Fundamental study of degradation of dichlorodiphenyltrichloroethane in soil by laccase from white rot fungi

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ABSTRACT: This research describes application of laccase from white-rot fungi (polyporus) to remove dichlorodiphenyltrichloroethane in soil. The degradation kinetics of dichlorodiphenyltrichloroethane in soil was also investigated by laboratory batch experiments. The results showed that laccase from white-rot fungi can effectively degrade dichlorodiphenyltrichloroethane and the degradation of total dichlorodiphenyltrichloroethane (the sum of the four dichlorodiphenyltrichloroethane compounds in a sample) was pseudo-first-order kinetics. The residues of almost all the dichlorodiphenyltrichloroethane components and total dichlorodiphenyltrichloroethane in soils treated with laccase decreased rapidly during first 15 days and then kept at a stable level during next 10 days. The residues of total dichlorodiphenyltrichloroethane in soils with different dosages laccase decreased by about 21-32 %, 29-45%, 35-51 % and 36-51 % after 5, 10, 15 and 25 days of incubation, respectively. The half-life of total dichlorodiphenyltrichloroethane in soils with different dosages laccase ranged from 24.75 to 41.75 days. The residues of total dichlorodiphenyltrichloroethane in three different types of soils decreased by 25-29 %, 39-43 %, 44-47 % and 47-52 % after 5, 10, 15 and 25 days of incubation with laccase, respectively. The half-life of total dichlorodiphenyltrichloroethane in different types of soil ranged from 24.71 to 27.68 days. The residues of total dichlorodiphenyltrichloroethane in soils with different pH levels decreased by 18-24 %, 29-39 %, 36-39 % and 39-50% after 5, 10, 15 and 25 days of incubation with laccase, respectively. The half-life of total dichlorodiphenyltrichloroethane ranged from 25.63 to 36.42 days. Laccase can be an efficient and safe agent for remediation of dichlorodiphenyltrichloroethane-contaminated soil.

Keywords: *Bioremediation; Enzyme; Half-life; Organochlorine pesticide; Residue*

INTRODUCTION

Dichlorodiphenyltrichloroethane (DDT) was one of the most widely used organochlorine pesticide. In 2001, the governing council of the United Nations environment programme issued a treaty to eliminate or restrict the production and use of persistent organic pollutants (POPs). Twelve substances were listed and DDT was among them. Although the use of DDT has been banned for wide agricultural use since 1983 in China, part of DDT was released to environment and this trend continues, because it was still used as an anti-malaria agent or a raw and processed material for dicofol production (Wong *et al.*, 2005). A large amount of DDT still remains in soils. In some area, the DDT concentration found in soil markedly exceeded the level set by the national soil quality standards (GB/T 18407-2001). For example, DDT concentration in the agricultural soils of suburb Shanghai of China severely exceeded the national soil quality standards (Li and Jiang, 2004). The detection rate of DDT in some vegetables produced by Nanjing suburb of China was up to 100 % (Gao and Jiang, 2005). Considering its potential hazard to the safety of agricultural products and human health via the food chain, effective methods of remediation must be developed (Nouri *et al.*, 2008). Recently, various physical (Tian *et al.*, 2009; Pang *et al.*, 2010), chemical (Gautam and Sumathi, 2006; Villa *et al.*, 2006) and biological (including phytoremediation and microbial bioremediation) (Kantachote *et al.*, 2004; Lunney *et al.*, 2004; Gaw *et al.*, 2008; Purnomo *et al.*, 2008) approaches have been utilized to remove DDT from the contaminated soil. Enzymatic remediation was a rapid and highly efficient method of removing pesticide residue from the environment (Sutherland *et al.*, 2004; Velmurugan *et al.*, 2009; Obire and Anyanwu, 2009). For

