

Biodegradation of 1,1,1-Trichloro-2,2-bis (4-chlorophenyl) ethane (DDT) by Mixed Cultures of White-Rot Fungus *Ganoderma lingzhi* and Bacterium *Pseudomonas aeruginosa*

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ABSTRACT

This study investigated the biodegradation of 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DDT) by mixed cultures of white-rot fungus *Ganoderma lingzhi* and bacterium *Pseudomonas aeruginosa*. Bacterium *P. aeruginosa* culture with various volumes 1, 3, 5, 7, and 10 ml (1 ml \approx 1.53 x 10⁹ bacteria cells/ml cultures) was added into 10 ml *G. lingzhi* culture for degrading DDT. After 7 d incubation, DDT was degraded about 100% with addition of 5, 7, and 10 ml of *P. aeruginosa* culture into *G. lingzhi*. Two metabolites; 1,1-dichloro-2,2-bis (4-chlorophenyl) ethane (DDD) and 1-chloro,2,2-bis (4-chlorophenyl) ethylene (DDMU) were detected from mixed cultures of *G. lingzhi* and *P. aeruginosa* as metabolic products of DDT. This research indicated that mixed cultures of white-rot fungus *G. lingzhi* and bacterium *P. aeruginosa* could be used to degrade DDT.

1. Introduction

The organochlorine pesticide 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DDT) was one of the first synthetic pesticides widely used in the world since the 1940s. The use of DDT was increased substantially since World War II for control agricultural pest and control insect borne diseases such as thypus and malaria (Curtis and Lines 2000; Turusov *et al.* 2002; Booij *et al.* 2016). Since the late 1970s, DDT has been banned for agricultural use in most countries because of its high toxicity, extremely hydrophobic and persistency lead to biological magnification and accumulation, and within the food chain bring on high degree of contamination (Curtis and Lines 2000; Turusov *et al.* 2002). Though the use of DDT has been banned, in several countries DDT still being used for essential public health purposes such as eradicate malaria (Curtis and Lines 2000).

In recent years, research on DDT has become an environmental priority because DDT and its metabolites have high resistance to degradation and relatively long half-life about 15 years up to 36 years in soil (Zhao *et al.* 2010). DDT has covered some health problems such as increase tumor production, DNA damage in

blood cells and disruption of synthesis and metabolism of endogenous hormones, interruption the functions of respiratory system, and attack on central nervous system. Therefore, DDT is still a serious environmental problem and efficient remediation method is required.

Many researchers have reported that microorganism fungi and bacteria have ability to degrade DDT and transform to another metabolite (Aislabie *et al.* 1997; Foght *et al.* 2001; Rubilar *et al.* 2013). Recently, biodegradation of DDT was performed using mixed cultures by microorganisms. Purnomo *et al.* 2017 reported that culture of white-rot fungus *Pleurotus ostreatus* has synergistic effect with biosurfactant-producing bacteria *Pseudomonas aeruginosa* and *Bacillus subtilis*. DDT was eliminated by mixed cultures of *P. ostreatus*-3 ml of *P. aeruginosa* (1 ml \approx 1.25 x 10⁹ bacteria cells/ml cultures) and *P. ostreatus*-5 ml of *B. subtilis* (1 ml \approx 1.25 x 10⁹ bacteria cells/ml cultures) about 85.74% and 43% respectively in PDB medium during 7 d incubation. In another study, DDT was degraded by mixed cultures brown-rot fungus *Fomitopsis pinicola*- and 10 ml of *P. aeruginosa* (1 ml \approx 6.7 x 10⁸ bacteria cell/ml cultures) and *F. pinicola*-10 ml of *B. subtilis* (1 ml \approx 1.53 x 10⁹ bacteria cell/ml cultures) approximately 68% and 86% during 7 d in PDB medium (Sariwati *et al.* 2017; Sariwati and Purnomo 2018).

Ganoderma lingzhi (before 2012 known as *Ganoderma lucidum*) is one of the most important and widely

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distributed white-rot fungus in the world that has the ability to degrade variety wood (D'Souza *et al.* 1999). Its capacity to degrade DDT is not well known. Recent studies, (Da Silva *et al.* 2010a; Da Silva *et al.* 2010b) reported *G. lingzhi* can remove herbicide bentazon in liquid and solid states condition approximately 55% and 90% after 10 d cultivation. In another report, *G. lingzhi* can enhance degradation processes of trichloroethylene, polycyclic aromatic and hydrocarbons (PAHs) and pentachlorophenol (Jeon *et al.* 2008; Punnapayak *et al.* 2009; Ting *et al.* 2011). This information lets us pay attention to *G. lingzhi* in its capability to degrade DDT.

