Abilities of Co-Cultures of White-Rot Fungus \textit{Ganoderma lingzhi} and Bacteria \textit{Bacillus subtilis} on Biodegradation of DDT

Erly Grizca Boelan\textsuperscript{1} and Adi Setyo Purnomo\textsuperscript{*2}

\textsuperscript{1} Department of Chemistry Education, Faculty of Teacher Training and Education Science, Universitas Katolik Widya Mandira, Kupang, Indonesia.
\textsuperscript{2} Department of Chemistry, Faculty of Natural Science, Institut Teknologi Sepuluh Nopember, Surabaya, Indonesia.

adi_setyo@chem.its.ac.id

Abstract. The insecticide 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) has been used extensively since the 1940s for controlling agricultural pest. Despite a ban on DDT use in most industrialized since 1972, DDT and its related residues (DDTr) still persist in the environmental and pose animal as worst as human health risk. In this study, abilities of co-cultures of white-rot fungus \textit{Ganoderma lingzhi} and \textit{Bacillus subtilis} on biodegradation of DDT was investigated. \textit{B. subtilis} at various volumes of 1, 3, 5, 7 and 10 mL (1 mL $\approx$ 6.7 x 10\textsuperscript{8} CFU) were mixed into 10 mL of \textit{G. lingzhi} culture for degrading DDT during a 7-days incubation period. The addition of 10 mL of \textit{B. subtilis} showed the highest DDT degradation about 82.30% during 7-days incubation period. DDD (1,1-dichloro-2,2-bis(4-chlorophenyl)ethane) and DDMU (1-chloro-2,2-bis(4-chlorophenyl)ethylene) were detected as metabolite products. This study indicated that co-cultures of \textit{G. lingzhi} and \textit{B. subtilis} can be used for degradation of DDT.

1. Introduction

1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) is an organochlorine pesticide that was extensively used for agricultural pest and control insect borne diseases such as malaria and typhus since World War II because low price (1,2). DDT and related compounds are highly hydrophobic and almost insoluble in water. These traits give rise to accumulation of DDT through the food chain. Although DDT had good capability as pesticides, in 1970 DDT was banned in most countries because their persistence (low biodegradability), bioaccumulation, carcinogenicity and negative impact on wildlife (2). Even though DDT has been banned, its residue still detectable in environmental samples and living organisms all over the world. DDT residues are lipophilic therefore high levels of DDT and its metabolites have been detected in human adipose tissues, blood plasma, liver, brain, placenta and even in breast milk along the food chain (2,3). DDT residues have been shown to persist in the environment predominantly in the form of DDT, 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene (DDE) and 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane. Because of negative impact of DDT, a method is needed to eliminate or reduction the residual of DDT and its metabolite products.

Biodegradation is one option treatment method to destroy or render harmless various contaminant using microorganisms (fungi and bacteria), because effective, economical and environment-friendly. There have been many studies about the ability bacteria and fungi to transform or degrade pollutants. White-rot fungi (WRF) have been studied extensively for their ability to transform or degrade a wide spectrum of hazardous chemical pollutants found in environment. \textit{Ganoderma lingzhi} (before 2012 known as \textit{Ganoderma lucidum}) is one of the most important and widely distributed WRF in the world and is associated with degradation of a wide variety of woods (4). Coelho\textit{e al} (5,6) reported that \textit{G. lingzhi} can degrade herbicide bentazon in solid and liquid state cultures approximately 90% and 55%
in 10 days incubation period respectively. Besides, *G. lingzhi* also have ability to degrade trichloroethylene and polycyclic aromatic hydrocarbon (PAHs) (7,8).