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example, the enzyme systems in the *P. australis* of wetland plant species were able to transform DDT effectively (Chu *et al.*, 2006); White-rot fungi Phanerochaete chrysosporium was used to metabolize DDT and its metabolites (Bumpus *et al.*, 1985; Gong *et al.*, 2006). They also suggested the degradation of DDT was initiated by ligninolytic enzymes, which comprise lignin peroxidase, manganese peroxidase and laccase. Laccase (EC 1.10.3.2) has been widely used to degrade pollutants from environment (Khlifi *et al.*, 2009; Steevensz *et al.*, 2009). Laccase can catalyze the oxidation of organic compounds (Baldrian, 2006). However, DDT and its metabolites have high resistance to degradation and relatively long half-lives. Howard (1991) estimated that the half-life of DDT was 15 years in soil. The half-life of DDT in soil was different, depending upon soil conditions (Alexander, 1994; Gong *et al.*, 2003). In this study, the effects of laccase dosage and soil conditions (different types of soil and different soil pH) on degradation of DDT in soil by laccase will be discussed. This study was carried out in Guangzhou, China during March 2007 to April 2008.

MATERIALS AND METHODS
Reagents
White-rot fungi used for the production of laccase were purchased from Guangzhou Chemical Company L.T.D of Chinese Academy of Sciences (Guangzhou, China). The standards for the four components of DDT (2,2-bis(p-chlorophenyl)-1,1-dichloroethylene, p,p'-DDE; 1,1,1-trichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethane, o,p'-DDT; 2,2-bis(p-chlorophenyl) 1,1-dichloroethane, p,p'-DDD; 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane, p,p'-DDT) were purchased from Supelco (Park Bellefonte, PA). The testing soils, lateritic red soil, latosol soil and red soil, were obtained from arboretum of South China Agricultural University, woodland of Shaoguan City of Guangdong Province in China and woodland of Zhanjiang City of Guangdong Province in China, respectively. The basic physical and chemical properties of the soils after air drying are listed in Table 1.

Total dichlorodiphenyltrichloroethane (DDTs) in this study stands for the total sum of p,p'-DDE, o,p'-DDT, p,p'-DDD and p,p'-DDT in each sample. DDT was not detected in these soils (the detection limit will be described later).

Preparation of laccase and enzymatic activity measurement
White-rot fungi was inoculated into a solid agar medium in which potato flour was used as carbon source for the cellulose decomposing microorganisms to grow. Once the white-rot fungi spread all over the agar plates, a 10 mm hole diameter puncher was used to inoculate a piece of the white-rot fungi lawn into 50 mL potato liquid medium. White-rot fungi was cultured in the temperature of 28 °C for 9 days under shaking condition (130 r/min). After centrifugation, the

Table 1: Physical and chemical properties of the experimental soil

Experimental soil	Soil texture	pH (H_2O:soil=2.5:1)	Fe (mg/kg)	Organic matter (g/kg)
Lateritic red soil	Silty soil	4.79	45	0.45
Latosol soil	Silty soil	5.22	22.77	3.15
Red soil	Silty soil	5.07	91.21	4.26

Table 2: Initial concentrations of four components of DDT and DDTs in soil (mg/kg)

Soil	p,p'-DDE	o,p'-DDT	p,p'-DDD	p,p'-DDT	DDTs
Lateritic red soil	0.351	0.775	1.403	2.334	4.863
Latosol soil	0.349	0.781	1.431	2.339	4.9
Red soil	0.351	0.781	1.432	2.333	4.897

Table 3: Kinetics parameters of degradation of DDTs in soil with different dosages laccase

Degradation experiment	Pseudo-first-order kinetics equation $lnC_t = -kt + lnC_0$	Half-life (days)	Degradation rate constant (days^{-1})	Regression coefficient R
Control	$lnC_t = -0.0083\,t + 1.536$	83.49	0.0083	0.9071
3 U/g soil	$lnC_t = -0.017\,t + 1.469$	41.75	0.017	0.8787
6 U/g soil	$lnC_t = -0.028\,t + 1.446$	24.75	0.028	0.9043
12 U/g soil	$lnC_t = -0.026\,t + 1.393$	26.25	0.026	0.8625

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Table 4: Kinetics parameters of degradation of DDTs in different types of soil

