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Determination of co-metabolism for 1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane (DDT) degradation with enzymes from *Trametes versicolor* U97

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***Trametes versicolor* U97 isolated from nature degraded 73% of the 1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane (DDT) in a malt extract liquid medium after a 40-d incubation period. This paper presents a kinetic study of microbial growth using the Monod equation. *T. versicolor* U97 degraded DDT during an exponential growth phase, using glucose as a carbon source for growth. The growth of *T. versicolor* U97 was not affected by DDT. DDT was degraded by *T. versicolor* U97 only when the secondary metabolism coincided with the production of several enzymes. Furthermore, modeling of several inhibitors using the partial least squares function in Minitab 15, revealed lignin peroxidase (98.7 U/l) plays a role in the degradation of DDT. *T. versicolor* U97 produced several metabolites included a single-ring aromatic compound, 4-chlorobenzoic acid.**

[**Key words:** Biodegradation; 1,1,1-Trichloro-2,2-bis(4-chlorophenyl) ethane (DDT); *Trametes versicolor*; Monod equation; Lignin peroxidase; Inhibitor]

Intensification of agriculture has resulted in the increased release of a wide range of aromatic compounds into the environment. While most developed countries have banned or restricted the production and usage of 1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane (DDT), some developing countries still use DDT to control agricultural pests and insects that carry diseases such as malaria (1). DDT and its major products 1,1-dichloro-2,2-bis(4-chlorophenyl) ethane (DDD) and 1,1-dichloro-2,2-bis(4-chlorophenyl) ethylene (DDE) have been found in water, soil, river sediment, fish and breast milk (2–4). The presence of DDT in the environment is of great concern due to its persistent, long-range transportable nature and toxic biological effects. Some studies have reported the presence of stable residues of DDT in air, water, soil, sediment, fish and birds more than 10 years after it was banned (5). Long-term exposure to small amounts of DDT (20–50 mg per kg of body weight every day) in animal studies can affect the liver, reproduction and the adrenal gland (6). Considering DDT's potential negative effects, it is necessary to address the environmental persistence of this pesticide and to develop effective methods for remediation (7).

Bioremediation is technology utilizing the metabolic potential of organisms, such as enzymes produced by microorganisms, i.e., yeast, fungi or bacteria, to destroy or render harmless various contaminants. It has been shown to be a viable, relatively low cost, low-technology technique with widespread use (8). White-rot fungi require a primary growth substrate such as glucose to mineralize aromatic pollutants (9). Metabolic activity and cell growth depend on environmental factors including pollutants and specific mechanisms of enzymatic regulation, which are very important for enzymatic production (10). Extracellular enzymes produced from white-rot fungi have very low substrate specificity and so are able to mineralize a wide range of highly recalcitrant organopollutants structurally similar to lignin (11). The tripartite relationships among the growth of microorganisms, glucose as an energy source and degradation of DDT must be clarified. Several fundamental factors should be elucidated including the amount of glucose required as a carbon source for the growth of fungi to degrade pollutants, the phase of growth in which the degradation process occurs, and the effect of pollutants on the growth of fungi. There are no previous reports about kinetic models of microbial growth and the degradation of DDT by fungi secreting enzymes and there have not been many reports on the degradation of DDT by *Trametes versicolor* compared with other fungi (12–17). Furthermore, the role of enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP), laccase, P-450 monooxygenase and dioxygenase in *T. versicolor* in the degradation of DDT and other pollutants is still unclear (18). To further confirm which enzymes are involved in the degradation of pollutants, enzyme inhibitors are used to investigate the effect on degradation. Decreased degradation of pollutants caused by inhibitors of ligninolytic enzyme, dioxygenase and P-450 monooxygenase such as EDTA, $CuSO_4$, NaN_3, $AgNO_3$ and piperonyl butoxide was observed (16,19–22).