P. aeruginosa is found in water and soil and can grow in areas that contain oil and other fuel oils. Thus, this bacterium can be used to degrade hydrocarbon pollutants in the aquatic environment as well as in the soil. *P. aeruginosa* can produce biosurfactant rhamnolipid which can increase dispersion or surface area for microbial attachment (Miller and Zhang 1994; Al-tahhan *et al.* 2000; Moussa *et al.* 2014). Because DDT is a hydrophobic compound, then the addition of biosurfactant can increase the solubility of DDT thereby optimizing the degradation process. The aims of this research were investigated the ability of mixed cultures *G. lingzhi* and *P. aeruginosa* to degrade DDT.

2. Materials and Methods

2.1. Chemicals

DDT and pyrene were purchased from Tokyo Chemical Industry Co (Tokyo, Japan). Acetone and *n*-hexane were purchased from Anhui Fulltime specialized solvent and reagent Co., Ltd (Anhui China). Dimethyl sulfoxide (DMSO), methanol and sodium sulfate anhydrous were purchased from Merck Millipore (Darmstadt, Germany).

2.2. Microorganisms Culture Conditions

Stock cultures of *G. lingzhi* BMC 9057 were grown on Potato Dextrose Agar (PDA, Merck Darmstadt, Germany) plates that had been incubated statically at 30°C. *G. lingzhi* mycelia (1 cm diameter) was inoculated into 10 ml of Potato Dextrose Broth (PDB, Merck Darmstadt, Germany) medium and then pre-incubated statically for 7 d at 30°C.

Bacterial cultures of *P. aeruginosa* NBRC 3080 (NITE Biological Resource Center, NBRC; Chiba, Japan) were grown on Nutrient Agar (NA; Merck Darmstadt, Germany) medium which was incubated at 37°C. The colony was inoculated into 60 ml of Nutrient Broth (NB, Merck, Darmstadt, Germany) medium and pre-incubated for 21 h at 37°C with shaker condition 180 rpm (Purnomo *et al.* 2008; Purnomo *et al.* 2017; Sariwati *et al.* 2017; Sariwati and Purnomo 2018).

2.3. Biodegradation of DDT by *G. lingzhi*

Cultures *G. lingzhi* after pre-incubation for 7 d, 10 ml of PDB medium and 50 µl of 5 mM DDT in DMSO as a substrate were added to each 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 to prevent the volatilization of the substrate. The cultures were incubated statically for 7 d at 37°C. As a control, the cultures were killed by autoclaving (121°C, 20 min) after pre-incubation (Purnomo *et al.* 2017; Sariwati *et al.* 2017; Sariwati and Purnomo 2018).

2.4. Biodegradation of DDT by *P. aeruginosa*

Culture bacterium *P. aeruginosa* (after pre-incubation for 21 h) was inoculated to PDB medium (final volume 20 ml) with a various volume 1, 3, 5, 7, and 10 ml (1 ml ≈ 1.53 × 10⁹ bacteria cell/ml cultures). As substrate, 50 µl of 5 mM DDT in DMSO was added to each inoculated flask. The headspace of each flask was flushed with oxygen and then sealed with a glass stopper. The sealing tape is used to prevent the volatilization substrate. The cultures were incubated statically for 7 d at 30°C. As the control, the cultures were killed by autoclave (121°C, 20 min) (Purnomo *et al.* 2017; Sariwati and Purnomo 2018).

2.5. Biodegradation of DDT by Mixed Cultures of *G. lingzhi* and *P. aeruginosa*

1, 3, 5, 7, and 10 ml of pre-incubated *P. aeruginosa* cultures were inoculated separately into 10 ml of *G. lingzhi* culture with final volume 20 ml. DDT (50 µl of 5 mM) in DMSO was added to each flask. The headspace of each flask was flushed with oxygen and sealed with glass stopper and then sealing tape to prevent the volatilization of substrate. The cultures were incubated statically for 7 d at 30°C. As a control, after incubation the cultures were killed by autoclave (120°C, 20 min) (Purnomo *et al.* 2017; Sariwati *et al.* 2017; Sariwati and Purnomo 2018).