Degradation experiment	Pseudo-first-order kinetics equation $lnC_t = -kt + lnC_0$	Half-life (days)	Degradation rate constant (days^{-1})	Regression coefficient R
Lateritic red soil control	$lnC_t = -0.0030\ t + 1.582$	231	0.003	0.9986
Latosol soil control	$lnC_t = -0.0031\ t + 1.592$	223.55	0.0031	0.9967
Red soil control	$lnC_t = -0.0033\ t + 1.589$	210	0.0033	0.9948
Lateritic red soil treatment	$lnC_t = -0.025\ t + 1.451$	27.68	0.025	0.9047
Latosol soil treatment	$lnC_t = -0.026\ t + 1.454$	26.61	0.026	0.9125
Red soil treatment	$lnC_t = -0.028\ t + 1.433$	24.71	0.028	0.9024

Table 5: Kinetics parameters of degradation of DDTs in soils with different pH levels

Degradation experiment	Pseudo-first-order kinetics equation $lnC_t = -kt + lnC_0$	Half-life (days)	Degradation rate constant (days^{-1})	Regression coefficient R
2.5 control	$lnC_t = -0.0028\ t + 1.583$	247.5	0.0028	0.999
3.5 control	$lnC_t = -0.0027\ t + 1.583$	256.67	0.0027	0.9978
4.5 control	$lnC_t = -0.0026\ t + 1.583$	266.54	0.0026	0.9967
5.5 control	$lnC_t = -0.0025\ t + 1.584$	277.2	0.0025	0.9965
6.5 control	$lnC_t = -0.0025\ t + 1.584$	277.2	0.0025	0.9965
2.5 treatment	$lnC_t = -0.024\ t + 1.461$	28.83	0.024	0.9106
3.5 treatment	$lnC_t = -0.027\ t + 1.463$	25.63	0.027	0.9265
4.5 treatment	$lnC_t = -0.025\ t + 1.468$	27.68	0.025	0.9194
5.5 treatment	$lnC_t = -0.021\ t + 1.476$	32.95	0.021	0.9092
6.5 treatment	$lnC_t = -0.019\ t + 1.497$	36.42	0.019	0.9241

supernatant was collected and to be used as laccase liquid for later processes. The laccase liquid was salt precipitated and further purified by Sephadex G-75 column to increase its activity (Wang and Zhou, 2002). Laccase activity was determined by monitoring the oxidation of 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonate (ABTS) at 420 nm. 1 U of activity was defined as the amount of enzyme able to oxidize 1 μmol ABTS per min.

Preparation of DDT-contaminated soils

0.0547 g commercial DDT was dissolved in 100 mL acetone with a volumetric flask. The DDT solution of 0.547 mg/mL was kept in a refrigerator under 4 °C for use. 5 mL of 0.547 mg/mL DDT solution was added into 50 g soil passed through a 2-mm sieve, stirred well and air-dried. Then the dried sample was put into 450 g soil passed through a 2-mm sieve, mixed well. That is to be experimented DDT-contaminated soil sample. The soil sample was kept in room temperature for four weeks, after that remediation experiments were carried out to assess the remedial potential of laccase. In all of the experiments, the soil samples water content were kept at approximately 15 %. The initial contents of DDT components in the soil samples are listed in Table 2.

Degradation experiments

Degradation of DDT in soil by different dosages laccase: Lateritic red soil was used as the experimental soil. The level of DDT pollution of Lateritic red soil was presented in Table 2. A total of four parallel experiments were conducted: one control experiment and three test experiments. In the control experiment, no laccase (0 U/g soil) was added; in the 3 test experiments, for each gram contaminated soil 3 U, 6 U and twelve U laccase, respectively, were added. Degradation of DDT in different types of soil: Three types of soils, lateritic red soil, latosol soil and red soil, were used in this experiment. The DDT pollution level for each type of soil was shown in Table 2. For each type of soil, one control experiment without laccase (0 U/g soil) and one degradation experiment with laccase (6 U/g soil) were conducted. Degradation of DDT in soils with different pH levels: Lateritic red soil was used in this experiment. The dosage of laccase was 6 U/g soil. The initial concentration of DDT pollutant was shown in Table 2. pH of soils were 2.5, 3.5, 4.5, 5.5 and 6.5, respectively. For each soil sample, one control experiment without laccase (0 U/g soil) and one degradation experiment with laccase (6 U/g soil) were conducted. Each experiment used 15 g soils. All experiments were conducted in beaker and replicated for 3 times. Soil samples were analyzed at 5 d, 10 d, 15 d and 25 d.