One kind of method to optimize the degradation of DDT by the fungus is with the addition of bacteria (9,10). Co-cultures of bacteria and fungus would be able to enhance the degradation of DDT with short incubation period. Purnomo *et al.* (9) reported DDT was degraded approximately 86% during 7 days incubation period by co-cultures of *Pleurotus ostreatus* and *Pseudomonas aeruginosa*. Sariwati *et al.* (10) reported co-cultures of brown-rot fungus *Fomitopsis pinicola* and *B. subtilis* can degrade DDT about 86% during 7 days incubation. In this study, the optimization degradation of DDT by *G. lingzhi* with addition of bacteria *B. subtilis* was investigated.

2. Materials and Methods

2.1 Chemicals

DDT and Pyrene were purchased from Tokyo Chemical Industry Co. Methanol, sodium sulfate anhydrous and dimethyl sulfoxide (DMSO) were purchased from Merck Milipore (Darmstadt, Germany). *n*-hexane and acetone were purchased from Anhui Fulltime Specialized Solvent and Reagent Co., Ltd (Anhui, China).

2.2 Culture Conditions

White-rot fungus *G. lingzhi* BMC9057 were grown on 9 cm diameter potato dextrose agar (PDA; Merck Darmstadt, Germany) plates and incubated statically at 30 °C. The mycelia (1cm diameter) from agar plate were inoculated into 10 mL of a liquid medium potato dextrose broth (PDB; Merck, Darmstadt, Germany) in 100 mL Erlenmeyer flasks and then pre-incubated at 30 °C for 7 days (9-11).

Stock cultures of *B. subtilis* NRBC3009 were grown on 9 cm diameter nutrient agar (NA, Merck, Darmstadt, Germany) plates and incubated at 37 °C. The colony was inoculated into 60 mL of Nutrient Broth (NB, Merck, Darmstadt, Germany) medium in 100 mL Erlenmeyer flasks and then were pre-incubated at 37 °C for 20 h with a shaker (180 rpm) (9,10).

2.3 Biodegradation by *G. lingzhi*

Cultures *G. lingzhi* after pre-incubation 7 days were added 10 mL of PDB medium (final volume 20 mL) and 50 µL of 5 mM DDT in DMSO. The headspace of each flask was flushed with oxygen. To prevent the volatilization of substrate Erlenmeyer was sealed with a glass stopper and sealing tape and then incubated at 30oC for 7 days. As a control, after pre-incubation cultures were killed by autoclaving (121 °C, 15 min) (9,10).

2.4 Biodegradation DDT by *B. subtilis*

Cultures after pre-incubation were inoculated into PDM medium with various volumes 1, 3, 5, 7 and 10 mL (1 mL ≈ 6.7 x 10^8 bacteria cells) (final volume 20 mL) and then 50 µL of 5 mM DDT in DMSO (final concentration 12.5 µM) added to each bacterial-inoculated flask. The headspace of each flask was flushed with oxygen. To prevent the volatilization of substrate Erlenmeyer was sealed with a glass stopper and sealing tape and then incubated at 30°C for 7 days. As a control, after pre-incubation cultures were killed by autoclaving (121 °C, 15 min) (9,10).

2.5 Biodegradation DDT by Co-Culture of *G. lingzhi* and *B. subtilis*

Culture *B. subtilis* after pre-incubation with various volumes of 1, 3, 5, 7 and 10 mL was inoculated separately into 10 mL of *G. lingzhi* culture (final volume 20 mL). The headspace of each flask was
flushed with oxygen. To prevent the volatilization of substrate, Erlenmeyer was sealed with a glass stopper and sealing tape. The cultures were incubated statically for 7 days at 30 °C. As a control, after pre-incubation cultures were terminated by autoclaving 121 °C for 15 min. The best degradation of co-cultures was selected for further experiments for identification of the metabolic products (9,10).