2.6. DDT Recovery

After incubation process, 50 µl of pyrene 5 mM in DMSO (final concentration 0.25 µmol) was added into the flask as internal standard, followed washed with 20 ml of methanol and 5 ml of acetone. The residual biomass was removed by centrifugation at 3,000 rpm for 10 min. The supernatant was filtrated using Whatman 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 and 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 (Sariwati and Purnomo 2018).

To identify the metabolites of DDT, samples were further diluted with *n*-hexane and then analyzed by GC-MS. 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 and increased to 200°C at 20°C min⁻¹ and increased again to 250°C at 10°C min⁻¹. The injection was splintedless approximately 1 µl.

3. Results

White-rot fungus *G. lingzhi* and bacterium *P. aeruginosa* have been investigated as a new mixed cultures for biodegradation of DDT. In this research, environmental parameter was manipulated using medium that allows growth and degradation process. *G. lingzhi* grew well at NB and PDB medium at 30°C

but at high temperatures, its growth was bad. While, *P. aeruginosa* grew well at NA and PDB medium at 30°C.

During 7 d incubation period, DDT was degraded about 52.52% by *G. lingzhi*. Degradation DDT by bacterium *P. aeruginosa* at 1, 3, 5, 7, and 10 ml volumes of bacteria (1 ml \approx 1.52 x 10⁹ bacteria cell/ml culture) during 7 d incubation is shown in Table 1. Degradation rate increased from 1 to 7 ml but decrease at 10 ml. Bacterial culture of *P. aeruginosa* at 10 ml degraded DDT about 65.98%, which was lower compared to 7 ml that was degrade DDT about 90.20%. This result indicated that *G. lingzhi* and *P. aeruginosa* have a greater potential to degrade DDT.

In the present study the ability of mixed cultures of white-rot fungus *G. lingzhi* and bacterium *P. aeruginosa* to degrade DDT was investigated. The analysis result of degradation of DDT by mixed cultures of *G. lingzhi* and *P. aeruginosa* are shown in Table 1 and Figure 1.

Table 1. Degradation of DDT by *G. lingzhi* and *P. aeruginosa* as well as by mixed cultures

Bacterial concentration (ml)	% Degradation of DDT	
	<i>P. aeruginosa</i>	Mixed cultures
<i>G. lingzhi</i> (only)	52.52±0.68	
1	75.17±2.06	59.11±0.81
3	82.13±2.34	91.34±0.73
5	86.15±0.51	100±0.00
7	90.20±0.16	100±0.00
10	65.98±0.87	100±0.00

