DEGRADATION OF DDT BY A NEWLY ISOLATED *TRAMETES VERSICOLOR* U97

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Introduction:

Dichlorodiphenyltrichloroethane (DDT) was one of the most widely used organochlorine pesticides. While most of the developed countries have already banned or restricted the production and usage this compound, some developing countries still use DDT for agricultural and the health purpose[1]. In the environment, DDT is very highly persistent, with a reported half life of between 2-15 years and is immobile in most soil[2]. In Indonesia, DDT was widely used as insecticides in 1950s-1982s. It has also been used to control insects that carried malaria during 1970s-1990s with annual capacity of 7000 tons/year[3]. Based on state of environment report of 2009[4], DDT and its derivatives were the predominant contaminants in water, soil and river sediment with concentrations from 0.005 to 0.4 ppb, from 23 to 170 ppb and 24300 ppb, respectively. Considering its potential negative effects, effective methods of remediation must be developed. Enzymatic remediation using microorganisms i.e. yeast, fungi or bacteria is a rapid, highly efficient relatively low cost and low-technology method for removing pesticide from the environment compare with physical and chemical methods[5]. In this study, the tripartite relationships among the growth of fungus screened from nature, its glucose consumption and degradation of DDT and the effects of enzyme activities to produce metabolic products will be discussed.

Materials and methods:

Degradation test of DDT in liquid medium: *Trametes versicolor* U97 inocula obtained from nature was maintained in malt extract agar medium at low temperature 4°C. Liquid culture experiments were conducted in 100 ml erlenmeyer flasks containing 20 ml of malt extract liquid medium (pH 4.8). The three 5 mm of actively growing fungus in agar plate were inoculated into the erlenmeyer flask. Inoculated flasks were pre-incubated statically at 25°C for several days. After pre-incubation, each inoculated flasks was supplemented with 300000 ppb of DDT in *N*,*N*-dimethylformamyde. Control treatment has been performed on erlenmeyer flasks only containing medium and pollutant without inoculated fungus. After additional incubation for 15 and 30 d, internal standard was added to the cultures. The culture was homogenized with 50 ml acetone at 10000 rpm for 10 min. The resulting supernatant was evaporated prior to extraction using ethyl acetate three times. The sample was dried over anhydrous sodium sulfate then evaporated to obtain concentrate extraction. It was purified on a column chromatography (5 g of C200 silica gel and anhydrous Na_2SO_4) eluted with hexane : dichloromethane (3:1) and was concentrated on rotary evaporator and under a stream of nitrogen.

Degradation test of DDT in soil: The soil used for experiment was collected from land farming area in Faculty of Agriculture, Ehime University. It was collected from surface layer (0-20 cm) with pH 6.16 (in distilled water ratio 1:5). The soil was air-dried, passed through a 3 mm sieve and homogenized. Prior to its use, 30 g soil was autoclaved 121°C for 180 min to eliminate exist of microorganisms and spiked with 1000 ppb DDT in *N*,*N*-dimethylformamyde. The water soil content was adjusted 30% with addition distilled water. The negative control was also used in this experiment. The container culture was incubated for 15 and 30 d at 25°C in the dark condition. In the fixed time, internal standard was added to the soil. The soil was extracted using a soxhlet apparatus for 16 h with dichloromethane and was concentrated on rotary evaporator. It was purified using column chromatography as same as with described above.

GC/MS analysis: The concentrate was analyzed by gas chromatography coupled with mass spectrometer (GC/MS Shimadzu QP-2010), equipped with a TC-1 column (30 m, id: 0.25 mm). The carrier gas was helium at constant flow rate 1.5 ml/min with column pressure 100 KPa and interface temperature 250°C. The temperature program was started at 60°C hold 2 min, 15°C/min to 150°C, 25°C/min to 280°C which maintained for 10 min to allow eluting peak to exit the column. The injection volume was 1 µl and the injector was maintained at 250°C. Identification of metabolic products was also performed using this method.