Sample pre-treatment

The preparation of sample and the measurement of DDT concentration were conducted according to the standards described in national standards of P. R. China (GB/T14550-93) set for soil quality-determination of BHC and DDT-gas chromatography. Briefly, 10 g soils were placed in a Soxhlet extraction apparatus and immersed with 100 mL petroleum ether-methanol (1:1) solution for 10 h, then extracted for 6 h. The extract liquid was transferred to a separating funnel, added with 10 % anhydrous sodium sulfate. After 1 min of shaking, the mixture stratified into two liquid phases on standing. The upper layer petroleum ether was obtained and added with concentrated sulfuric acid with a volume equivalent to 10 % of the petroleum ether solution. The mixture of petroleum ether and sulfuric acid was allocated for 3-4 times until both layers became colorless. 10 % anhydrous sodium sulfate solution with a volume equivalent to half of the petroleum ether layer was added to the remaining petroleum ether solution for washing up until the petroleum ether solution became neutral. At the end, the petroleum ether solution was dehydrated through anhydrous sodium sulfate, concentrated and suspended in 10 mL volume for DDT measurement.

Gas chromatographic analysis of DDT

The DDT standard solution contained components of p,p'-DDE0o,p'-DDT0p,p'-DDD and p,p'-DDT, their concentration was 20, 20, 60.08, 60 mg/L, respectively. Chromatographic grade acetone was used to dilute the DDT solution to make four concentration levels for each component. Gas chromatographic instrument deployed was HP5890II. Column HP-5, 30 m×0.320 mm (id) × 0.25 um. High purity nitrogen (99.999 %) was used as the carrier gas. Gasification temperature was 220 °C, column temperature was 195 °C, detector temperature (ECD) was 245 °C. The speed of gas flow was 2 mL/min. Splitless injections (2 μL) were made. 2, 4, 5, 6-Tetrachloro-m-xylene was used as the recovery indicator to control the recovery rate during the whole operation procedure. The recovery standard was used to control the sample recovery rate. The recovery rate of the indicator ranged between 78 % and 88.6 %. The recovery rate of DDT ranged between 88.4 % and 98.7 %. The detection limit for p,p'-DDE, o,p'-DDT, p,p'-DDD and p,p'-DDT was 0.902, 3.869, 2.847l and 0.756 μg/L, respectively.

RESULTS AND DISCUSSION

Degradation of DDT in soil by different dosages laccase

The residues of DDTs in soils with different dosages laccase decreased significantly with the increase of the incubation time while the soils without laccase showed less disappearance of DDTs (Fig. 1). The residues of DDTs in the soils with different dosages laccase decrease by 21-32 %, 29-45 %, 35-51 % and 36-51% after 5, 10, 15 and 25 days of incubation, respectively. Wu *et al.* (2008) reported the removal of polycyclic aromatic hydrocarbons (PAHs) in soil by fungal laccase, which increased with the increase of laccase dosage.

The residues of most of the DDT components (except for p,p'-DDE) in soils with different dosages laccase decreased significantly with the increase of the treatment time. The residues of p,p'-DDE increased in the first five days and then decreased slowly after five days. The residues of p,p'-DDE, o,p'-DDT, p,p'-DDD and p,p'-DDT in soils with different dosages laccase reduced by -1.79-0.96 %, 34.83-42.90 %, 35.53-49.19 % and 51.09-62.60 % after incubation for twenty-five days, respectively. Although the amount of laccase in the experiment with laccase of 6 U/g soil was half of that in the experiment with laccase of 12 U/g soil, similar percentages of DDTs degradation were observed after incubation for 25 days, which were 50.62 % and 50.87 %, respectively.

Therefore, the 6 U/g soil was the best dosage for remediation of DDT-contaminated soil in this experiment.