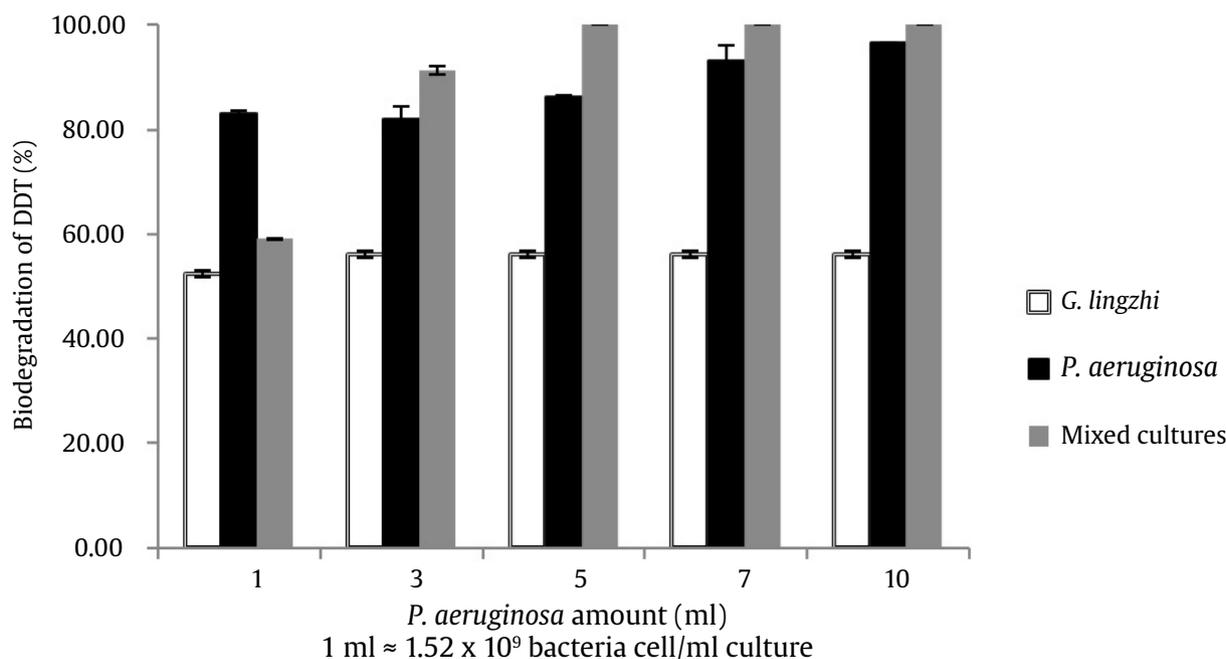


Figure 1. Biodegradation of DDT by mixed cultures of *G. lingzhi* and *P. aeruginosa*

The addition of bacterium *P. aeruginosa* into cultures of *G. lingzhi* gave significant effect on degradation of DDT, where the degradation rate increases 100% in addition of 5, 7, and 10 ml cultures bacteria after 7 d incubation. This result indicates that the mixed cultures of *G. lingzhi* and *P. aeruginosa* can increase the DDT degradation rate.

Based on GC-MS analysis, DDD, and DDE were detected as metabolites of DDT degradation by *G. lingzhi* while DDD and DDMU were detected as metabolites from degradation of DDT by *P. aeruginosa* (data not shown). Figure 2 showed DDE and DDMU, which are the metabolites obtained from degradation of DDT by mixed cultures of *G. lingzhi* and *P. aeruginosa* after extraction and analysis by GC-MS with comparing their retention time and mass spectra with those of available standard compounds. The peak at retention time of 11.3 min had m/z 212, which identified as DDMU. The peak at retention time of 13.4 min had m/z 235, which identified as DDD. The internal standard peaks appeared in control and treatment samples at m/z 202, which identified as pyrene. In this result, DDT was not detected, its equivalent to the degradation result. DDT possibly transformed into DDD and DDMU.

4. Discussion

Biodegradation is a good alternative to clean-up contaminants using biological activity. In this method, contaminant can be metabolized by microorganisms

and can be effective only where environmental condition permit microbial growth and activity. Punnapayak *et al.* 2009 reported that *G. lucidum* Chaaim-001 BCU grew well at 30°C but at temperatures above 30°C the growth was poor. While, *P. aeruginosa* that can be grown easily in a wide variety of condition and temperatures.

A successful biodegradation technique requires an efficient microorganism that can degrade the largest pollutants to minimum level. Fungi and bacteria are very good degrader of pollutants. They use the contaminant as the source of carbon and energy for growth and metabolism. *G. lingzhi* is a white-rot fungus that has potential ability as traditional medicine. Beside that, *G. lingzhi* could degrade the major components of plants cell wall including lignin, cellulose and hemicelluloses (Liu *et al.* 2012). In general, the ability of *G. lingzhi* to degrade DDT is related to the ability to degrade cellulose and lignin. According to Liu *et al.* 2012, *G. lingzhi* has many genes might be involved in degradation process including 216 putative glycoside hydrolases, 56 putative glycosyl transferase, 16 laccase, 7 peroxidase, and 9 glyoxal oxidase genes. Moreover, *G. lingzhi* showed rich P450 family which enzymes catalyze of variety reaction.

Ligninolytic enzymes such as Mangan Peroxidase (MnP), Lignin Peroxidase (LiP), and Laccase have played an important role in degradation or transformation of several xenobiotic compounds. Some reports mentioned that laccase is one of the most applied in biodegradation because its board substrate specificity