Growth of T. versicolor U97 test: For determination of cell concentration, the cells were separated from 20 ml sample using filter paper, which were then dried at 55°C for 28 h. The weight of cells on the filter was measured to determine the concentration of *T. versicolor* U97 cells (drying method). The concentration of glucose was determined according to a slightly modified method of Ishii et al[6]. A 200 µl volume of phenol solution (5% (w/w)) was added to 200 µl of sample. Concentrated sulfuric acid (1 ml) was added to the mixture and the mixture was shaken vigorously. The concentration of glucose was determined by absorption (492 nm) after resting the mixture at 30°C for 30 min.

Enzyme activity of T. versicolor U97: Each sample treated in the same procedure with previous was collected, blended at 10000 rpm for 10 min and filtered through a 0.2 µm membrane filter. Manganese peroxidase (MnP) activity was measured by monitoring the oxidation of 20 mM 2,6-dimethoxyphenol (2,6-DMP) at 470 nm in 50 mM malonate buffer (pH 4.5) containing 20 mM $MnSO_4$ in the presence of 2 mM H_2O_2[7]. Lignin peroxidase (LiP) activity was measured by monitoring the formation of 2 mM H_2O_2 and LiP buffer at 310 nm[8]. Laccase activity was measured by monitoring the oxidation of syringaldazine to its quinone form at 525 nm in 0.1 M sodium acid buffer[9]. 1,2-dioxygenase and 2,3-dioxygenase was measured by monitoring the oxidation of 0.01 M catechol in 0.1 M phosporic acid at 260 nm and 375 nm, respectively[10]. All the enzyme activites were measured at 25°C by Spectrophotometer Shimadzu UV-1600 and expressed in U/l, defined as the amount of enzyme required to oxidize 1 µmol of substrate in 1 min.

Results and discussion:
The ability of *T. versicolor* U97 to degrade DDT was determined in a defined medium as shown in Fig 1. More than a half of degradation was obtained for 15 d of incubation in liquid medium. After 30 d of incubation, the percentage of degradation was 71%. In the soil, DDT was degraded by 49% during the 30 d incubations. The degradation activity of DDT was observed in white rot fungi, *Phlebia lindteri* as 70% during the 21 d incubations[11].

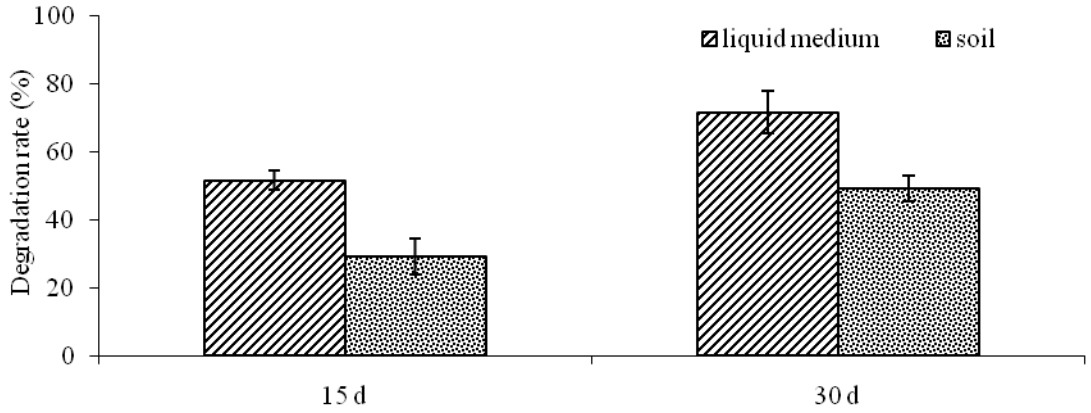

Figure 1. Degradation rate of DDT by *T. versicolor* U97 in several media

Therefore, the lower remediation values obtained in soil than liquid medium can be explained by sorption phenomena. Pollutant is retained in the soil pores thereby impeding transfer to the liquid phase[12,13]. In environment, due to its extremely low solubility in water, DDT will be retained to a greater degree by soils and soil fraction with higher proportions of soil organic matter. It may accumulate in the top soil layer in situations where heavy applications were made annually.

