Degradation Kinetics of Pentachlorophenol by *Phanerochaete chrysosporium*

Jian-Er Lin and Henry Y. Wang*
Department of Chemical Engineering, The University of Michigan, Ann Arbor, Michigan 48109

Robert F. Hickey
Michigan Biotechnology Institute, Lansing, Michigan 48909

Accepted for publication October 31, 1989

The extracellular enzymes and cell mass from the pregrown *Phanerochaete chrysosporium* cultures were used for the degradation of PCP. The use of both extracellular enzymes and cell mass resulted in extensive mineralization of PCP, while the action of only the crude extracellular enzymes led to the formation of a degradation intermediate (TCHD). A kinetic model, which describes the relationship among PCP degradation, initial PCP concentration, dosage of extracellular enzymes, and cell mass concentration, was developed. Based on this model, various effects of initial PCP concentration, dosage of extracellular enzymes, and cell mass concentration were evaluated experimentally. It was found that when initial PCP concentration is lower than 12 μmol/L, the model of a parallel-series first-order reaction is sufficient to describe the degradation process. PCP disappearance and mineralization were enhanced by increasing either the extracellular enzyme concentration or the cell mass concentration. As high as 70% of PCP mineralization could be obtained by using a higher dosage of extracellular enzymes and cell mass. Various parameters of the kinetic model were determined and the model was verified experimentally. Simulation using this model provided the criteria needed to choose rational dosages of extracellular enzymes and cell mass for the degradation of PCP. Data reported allow some insight into the function of the extracellular enzymes and cell mass of *P. chrysosporium* in degradation processes of toxic pollutants and assist in the design and evaluation of practical bioremediation methods.

INTRODUCTION

Disposal of toxic organic chemicals is a major environmental concern because these compounds are recalcitrant and can pose a serious health problem. Much effort has recently been directed toward effecting biodegradation of toxic compounds in the environment. A white rot fungus, *Phanerochaete chrysosporium*, has been shown to have nonspecific ability to degrade many persistent toxic organic chemicals, such as polychlorinated biphenyls (PCBs), pentachlorophenol (PCP), DDT, several polycyclic aromatic hydrocarbons, etc.[1–9] Much attention has been directed toward the use of this white rot fungus at various contaminated sites for groundwater and soil remediation.[10–13] In order to utilize this white rot fungus effectively, kinetic characteristics of this degradation process need to be more thoroughly understood.

This white rot fungus produces several extracellular lignin peroxidases and Mn–peroxidases upon the depletion of some substrates, such as nitrogen, in the culture medium.[14] It has been suggested that this extracellular lignin-degrading enzyme system plays a role in the degradation of toxic compounds.[1,2] Recently, Mileski et al.[3] used purified ligninases to convert PCP into an oxidation product, 2,3,5,6-tetrachloro-2,5-cyclohexadiene-1,4-dione (TCHD). Also several researchers reported that the extracellular ligninases or the crude extracellular enzymes could oxidize several polycyclic aromatic hydrocarbons to some degree.[5,8,9] These results imply that a common function of the extracellular enzymes is to catalyze the initial step of the degradation process. So far the quantitative relationship between the degradation and the extracellular enzymes has not been demonstrated.

Most of the reports on degradation by *P. chrysosporium* were based on determination of $^{14}CO_2$ release when adding ^{14}C-labeled toxic compounds to growing cultures. The release of $^{14}CO_2$ indicates involvement of the complex cellular metabolism in the complete degradation process.[15] It has been suggested that the biodegradation begins at the onset of secondary metabolism.[1] Because the role of the cells was not clearly defined in the growing cultures, it was difficult to distinguish the precise functions of the cell mass and the extracellular enzymes in the degradation process using these growing cultures. Employment of the pregrown cell mass may assist in investigating the interaction between the cell mass and the extracellular enzymes.

It was also shown that some toxic chemicals, such as PCP, had an inhibitory effect on the growth of

* To whom all correspondence should be addressed.

Biotechnology and Bioengineering, Vol. 35, Pp. 1125–1134 (1990)

CCC 0006-3592/90/01101125-010$04.00

P. chrysosporium culture.[3] However, after a certain period of pregrowth without the toxic chemical, the inhibitory effect became insignificant. Further investigation into the relationship between degradation rates and concentrations of toxic compounds is necessary.