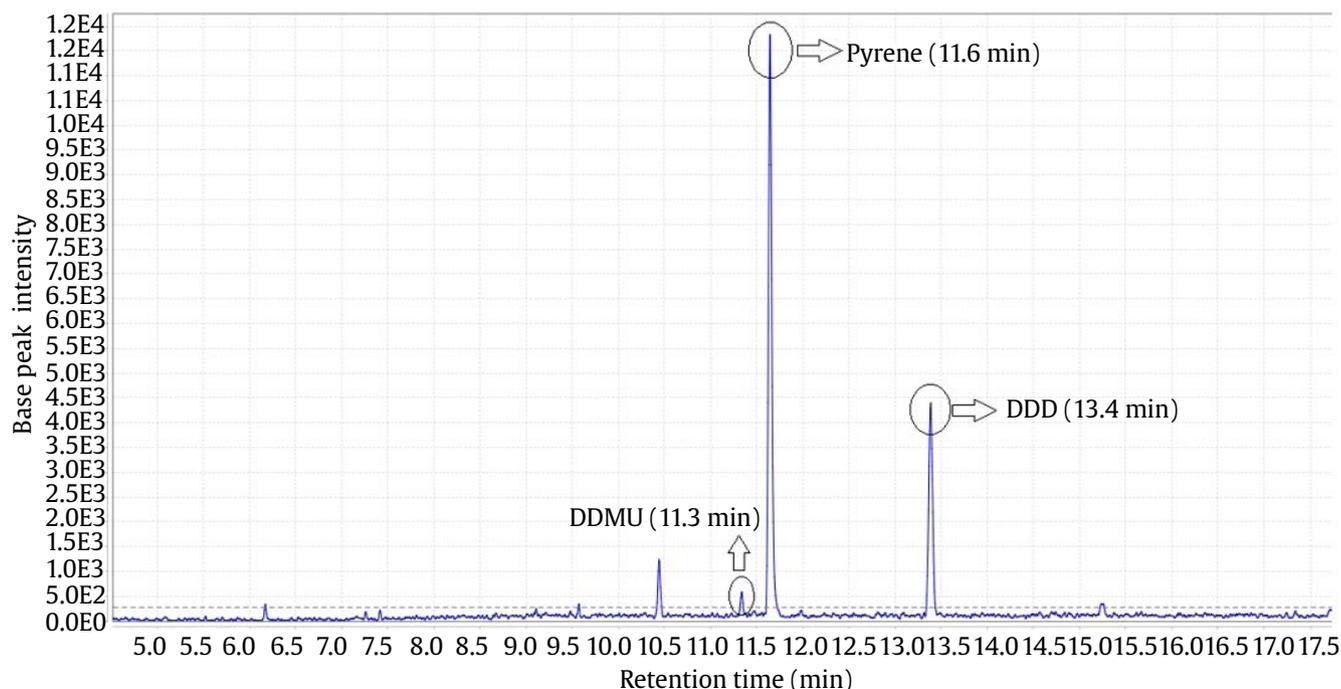


Figure 2. Chromatogram of DDT biodegradation by mixed cultures of *G. lingzhi* and 5 ml of *P. aeruginosa* in 7 d incubation

(Canas and Camarero 2010). Da Silva *et al.* 2010 reported herbicide bentazon was degraded by *G. lingzhi* approximately 90% and 55% in solid state and liquid cultures respectively.

Degradation of DDT by *P. aeruginosa* at various volumes is shown in Table 1. After 7 d incubation period, DDT was degraded approximately 75%, 82%, 86%, 90%, and 66% at 1, 3, 5, 7, and 10 ml of bacteria in PDB medium respectively. *P. aeruginosa* is bacterium that has potential for degradation of DDT. The highest degradation rate was recorded at 7 ml volume of bacteria approximately 90%. While at 10 ml degradation rate was decrease approximately 66%. It indicated, in excess concentration of bacterium *P. aeruginosa* can decrease degradation of DDT. In stationary phase, bacteria can produce some secondary metabolites that may be toxic for other in order to survive.

In previous report, *P. aeruginosa* DT-Ct1 can degrade 5 ppm of DDT completely in 4 days incubation (Bidlan 2003). Furthermore, Kamanayalli and Ninnerkar 2004 reported *Pseudomonas* species has the ability to transform DDT to 4-chlorobenzoic acid.

Biodegradation process will work fine depends on the nature of organisms, the enzyme involved, its concentration and availability in its survival microorganism. According to Das 2014, microorganisms can grow by utilizing pesticides as an energy source at the biodegradation of pesticides.