2.6 DDT Recovery
The culture flask was added 50 µL of 5 Mm pyrene in DMSO (final concentration 12.5 µM) as an internal standard, followed washed with 20 mL of methanol and 5 mL of acetone. The mixtures were homogenized and then centrifugated at 3000 rpm for 10 min. The supernatant was filtrated using Whatman filter paper 41 (GE Healthcare Life Science, UK). The filtrates were evaporated at 64 °C and extracted with 200 mL n-hexane and then the organic fraction was collected and over anhydrous sodium sulfate. The extracts were evaporated at 68 °C and concentrated to dryness under reduced pressure. The concentrate was diluted with methanol and the analysed by high-performance liquid chromatography (HPLC; Jasco, Japan) to quantify the amount of DDT and its metabolic products. The multiwavelength detector (Jasco, Japan) fitted with an Inertsil ODS-3 column (150 mm) with an inner diameter of 4.6 mm (GL Science, Japan). The samples were eluted by 82% methanol in 0.1% trifluoroacetic acid aqueous solution at a flow rate of 1 mL min⁻¹ (9–11).

2.7 Metabolic Detection
To identity metabolic products, samples were further diluted with n-hexane and the analysed by gas chromatography/mass spectrometry (GC/MS; HP, USA). The oven temperatures were programmed at 80 °C for 3 min, followed by a linear increase to 320 °C at 15 °C min⁻¹ and held for 5 min the injector temperature was set at 250 °C. injection was splinted less approximately 1 µL (10).

2.8 Statiscal Analysis
Student’s test was used to detect any significant differences between or within groups during DDT degradation. Differences between mean values at a confidence level of 5 % (P < 0.05) were considered to be statically significant. The results were calculated as average of triplicate determination.

3. Results and Discussion

3.1 Degradation of DDT by G. lingzhi
Fungus is a strong organism that has a longer life and can tolerate higher pollutant concentration more than bacteria. Many fungi especially white-rot fungi have been reported capable of degrading DDT. Phanerocheate chrysosporium, Phlebia lindtneri and Phlebia brevispora, can degrade DDT by approximately, 50% (30 days incubation period), 70% (21 days incubation period) and 30% (21 days incubation period), respectively (12,13). G. lingzhi is one of the most broadly distributed white-rot fungus. The ability of G. lingzhi BMC9057 to degrade DDT was determined in PDB medium. Selection of the PDB as a media inoculation was due to consists of potato extract and dextrose, which source of carbon and energy for fungus growth. DDT was eliminated by approximately 53% during the 7 days incubation period.

The ability of G. lingzhi to degrade pollutants was based on their ability to produced ligninolytic enzymes. These enzymes have been applied in various field including biodegradation of xenobiotic compound. Besides having the ligninolytic enzymes, G. lingzhi also has other enzymes involved in biodegradation, such as glyoxal oxidase, catalase, cellobiose dehydrogenase, endoglucanase,
cellobiohydrolase, alginate, lyase, chitinase, carbohydrate esterase, glycoside hydrolase and exo-1,3-beta-glucanase (12,14).

3.2 Degradation of DDT by Co-cultures G. lingzhi and B. subtilis

In this study G. lingzhi was used as degrading fungus that was optimized by addition of biosurfactant-producing bacteria B. subtilis. This bacterium has been reported as biosurfactant as biosurfactant producing bacteria (15,16). For optimization process B. subtilis was added into G. lingzhi culture at 1, 3, 5, 7 and 10 mL (1 mL ≈ 6.7 x 10⁸ bacteria cells). From HPLC analysis, the amount of degradation of DDT by G. lingzhi with addition of B. subtilis at 1, 3, 5, 7 and 10 mL volumes of bacteria were 14.35%, 37.61%, 71.79%, 32.43% and 82.30% respectively. Figure 1 showed the ability of G. lingzhi and co-cultures to degrade DDT. Based on this Figure, the addition of 5 and 10 mL of B. subtilis to G. lingzhi cultures enhanced the degradation of DDT about 71.79% and 82.30%. Because of DDT was hydrophobic compound, surfactin biosurfactant which produced from addition B. subtilis, can increase the solubility of DDT to optimize the DDT degradation process. These results indicated that addition of B. subtilis enhanced the biodegradation of DDT by G. lingzhi. The level of biodegradation of a pesticide depends on the rapid speed of microorganisms able to grow by utilizing pesticides as an energy source.