This study aims to clarify kinetic models of microbial growth and the degradation of DDT by *T. versicolor* U97. This study also investigates the enzymatic activities that play a role in the degradation of DDT related to metabolic products obtained by

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T. versicolor U97 using the modeling of inhibitors. We believe this study will improve understanding of the co-metabolism between the growth and enzymatic activities of *T. versicolor* U97.

MATERIALS AND METHODS

Culture condition *T. versicolor* U97 taken from nature was maintained on malt extract agar plates (MEA) at 25°C. Three 5-mm disks of an actively growing fungus in agar were transferred to 20 ml of sterilized malt extract liquid medium (malt extract 20 g/l, glucose 15 g/l, agar 20 g/l, polypeptone 1 g/l). Inoculated flasks were pre-incubated at 25°C for 7 d under static condition to obtain similar radial growth and to minimize growth variation.

Degradation test with DDT After pre-incubation for 7 d, each inoculated flask was supplemented with 0.1 mM of DDT diluted in *N,N*-dimethylformamide (DMF), Tween 80 and water. The control treatment was performed in an Erlenmeyer flask containing only 20 ml of medium and 0.1 mM of DDT without inoculated fungus. After additional incubation for 10, 20, 30 and 40 d, several analyses were conducted.

Mycelial mass of *T. versicolor* U97 The mycelial were separated from the medium using filter paper, then dried at 55°C for 28 h. The weight of mycelial on the filter was measured to determine the mycelial mass of *T. versicolor* U97.

Concentration of glucose The concentration of glucose was determined according to a slightly modified method of Ishii et al. (23). A 200-µl volume of phenol solution (5% (w/w)) was added to 200 µl of sample. Concentrated sulphuric acid (1 ml) was added and the mixture was shaken vigorously. The concentration of glucose was determined by absorption (492 nm) using a spectrophotometer (Shimadzu UV-1600) after incubation at 30°C for 30 min.

Monod analysis The Monod equation was used to express the relationship between the specific growth rate of the cells and the substrate concentration, given as:

$$X_{n+1} = X_n\, S_n/(K_s + S_n) \quad (1)$$

where, X_{n+1} is specific growth rate (1/d), X_n is maximum specific growth (1/d), S_n is substrate concentration (g/l), and K_s is saturation constant for substrate (g/l).

Residual DDT analysis The initial extraction was conducted by adding 50 ml of acetone to the culture and homogenizing at 10,000 rpm for 10 min. Samples were evaporated prior to extraction using ethyl acetate by funnel separation. Each extract was evaporated to obtain a concentrate. It was purified by column chromatography (5 g of C200 silica gel and anhydrous Na_2SO_4) eluted with hexane:dichloromethane (3:1), then concentrated on a rotary evaporator and under a stream of nitrogen. The concentrate dissolved in toluene was analyzed by gas chromatography coupled with mass spectrometry (GC-MS Shimadzu QP-2010), equipped with a TC-1 column (30 m, id: 0.25 mm). The carrier gas was helium delivered at a constant flow rate of 1.5 ml/min with column pressure of 100 kPa and interface temperature of 250°C. The temperature program was started at 60°C for 2 min, raised 15°C/min to 150°C, then 25°C/min to 280°C, and maintained at 280°C for 10 min to allow the eluting peak to exit the column. The injection volume was 1 µl and the injector was maintained at 250°C. The identification of metabolic products was performed in comparison with standard samples.

Analysis on enzymatic activity Enzymatic activity was measured at 15 and 30 d. After the culture period, the extracellular fluid was collected, blended at 10,000 rpm for 10 min and filtered through a 0.2-µm membrane filter. The medium was measured for enzymatic activities by absorption. 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 (24). Lignin peroxidase (LiP) activity was measured by monitoring the formation of 2 mM H_2O_2 and LiP buffer at 310 nm (25). Laccase activity was measured by monitoring the oxidation of syringaldazine to its quinone form at 525 nm in 0.1 M sodium acid buffer (26). 1,2-Dioxygenase and 2,3-dioxygenase were measured by monitoring the oxidation of 0.01 M catechol in 0.1 M phosphoric acid at 260 nm and 375 nm, respectively (27). The reaction was performed at 20°C and after 1 min the numerical value was measured. All activities were expressed in U/l, defined as the amount of enzyme required to oxidize 1 µmol of substrate in 1 min.