In this research, we carried out PCP degradation using extracellular enzymes and cell mass obtained from pregrown *P. chrysosporium* cultures in DMS buffer. The goal of this investigation was to examine effects of PCP, extracellular enzyme, and cell mass concentrations on the disappearance and mineralization of PCP. A kinetic model which can describe the time dependence of PCP degradation on initial PCP concentration and dosages of extracellular enzymes and cell mass was developed.

MATERIALS AND METHODS

Microorganism and Culture Procedure

The fungus *Phanerochaete chrysosporium* (BKM-F-1767) was obtained from Dr. T. K. Kirk (USDA Forest Products Lab., Madison, WI). The fungus was maintained on 2% malt agar slants.

A 10-mL inoculum consisting of glass-wool filtered conidial suspension[16] from 3-week-old agar slants was introduced into 200 mL of malt broth (1.5% w/v). This seed culture was incubated in a stirred flask at 38°C for 12 h. The inoculum (2 mL) from the seed culture was then added into 20 mL DMS growth medium as described by Reid et al.[17] The culture flasks were maintained in a stationary condition at 38°C and at air atmosphere for 7 days. The mycelial mat and extracellular enzymes were then harvested for use in various degradation experiments.

Assay for PCP in Aqueous Solution

PCP in solution was assayed by HPLC using a modification of the technique of Mikesell et al.[18] Samples (0.5 mL) were mixed with 0.25 mL of acetonitrile on a vortex mixer, centrifuged for 10 min at 10,000*g*, and filtered through 0.45-μm membrane filters (ACRO LC 13, Gelman Sciences Co., MI). A Waters HPLC system, consisting of the Waters 501 pump, Waters Lambda-Max Model 481 UV detector, and Waters 740 data module, was used. Samples were injected using a Rheodyne 7010 injector fitted with a 20-μL loop. Separation was accomplished using a Waters Radial-Pak C-18 cartridge held in a Waters Zmodule radial compression separation system. The mobile phase consisted of acetonitrile and 5% aqueous acetic acid (8 : 2). The flow rate of the mobile phase was 1.2 mL/min. PCP was measured at an absorbance of 300.5 nm.

Assay for PCP Mineralization ($^{14}CO_2$ Release)

Evolved $^{14}CO_2$ was assayed by trapping in 10 mL of an ethanolamine-containing scintillation fluid.[19] The trapping procedure was conducted as described by Kirk et al.[16] Degradation mixture was contained in flasks fitted with ports that permitted periodic flushing for $^{14}CO_2$ release. Air was used for flushing at a flow rate of 100–200 mL/min for a total of 20 min. Data of $^{14}CO_2$ release were corrected for background radioactivity and for counting efficiency. The latter was determined with an internal standard of ^{14}C-toluene. The average total recovery of ^{14}C by this procedure was 91.5 ±9.97%. The data for PCP mineralization were calculated from the trapped $^{14}CO_2$. The reported data were after correction for the recovery efficiency of the assay procedure, except for those mentioned in the text.

Experiments for PCP Disappearance and Mineralization

After 7 days of incubation, the mycelial mat and the extracellular fluid (supernatant containing crude extracellular enzymes) were harvested and separated by centrifugation. Wet volume of the mycelia was determined and the mycelia were washed with 10 m*M* 2,2-dimethyl succinate (DMS) buffer (pH 4.5). The mycelia were then homogenized using a glass homogenizer. The extracellular fluid was concentrated 10- to 20-fold by a membrane filter (10,000 MW cutoff, Amicon, W. R. Grace & Co., CA). The protein concentration and ligninase activity of the crude enzyme concentrate were then determined by the Bio-Rad method (Bio-Rad Laboratories, CA) and the veratryl alcohol method,[20] respectively. The cell mass concentration reported was expressed as dry cell weight per unit volume, which was converted from the wet volume of the mycelia using a calibration curve. Since crude mixtures of enzymes were used for the experiments, the total extracellular protein was used to quantify the extracellular enzymes rather than any single enzyme activity in the crude enzyme fluid. For 11 groups of data, the specific ligninase activity of the extracellular fluid was 0.0951 A_{310}/min-μg protein. The correlation coefficient for this linear relation was 0.966.