Fig 2 shows the cell concentration and the growth of kinetics of *T. versicolor* U97 when it was cultivated with absence and presence of DDT.

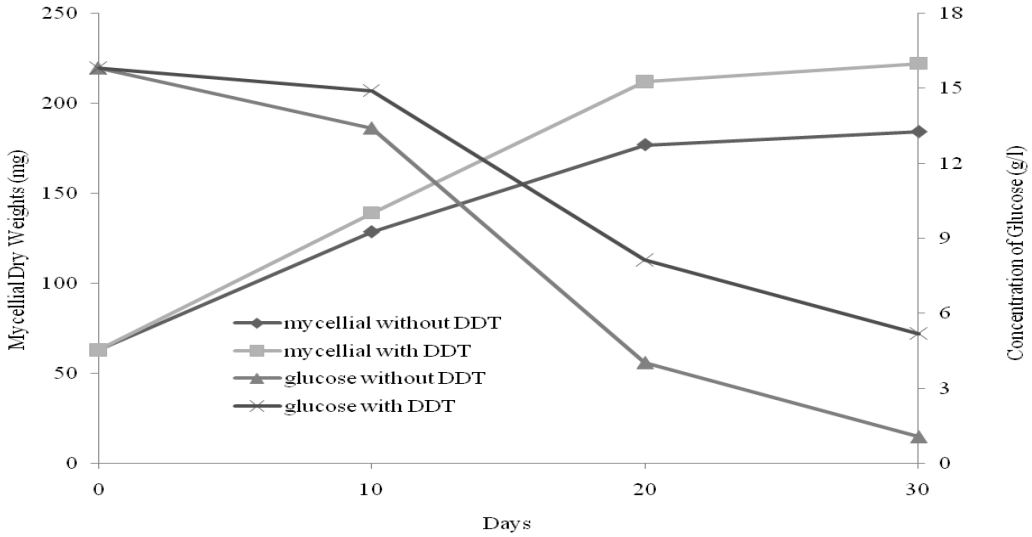

Figure 2. Growth curve of *T. versicolor* U97 during the incubation with or without addition of DDT

Related with degradation, the growth of *T. versicolor* U97 coincided with a decrease in the concentration of glucose. When it was cultivated in the presence of both glucose and DDT, the concentration of DDT decreased at the same rate as the concentration of glucose. This observation indicates that the decrease in DDT concentration may coincided with the growth of *T. versicolor* U97, as it grew using glucose and DDT. This figure also shows the growth kinetics of *T. versicolor* U97 in presence and absence of DDT. The metabolism of DDT by *T. versicolor* U97 was indicated by a visible increase in mycelial mass with the time. In the first of 3 d, the growth was same between the presence and the absence of DDT. After the adaptation to DDT and initial transformation of DDT, the culture grow rapidly exhibiting growth rate. In later stage the amount of biomass produced in the medium containing DDT was much higher as compared to the growth in the absence of DDT. This could be due to avaibility of additional carbon upon degradation of DDT, in the medium. Furthermore, to know exactly the kinetic analysis of the biodegradation of DDT using *T. versicolor* U97, the monod equation was used as described later. *T. versicolor* U97 with expressive DDT degradation capacity was tested for enzyme activities as shown in Fig 3.