Inhibition of enzymatic activity After pre-incubation of the fungus for 7 d, EDTA, $CuSO_4$, $AgNO_3$, NaN_3 and piperonyl butoxide were added at 0.1 mM, 1 mM and 10 mM to the cultures of *T. versicolor* U97. After 2 h, 0.1 mM DDT was added and incubated again for 15 and 30 d. The enzymatic activity and degradation of DDT were then measured as described above. To determine the regression equation for the degradation of DDT, the partial least squares method in Minitab 15 was used.

$$X = TP^T + E \quad (2)$$

$$Y = TQ^T + F \quad (3)$$

where X is an $n \times m$ matrix of predictors (enzymatic activity), Y is an $n \times p$ matrix of responses (degradation rate), T is an $n \times l$ matrix (the score, component or factor matrix), P and Q are $m \times l$ and $p \times l$ loading matrices, respectively, and matrices E and F are the error terms. All the experiments were conducted in triplicate samples. The results are reported as the average of triplicate determinations.

RESULTS AND DISCUSSION

Degradation of DDT by *T. versicolor* U97 The ability of *T. versicolor* U97 to degrade DDT was determined in malt extract medium as shown in Fig. 1. DDT was eliminated by approximately 65% during the 20 d incubation period. After 40 d of incubation, the rate of degradation was 73%. This result indicates that a nutrient-rich medium like the malt extract medium can be used for DDT degradation. A report has shown that the degradative activity of *T. versicolor* is more efficient in nutrient-rich cultures (28). Fig. 1 also shows the growth kinetics of *T. versicolor* U97 cultivated either without addition or with addition of DDT. The metabolism of DDT is indicated by a visible increase in mycelial mass with time and the increasing of glucose consumption. The time course of the degradation shows a slow start in the first two days, there was an exponential phase in the rate of degradation, which coincided with an increase in mycelial mass and

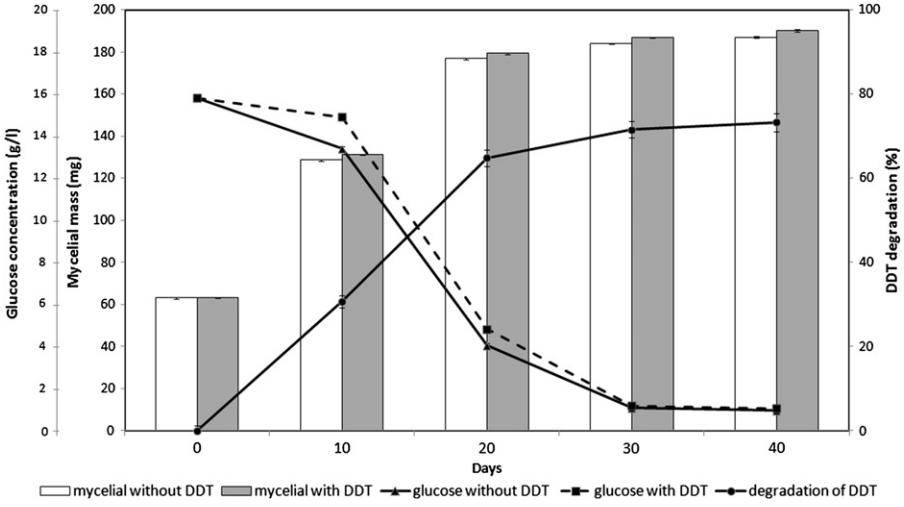

FIG. 1. Growth curve of *T. versicolor* U97 without and with DDT.