Experiments examining both PCP disappearance and PCP mineralization were carried out in 250-mL flasks at air atmosphere shaken at 38°C and 80 rpm. A 25 m*M* DMS buffer solution (pH 4.5) containing 7 g/L glucose was added to each flask (preliminary experiments demonstrated that 7 g/L glucose was sufficient to support PCP degradation without limitation due to depletion of the cosubstrate for all the experimental conditions). The required extracellular enzymes and cell mass prepared by the above procedure were then added to the DMS buffer. For PCP disappearance experiments, a 200-mg/L PCP stock solution was added to the flasks at the necessary volume to attain the desired initial PCP concentrations. The stock solution was prepared by dissolving PCP in double distilled water and adjusting the pH to 11.0 with 1*N* NaOH to increase PCP solubility. PCP concentration during the degradation was monitored using high-performance liquid chromatography (HPLC). For PCP mineralization experiments, the PCP stock solution was mixed with a required amount of ^{14}C-labeled PCP before addition to the flasks.

The total radioactivity contained in each flask was about 6.5×10^4 DPM. Release of $^{14}CO_2$ from these flasks was detected by following the assay procedure detailed above. The total volume of the degradation mixture contained in each flask was 20 mL. All experimental conditions were kept constant except the parameters of interest: PCP, extracellular enzyme, and cell mass concentrations.

Experiment for ^{14}C Mass Balance

The ^{14}C–PCP mixture was added to flasks containing different amounts of extracellular enzymes and cell mass up to a concentration of 10.45 μmol/L. The $^{14}CO_2$ release was measured every 2 days. After 22 days, the whole degradation mixture was extracted using 50 mL of hexane and strong agitation. Following the hexane extraction, the aqueous phase was acidified to pH 2.0 with concentrated HCl and extracted with 50 mL of methylene chloride. The mycelium was then separated from the aqueous fraction by filtration. Ready-Safe scintillation liquid (10 mL) (Beckman Co., CA) was added to 1-mL samples of the hexane, methylene chloride, and aqueous fractions. The radioactivity of these three fractions was determined using a liquid scintillation spectrometer (Beckman LS 3801). The radioactivity in the mycelium fraction was measured using a Biological Oxidizer (OX200, R. J. Harvey Instrument Corp., NJ). This oxidizer under high temperature converted the residual ^{14}C in the mycelium to $^{14}CO_2$, which was then trapped in scintillation liquid and measured by the scintillation spectrometry.

Table I. ^{14}C balance after 22-day degradation of ^{14}C–PCP by different amounts of extracellular enzymes and cell mass from *P. chrysosporium* culture.

Items	Condition 1: P = 2.61 mg/L C = 0.57 g/L	Condition 2: P = 6.42 mg/L C = 2.21 g/L
% $^{14}CO_2$ released	33.15	70.11
% ^{14}C in hexane extract	0.96	0.927
% ^{14}C in acidic CH_2Cl_2 extract	8.62	3.95
% ^{14}C remaining in aqueous phase	32.61	20.23
% ^{14}C remaining in cell mass	24.66	4.79
% Total recovery	84.20	93.19

Note: After 7-day incubation, the extracellular enzymes and cell mass were added to DMS buffer with 7 g glucose/L for PCP degradation. Initial PCP concentration was 10.45 μmol/L. The percentage of ^{14}C for the samples was corrected with the total recovery of assay procedure, respectively. Symbols: P, extracellular enzyme concentration, expressed as protein concentration; C, cell mass concentration, expressed as dry cell weight per unit volume.

Identification of PCP Degradation Product by the Extracellular Enzymes

The extracellular fluid from 7-day-old culture was concentrated and dialyzed in 25 mM DMS buffer (pH 4.5) for 17 h at 4°C. Then the dialyzed crude enzyme solution (36 mg protein/L) was mixed with PCP (24μmol/L), H_2O_2 (50 μmol/L), and the DMS buffer to attain a total volume of 4.2 mL. After 72 h of incubation at 38°C, the reaction was terminated by addition of 1.4 g NaCl and 1 mL of hexane. PCP and TCHD were extracted by mixing vigorously on a vortex mixer for 30 s. The hexane extract was analyzed by using HPLC and a UV detector set at 290 nm according to Mileski et al.[3]

Chemicals

Unlabeled PCP was obtained from Aldrich Co. Inc. (Milwaukee, WI). ^{14}C-labeled (UL) PCP was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used for assays were reagent grade.