Degradation of DDT in different types of soil

The residues of DDTs in different types of soil incubated with laccase decreased with the increase of treatment time, while the soils without laccase showed less disappearance of DDTs (Fig. 2). The residues of DDTs in three different types of soils decreased by 25-29 %, 39-43 %, 44-47 % and 47-52 % after 5, 10, 15 and 25 days of incubation with laccase, respectively.

The residues of most of the DDT components (except for p,p'-DDE) in different types of soil incubated with laccase decreased with the increasing the incubation period time. The dynamic changes of residues of p,p'-DDE in different types of soil were similar to that in soils with different dosages laccase. After twenty five days of incubation with laccase, the residues of p,p'-DDE,

o,p'-DDT, p,p'-DDD and p,p'-DDT in different types of soil declined by 2.56-3.39 %, 38.49-52.20 %, 45.25-47.30 % and 50.54-54.28 %, respectively. The results of some studies suggest that Zero-valent iron (Fe^0) can enhance pesticide transformation in soil. For example, Shea *et al.* (2004) presented Zero-valent iron (Fe^0) can promote the remediation of pesticide-contaminated soil and Yao *et al.* (2006) reported that Zero-valant iron can effectively promote reductive dechlorination of p,p'-DDT and its metabolites. However, the results from other studies showed that Fe^{2+} and Fe^{3+} may strongly inhibit the activity of laccase (Kang *et al.*, 2002; Niladevi *et al.*, 2008). In this study, red soil, latosol soil and lateritic red soil were three typical soils in Guangdong Province China, which contain a larger amount of free iron oxides (Li *et al*, 2006). For three different types of soils, the iron content in the red soil was the highest and the lowest in the latosol soil (Table 1). After twenty five days of incubation with laccase, the residues of o,p'-DDT, p,p'-DDT and DDTs were lowest in red soil, the residue of p,p'-DDD was the lowest in lateritic red soil and the residue of p,p'-DDE was not significantly different in three different types of soils. Therefore, there was no evidence to show that there is a correlation between the degradation of DDT by laccase and the iron content of soil.

Degradation of DDT in soils with different pH levels

The residues of DDTs in soils with different pH levels declined rapidly in the first 15 days of incubation with laccase and then slowed down after 15 days of incubation (Fig. 3). This result indicated DDT was degraded mainly within the first 15 days of incubation.
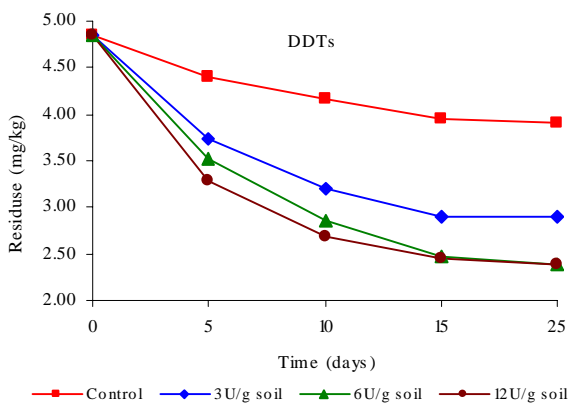
The residues of DDTs in soils with different pH levels decreased by 18-24 %, 29-39 %, 36-39 % and 39-50 % after 5, 10, 15 and 25 days of incubation with laccase, respectively. Gianfreda *et al.* (1999) reported that pH can affect the activity of laccase. The activity of laccase from polyporus sp., pleurotus sp. and lentinus edodes was the highest in a pH range of 3.2-4.5 (Huang and Zhang, 2006). The activity of laccase from polyporus sp. was the highest at the pH of 4.2 (Wang and Zhou, 2002). The pH optima of fungal laccases were in the acidic pH range (Baldrian, 2006). For example, different fungal enzymes for hydroquinone and catechol exhibited pH optima in the range of 3.6-4.0 and 3.5-6.2, respectively (Lalitha Kumari and Sirsi, 1972; Shleev *et al.*, 2004). In this study, the residues of most of the DDT components (except for p,p'-DDE) and DDTs in soil were lower at a pH range of 2.5-4.5 and the lowest at pH 3.5 during the period of twenty five days incubation. The residues of most of the DDT components (except for p,p'-DDE) in soils with different pH levels decreased with increasing the treatment time. The residues of p,p'-DDE in soils treated with laccase increased during the first 5 days and then decrease slowly after 5 days, which could be similar to the results of p,p'-DDE degradation in different types of soil. Te residues of p,p'-DDE, o,p'-DDT, p,p'-DDD and p,p'-DDT in soils with different pH levels decreased by 4.79-5.73 %, 30.37-42.89 %, 39.53-50.88 % and 46.09-57.87 % at the end of the incubation period, respectively.