Study about mixed cultures of microorganisms to degrade xenobiotic compounds was reported. Endosulfan was eliminated efficiently by co-cultures of bacteria were collected from mixed and pure cultures in aerobic and facultative aerobic condition (Awasthi *et al.* 1997; Kumar and Philip 2006). Other studies reported mixed

cultures fungi and bacteria have an ability to degrade xenobiotic compound. Hai *et al.* 2012 reported that effect of combining fungus and bacteria cultures can improve the removal rates of pesticides aldicarb, antrazine and alachlor approximately 47.98% and 62% respectively. Purnomo *et al.* 2017 reported mixed cultures of white-rot fungus *P. ostreatus*-5 ml of *B. subtilis* and *P. ostreatus*-3 ml of *P. aeruginosa* can degrade DDT about 43.00% and 85.74% respectively during 7 d incubation. In another research, Sariwati *et al.* 2017 reported that co-cultures of brown-rot fungus *F. pinicola* and 10 ml of *B. subtilis* removed DDT about 85.87% during 7 d incubation.

In comparison with several previous studies, we reported that degradation of DDT by mixed cultures of *G. lingzhi* and *P. aeruginosa* is more effective than mixed cultures *P. ostreatus*-5 ml of *P. aeruginosa* and also *F. pinicola*-10 ml of *P. aeruginosa* were approximately 69% and 67% respectively (Purnomo *et al.* 2017; Sariwati and Purnomo 2018). This might be caused by the ability of *G. lingzhi* to produce many genes that can involve in degradation process and also the ability *P. aeruginosa* to produce a rhamnolipid biosurfactant, which can improve the solubility of DDT.

G. lingzhi metabolized DDT to DDD and DDE, while *P. aeruginosa* produced DDD and DDMU. Based on identification of metabolic products by GC-MS, a DDT transformation pathway is proposed (Figure 3.). This result indicated bacteria have an important role in DDT transformation. The mechanisms of microbial attack have already been described. Most reports indicate that DDT is reductive dechlorinated to DDD involving substitution of an aliphatic chlorine for a hydrogen atom. DDMU was also produced from reductive dechlorination of DDD.

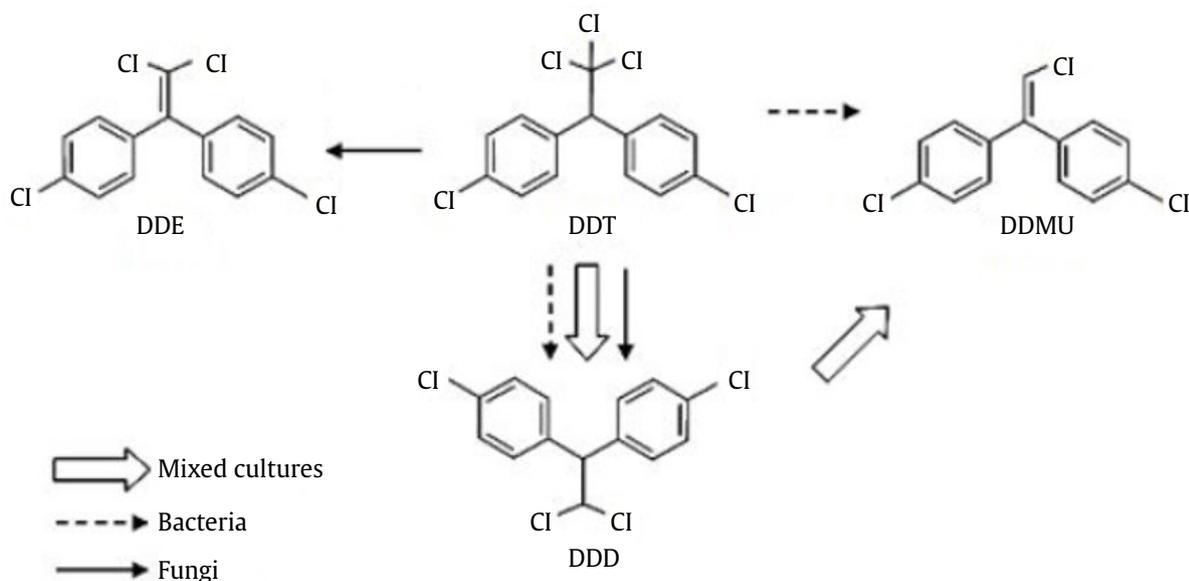


Figure 3. Proposed DDT degradation pathways by particular *G. lingzhi* and *P. aeruginosa* as well as by mixed cultures

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