RESULTS

Proposed Reaction Scheme and Kinetic Model

Experimental Bases of the Degradation Mechanisms

Table I shows the ^{14}C balance after 22-day degradation of ^{14}C–PCP by different concentrations of extracellular enzymes and cell mass. The effect of different amounts of extracellular enzymes and cell mass on PCP degradation was significant. By using higher concentrations of both enzymes and cell mass (P = 6.42 mg/L, C = 2.21 g/L), about 70% of the initially added PCP was mineralized during 22 days, with the residual labeled compound in cell mass remaining quite low (4.79%). This high percentage of PCP mineralization has not been reported for work using growing cultures of *P. chrysosporium*.[3]

When PCP, with H_2O_2, was added to the dialyzed extracellular fluid from 7-day-old culture (a crude enzyme mixture), a degradation product was observed. This was identified as TCHD (Fig. 1), the same oxidation product of PCP found by Mileski et al.[3] using purified lignin peroxidases in tartrate buffer. Our result confirms that the function of extracellular enzymes, from which the lignin peroxidases are purified, is to degrade PCP into some degradation intermediate(s) under the present experimental conditions.

We also observed direct mineralization of PCP by only using cell mass (without extracellular enzymes) (data not shown), but the degree of mineralization was significantly reduced from that observed using both extracellular enzymes and cell mass.

It is suggested by these data that both the extracellular enzymes and the cell mass (or cell-bound enzymes) are

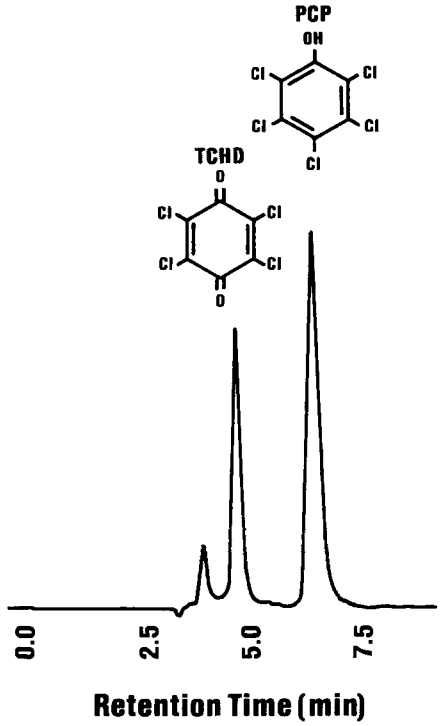

Figure 1. HPLC chromatogram of the hexane extract of the PCP degradation mixture after 72-hour incubation with the dialyzed extracellular enzymes. TCHD and PCP were identified by comigration with authentic standards.

required for the effective PCP mineralization, and the rational use of both extracellular enzymes and cell mass is a key to reaching the higher PCP mineralization.

Reaction Scheme and Kinetic Model

A metabolic model for PCP degradation by *P. chrysosporium* is proposed based on the experimental results (see Fig. 2). In this model, PCP can be mineralized to CO_2 through two mechanisms. First, PCP can be degraded by the extracellular enzymes to some intermediate, with the intermediate being subsequently mineralized to CO_2 by cell mass (or cell-bound enzymes). Second, the cell mass (or cell-bound degrading enzymes) can directly mineralize PCP to CO_2 without requiring the initial reaction that is mediated by the extracellular enzymes. In addition to our results, Mileski et al.[3] also found PCP mineralization by using the culture broth without the active extracellular lignin–peroxidase system (inhibited by a high nitrogen concentration). This observation circumstantially supports the existence of the second mineralization mechanism. The extracellular enzymes require the presence of H_2O_2 for activity. The H_2O_2 may be produced by intracellular glucose oxidase or by extracellular oxidases (including the Mn–peroxidase in the oxidizing state).[21–24] For given concentrations of cell mass and enzymes, the existing concentration of H_2O_2 may be controlled by manipulating the levels of O_2 and cosubstrates. In the following model, it is assumed that the concentration of H_2O_2 is sufficient not to limit the enzyme activity. When a mixture of extracellular enzymes and cell mass are used concurrently, this assumption would appear reasonable. The extracellular oxidases probably exist in proportion to the peroxidases, and the cell mass (intracellular glucose oxidase) also likely provides the H_2O_2.