Kinetic studies

Dynamic analysis of residues of DDTs showed that the degradation of DDTs fits pseudo-first-order kinetics. Kinetics equation was $lnC_t = -kt + lnC_0$, where t is the

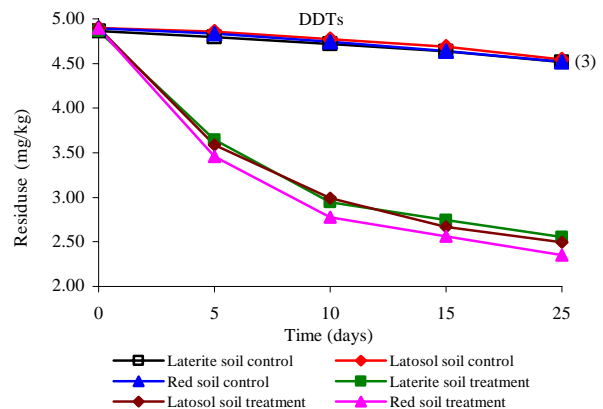Fig. 1: Dynamic changes of residues of 4 components of DDT and DDTs in soil with different dosages laccase

Fig. 2: Dynamic changes of residues of 4 components of DDT and DDTs in different types of soil
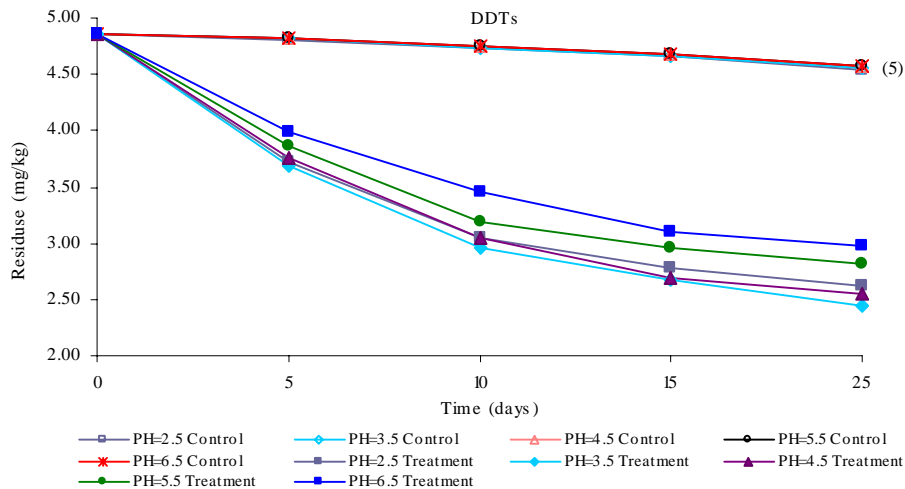
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Fig. 3: Dynamic changes of residues of each component of DDT and DDTs in soils with different pH levels

