by GCMS analysis which revealed no metabolic products from DDD and DDE degradation by co-cultures of *D. dickinsii* and *R. pickettii* (10 mL) (data not shown).

Conclusion

In this study, the ability of brown rot fungus (BRF) *D. dickinsii* to degrade DDT with the addition of *R. pickettii* was investigated. The addition of 10 mL of *R. pickettii* into *D. dickinsii* culture showed the highest DDT degradation of about 68.62%. This result indicated that addition of *R. pickettii* enhanced the biodegradation of DDT by *D. dickinsii*. DDD and DDMU were detected as metabolic products of DDT degradation by the co-cultures.

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