According to the proposed metabolic model, each of the two parallel PCP mineralization mechanisms includes a multienzyme series reaction. To simplify the description, we assume representative key enzymes and representative key intermediates exist in the process. The lumping technique, in which a single imaginary step is used to represent a multireaction process, has been used elsewhere.[25] For the first mechanism, in which the initial degradation of PCP is catalyzed by the extracellular enzymes and the reaction converting the degradation intermediate to CO_2 is carried out by cell-bound enzymes (or cell mass), a Michaelis–Menten model is applied. Adsorption of PCP by the fungal mycelium has been observed (Fig. 4). A quasi-steady-state assumption does not describe these adsorption results. For simplicity in the model, therefore, adsorption of PCP to the mycelia is assumed as the first step of the second mechanism, and then the mycelium-bound PCP is mineralized to CO_2. As a result, the second mechanism is described as a process combining an irreversible adsorption and a surface reaction.

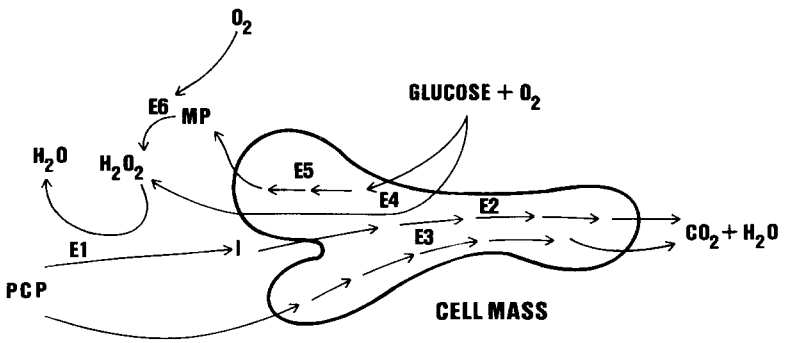

Figure 2. Metabolic model for PCP degradation by *P. chrysosporium*. E1: Extracellular enzymes for conversion of PCP to intermediate(s). E2: Cell-bound enzymes for conversion of the intermediate to CO_2. E3: Cell-bound enzymes for direct conversion of PCP to CO_2. E4: Intracellular glucose oxidase. E5: Enzymes for generation of the metabolic products used as substrates of E6. E6: Extracellular oxidases for H_2O_2 production. I: Major degradation intermediate. MP: Metabolic products used for H_2O_2 production.

The first mechanism can be expressed as follows:

$$A + E_0 \rightleftharpoons E_0A \longrightarrow X + E_0$$

$$X + E_{ik} \rightleftharpoons E_{ik}X \longrightarrow 6F + E_{ik}$$

For the second mechanism, in which cell mass directly mineralizes PCP to CO_2, the expression can be written as

$$A + S \longrightarrow A_b$$

$$A_b \longrightarrow 6F + S$$

where A, X, A_b, and F stand for PCP, the representative intermediate of extracellular enzyme degradation, the mycelium-bound PCP, and CO_2, respectively; E_0, E_{ik}, and S represent the extracellular enzymes, the key cell-bound enzyme, and the cellular active surface.

If the concentration of PCP is low, the rate expression can be approximated by the quasi-first-order mode as follows:

$$V(a)_1 = k_1Pa \quad (1)$$

$$V(a)_2 = k_2Ca \quad (2)$$

$$V(x) = k_3Cx \quad (3)$$

$$V(a_b) = k_4Ca_b \quad (4)$$

where $V(a)_1$ and $V(a)_2$ represent the rate for PCP disappearance by the extracellular enzymes and by the cell mass, respectively; $V(x)$ stands for the rate of conversion from the key intermediate to CO_2, while $V(a_b)$ indicates the rate for mineralization of the mycelium-bound PCP.