incubation time, C_0 is the initial concentration of DDTs, C_t is the residues of DDTs at t days, k is the degradation rate constant. The half-life of DDTs is 0.693/k. Some reports on the degradation of insecticide and pesticide showed the degradation of insecticide and pesticide in soil followed first-order kinetics. For instance, the degradation of novaluron in tropical soil followed first-order reaction kinetics (Das *et al.*, 2008). Chiu *et al.* (2004) has showed degradation of DDT under anaerobic condition was fitted to a first-order kinetic equation. The results from this study indicated that the degradation of DDT in soil followed pseudo-first-order kinetics, which were in accordance with many investigations reported. Zhao *et al.* (2002) found that the anaerobic dechlorination of p,p'-DDT in the sediment by adding short chain organic carbon sources was pseudo-first-order kinetics. Boussahel *et al.* (2007) used Fenton oxidation with Zero-valent iron method to degrade DDT found the degradation of DDT was also pseudo-first-order kinetics. The residues of DDTs in soils treated with different dosages laccase decreased rapidly in the first 15 days and then slowed down after 15 days (Fig. 1). The results indicated that degradation of DDTs by laccase mainly takes place during the period of first 15 days of incubation. The increase order of half-life of DDTs was 6 U/g soil (24.75 days) < 12 U/g soil (26.25 days) < 3 U/g soil (41.75 days) (Table 3). For three different types of soils, the residues of DDTs decreased rapidly during first 15 days, steady state was reached after 15 days of incubation with laccase (Fig. 2). The increase order of half-life of DDTs was red soil (24.71 days) < latosol soil (26.61 days) <laterite soil (27.68 days) (Table 4). The

residues of DDTs in soils with different pH levels decreased rapidly during the first 15 days and followed reached a steady state during the periods of 16-25 days incubation with laccase (Fig. 3). The increase order of half-life of DDTs was pH 3.5 (25.63 days) < pH 4.5 (27.68 days) < pH 2.5 (28.83 days) < pH 5.5 (32.95 days) < pH 6.6 (36.42 days) (Table 5). The finding from this study was consistent with the results from other studies, which reported that the kinetics of pesticides degradation in soils was biphasic with a fast degradation at initial stage followed a slow process (Rigas *et al.*, 2007; Osman *et al.*, 2008). One possible explanation for the kinetics of DDT degradation was that the activity of laccase quickly decreased during a 14 days of incubation with soil (Ahn *et al.*, 2002; Wu *et al.*, 2008), while the degradation capacity of laccase is high correlated with the activity of laccase, suggesting that the degradation rate of DDT reduce with the decreasing of laccase activity in soil. In this study, the residues of p,p'-DDE increased during the first 5 days and thereafter decreased slowly. Chu *et al.* (2006) has reported enzymes that produced by plants (Phragmites australis) can degrade DDT into DDD with some DDE being forming. You *et al.* (1996) found that DDE degradation was lower than DDT and DDD, which was due to DDE strongly bound to the soil particles. In this study, the changes of residues of p,p'-DDE were divided into two phases: First, part of p,p'-DDE, was a transformation product of the laccase degradation of other components of DDT. Also, the amount of p,p'-DDE transformed by the other components of DDT was higher than the amount of p,p'-DDE degraded by laccase in the first 5 days.

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Second, the amount of p,p'-DDE degraded by laccase was higher than the amount of p,p'-DDE transformed by the other components of DDT after five days of incubation.

This study showed that the residue of p,p'-DDD in soil incubated with laccase decrease with the increasing of incubation time, suggesting that laccase can effectively degrade p,p'-DDD.

CONCLUSION

Laccase from white-rot fungi can effectively degrade DDT and the degradation of DDTs fits pseudo-first-order kinetics. The residues of DDTs in soils with different dosages laccase decreased by about 21-32%, 29-45%, 35-51% and 36-51% after 5, 10, 15 and 25 days of incubation, respectively. The increase order of half-life of DDTs was 6 U/g soil (24.75 days) < 12 U/g soil (26.25 days) < 3 U/g soil (41.75 days). The residues of DDTs in three different types of soils decreased by 25-29 %, 39-43 %, 44-47 % and 47-52 % after 5, 10, 15 and 25 days of incubation with laccase, respectively. The increase order of half-life of DDTs was red soil (24.71 days) > latosol soil (26.61 days) > lateritic red soil (27.68 days). The residues of DDTs in soils with different pH levels decreased by 18-24 %, 29-39 %, 36-39 % and 39-50 % after 5, 10,15 and 25 days of incubation with laccase, respectively. The residue of DDTs in soil treated with laccase decreased by 50 % at an optimum pH 3.5. The increase order of half-life of DDTs was pH 3.5 (25.63 days) < pH4.5 (27.68 days) < pH2.5 (28.83 days) < pH5.5 (32.95 days) < pH 6.5 (36.42 days).

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