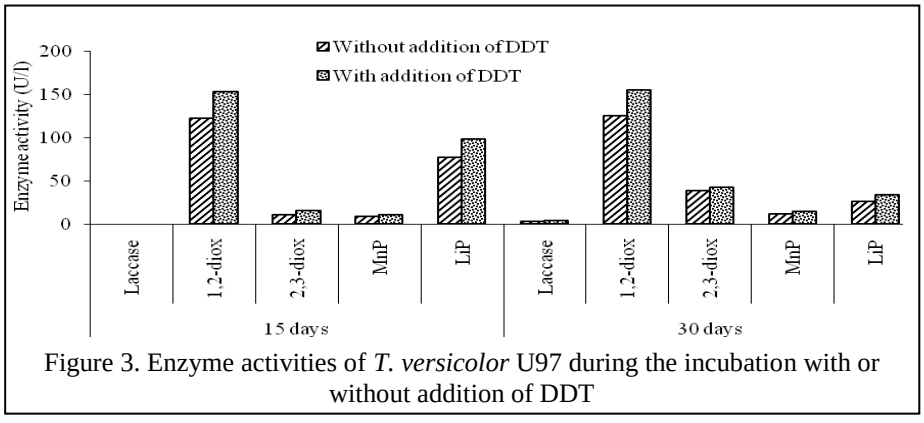

Figure 3. Enzyme activities of *T. versicolor* U97 during the incubation with or without addition of DDT

Fig 3 shows that *T. versicolor* U97 secreted more all extracellular enzymes except laccase when under addition of DDT in cultures on 15 and 30 d. The high enzyme activity of *T. versicolor* U97 in the liquid medium during addition of DDT was 1,2-dioxygenase (115.7 U/l) at 30 d and LiP (98.7 U/l) at 15 d, respectively. This enzyme activity may play important role for DDT degradation. Catalysis of the decomposition of the recalcitrant aromatic compounds in white rot fungi is affected by extracellular enzyme.

The Double Monod equation was applied to the growth rate of *T. versicolor* U97 and the rate of glucose and DDT consumption. Since the growth rate of *T. versicolor* U97 was not affected by the presence of DDT in this study, the growth value equation can be shown in Eq. (1).

$$X_{n+1} = X_n + 0.98125\frac{S_n}{0.611711 + S_n}X_n \quad (1)$$

where X is the cell mass (g/l), n is the time (d), S is the concentration of glucose (g/l). The maximum specific growth rate (1/d) is 0.98125, and the half saturation coefficient is 0.611711.

To obtain metabolite products of DDT by *T. versicolor* U97, culture extracts from liquid medium were analyzed with GC/MS by comparing their retention times and mass spectra with those of standard compounds. Several biodegradation products were detected. Mineralization of DDT began to occur in 7 d and continued to occur throughout the incubation period (30 d). The culture of *T. versicolor* U97 resulted *p,p'*-DDE as a major metabolite and trace amounts of *p,p'*-DDD, *p,p'*-DDMU, DDOH and 4-chlorobenzoic acid. This result suggests that DDT might have been reductively dechlorinated to *p,p'*-DDD, which was then degraded to DDOH by dechlorination and oxidation. This compound might have been formed to single-ring aromatic compounds via hydroxilation of the aromatic ring and subsequent *meta*-ring cleavage. Moreover, 4-chlorobenzoic acid has less toxic than DDT since the lethal dose 50 (LD50) ranges of 4-chlorobenzoic acid and DDT in rats are $1.17x10^6$ ppb[14] and $1.5x10^5$-$4x10^5$ ppb[15], respectively.

In general, there was a significant decrease in the rate of DDT degradation by newly isolated *T. versicolor* U97 in liquid medium and soil. *T. versicolor* U97 degraded DDT during its logarithmic growth phase, using glucose as a carbon source for growth, and that the growth of *T. versicolor* U97 was not affected by DDT. 1,2 dioxygenase and LiP produced by *T. versicolor* U97 play an important role to metabolize DDT into *p,p'*-DDE as a major metabolite and trace amounts of *p,p'*-DDD, *p,p'*-DDMU and DDOH and 4-chlorobenzoic acid.

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