A mass balance for the degradation process yields the following set of equations:

$$-da/dt = k_1Pa + k_2Ca \quad (5)$$

$$dx/dt = k_1Pa - k_3Cx \quad (6)$$

$$da_b/dt = k_2Ca - k_4Ca_b \quad (7)$$

$$(1/6)df/dt = k_3Cx + k_4Ca_b \quad (8)$$

With the initial conditions $t = 0$, $a = a_0$, $x = a_b = f = 0$, the solutions for Equations (5)–(8) are as follows:

$$a = a_0\exp[-(k_1P + k_2C)t] \quad (9)$$

$$x = \frac{k_1Pa_0}{k_3C - (k_1P + k_2C)}\{\exp[-(k_1P + k_2C)t] - \exp(-k_3Ct)\} \quad (10)$$

$$a_b = \frac{k_2Ca_0}{k_3C - (k_1P + k_2C)}\{\exp[-(k_1P + k_2C)t] - \exp(-k_4Ct)\} \quad (11)$$

$$\frac{f}{6} = \frac{k_1Pk_3Ca_0}{k_3C - (k_1P + k_2C)}\left\{\frac{\exp(-k_3Ct)}{k_3C} - \frac{\exp[-(k_1P + k_2C)t]}{k_1P + k_2C}\right\} + \frac{k_2Ck_4Ca_0}{k_4C - (k_1P + k_2C)} \times \left\{\frac{\exp(-k_4Ct)}{k_4C} - \frac{\exp[-(k_1P + k_2C)t]}{k_1P + k_2C}\right\} + a_0 \quad (12)$$

According to Equations (9) and (12), the degree of PCP disappearance and PCP mineralization is determined by four independent variables: initial PCP concentration, extracellular enzyme concentration, cell mass concentration, and degradation time. If PCP disappearance and PCP mineralization are expressed as fractions of the initial PCP concentration, a/a_0 and $f/6a_0$, they are determined by extracellular enzyme concentration, cell mass concentration, and degradation time. It is also seen from Equation (12) that for a fixed degradation time, extracellular enzyme concentration, and cell mass concentration, PCP mineralization is proportional to initial PCP concentrations.

The above analyses were used as a basis for design of a second set of experiments. The purpose of these experiments was twofold: verification of the kinetic analyses and determination of the model parameters.

Effects of PCP, Extracellular Enzyme, and Cell Mass Concentrations

Effect of Initial PCP Concentration

Figure 3 demonstrates the relationship between PCP mineralization (for 24 h) and initial PCP concentrations. The experiment was conducted at different ratios of extracellular enzymes to cell mass (P/C) for a given cell mass concentration. At initial PCP concentrations of less than 12 μmol/L, PCP mineralization for a certain P/C ratio increased with the PCP concentration as expressed by Equation (12). Thus the combination of first-order reactions appears to accurately describe the degradation process over the low PCP concentration range. When initial PCP concentrations were higher than 30 μmol/L, PCP mineralization was not dependent on the initial PCP concentration but approximated zero-order reaction kinetics.

Effects of Extracellular Enzyme and Cell Mass Concentrations on PCP Disappearance

PCP disappearance may be due to both transformation by extracellular enzymes and adsorption to mycelia. For the low-PCP concentration range (<12 μmol/L), these two cases can be characterized by Equation (9). To study the separate effects, experiments were conducted by using only cell mass (without the extracellular enzymes) and by using both the extracellular enzymes and the cell mass.

Figure 4 shows PCP disappearance using only cell mass as a function of time (data are plotted as the logarithm of residual PCP concentration vs. time). For the experiment with a higher initial PCP concentration (21.88 μmol/L), PCP concentration continually decreased during the first 33 h, and thereafter the disappearance rate became greatly reduced. This indicated adsorption of PCP to mycelium was likely the major reason for PCP disappearance for the case shown, although the direct mineralization by the cell mass was also occurring. Once the cellular binding surface became saturated, the adsorption rate was controlled by the direct mineralization rate. For the low initial PCP concen-