Degradation of DDT by co-cultures fungus-bacterium has been reported. Purnomo et al (9) reported that degradation of DDT by white-rot fungus P. ostreatus only about 19.34%, but co-cultures P. ostreatus-B. subtilis (5 mL) and P. ostreatus - P. aeruginosa (3 mL) enhanced degradation of DDT about 43.00% and 85.74 % respectively, during 7 days incubation. Sariwati et al (10) reported that co-cultures of brown-rot fungi F. penicola and B. subtilis 10 mL removed DTT about 85.87% during 7 days incubation.

![Graph showing degradation rate of DDT](image.png)
Figure 1. Percent Degradation of DDT by *G. lingzhi* and co-cultures *G. lingzhi* and *B. subtilis*

3.3 Identification Metabolite Product

To obtain trace metabolite of DDT by co-cultures *G. lingzhi* and *B. subtilis*, cultures extracts were analysed with GC/MS by comparing their retention times and mass spectra with those of available standard compounds. GC/MS analysis of samples co-cultures *G. lingzhi* and *B. subtilis* yielded two metabolites. Retention time (rt) of them were 11.3 and 13.4 min (Figure 2). The metabolite at rt = 11.3 min had a base peak $m/z = 212$ and three ionization peaks at $m/z = 282, 247, 212$ and 176 (Table 1), that correspond to DDMU (1-chloro-2,2-bis(4-chlorophenyl)ethylene). Since DDT have a base peak ion at $m/z = 235$ (M+ - CCl₃ or -CHCl₃). The base peak ion at rt = 13.4 min corresponds to DDD, that metabolite had $m/z = 318, 235$ (base peak), 199, and 165 (Table 1). The standard internal peak was appeared in control and treatment samples at rt = 11.6 which had $m/z = 202$, which identified as pyrene. In biodegradation of DDT by *G. lingzhi*, DDD and DDE were detected as metabolites products. In other research, white-rot fungus *P. lindtneri* and *P. brevispora* metabolized DDT to DDD, DDA (2,2-bis (4-chlorophenyl) acetic acid) and DBP (12). Purnomo *et al* (9) reported that degradation DDT by co-cultures *P. ostreatus*-*B.subtilis* yielded some metabolite product such as DDD, DDE and DDMU.

On the basis of the information obtained from the results, a degradation pathway co-cultures *G. lingzhi* and *B. subtilis* is proposed, DDT was transformed to DDD and DDMU. The mechanisms of microbial attack have already been described. Most study have been reported that DDT is reductively dechlorinated to DDD and then to DDMU. The results presented in this paper clearly demonstrate the capability of the co-cultures *G. lingzhi* and *B. subtilis* to degrade DDT in vivo. This research showed that co-cultures can be useful in the environmental pollution caused by DDT.

| Substrate or metabolite | GC retention time (min) | Mass spectrum $m/z$ (relative intensity) |
|-------------------------|-------------------------|------------------------------------------|
| DDMU                    | 11.30                   | 282, 247, 212, 176,                       |
| Pyrene                  | 11.60                   | 202,174, 150, 101,                       |
| DDD                     | 13.40                   | 318, 235, 199,165                        |
| DDT                     | 14.80                   | 354, 282, 235, 165                       |
Figure 2. GC/MS chromatogram of DDT degradation by co-cultures of *G. lingzhi* and *B. subtilis*

### 4. Conclusion
Co-cultures of 10 mL of *B. subtilis* and *G. lingzhi* showed the highest DDT degradation about 82.30% on 7 days incubation period. The addition of *B. subtilis* 10 mL were higher than DDT degradation by *G. lingzhi* without addition of *B. subtilis*, which only 52.52%. DDD and DDMU were detected as metabolite products from DDT degradation by co-cultures of *G. lingzhi* and *B. subtilis*. This study indicated that addition of *B. subtilis* can be used for optimization of DDT degradation by *G. lingzhi*.

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