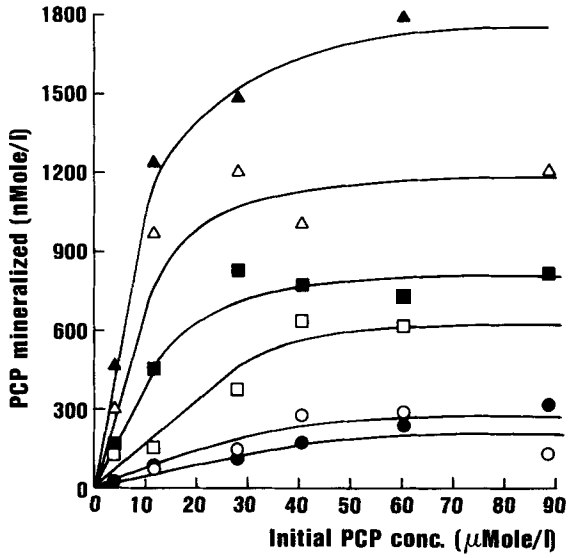

Figure 3. PCP mineralization during 24 hours vs. initial PCP concentrations at different ratios of extracellular enzymes to cell mass (for a given cell mass concentration = 0.57 g/L). ▲, P/C = 0.0172; △, P/C = 0.0128; ■, P/C = 0.00448; □, P/C = 0.0026; ●, P/C = 0.000484; ○, P/C = 0.

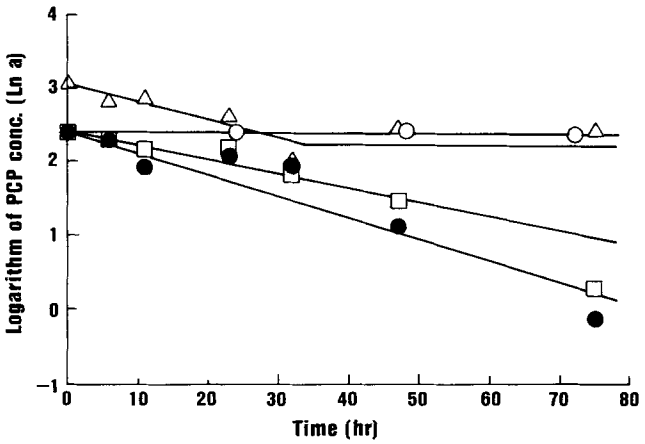

Figure 4. PCP disappearance profile (in semilogarithmic plot) by using cell mass only (without extracellular enzymes). △, C = 0.91 g/L; □, C = 0.46 g/L; ●, C = 0.91 g/L; ○, no cell mass.

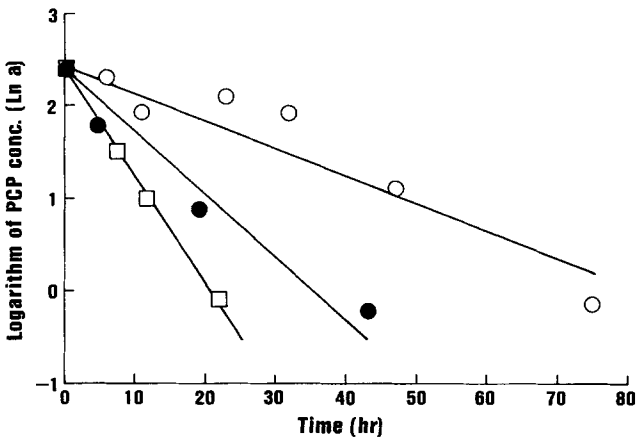

Figure 5. PCP disappearance profile (in semilogarithmic plot) by using cell mass with different amount of extracellular enzymes; C = 0.57 g/L. ○, No enzymes: ●, P = 2.76 mg/L; □, P = 6.81 mg/L.

tration (10.45 μmol/L), PCP disappearance followed the first-order reaction mode with the rate of disappearance increasing with increased cell mass concentrations.

When both cell mass and extracellular enzymes were added into the DMS buffer, PCP disappearance rates were much more rapid than using only cell mass (Fig. 5). In addition, the rates increased with increased extracellular enzyme concentrations for a given cell mass concentration. For the low PCP concentration, PCP disappearance in the presence of both extracellular enzymes and cell mass was first order.

The disappearance of PCP in the presence of the extracellular enzymes and cell mass is a competitive process. The fractions of the intermediate from the action of extracellular enzymes and the mycelium-bound PCP are determined by the ratio of extracellular enzymes to cell mass.

Effects of Extracellular Enzyme and Cell Mass Concentrations on PCP Mineralization

The extracellular enzymes degrade PCP to the intermediate, while the cell mass is involved in both mineralization mechanisms. Either of these processes can be rate limiting for PCP mineralization depending on the relative concentrations of extracellular enzymes and cell mass.

The effect of extracellular enzyme concentrations on PCP mineralization for a given cell mass concentration is shown in Figure 6. The data are the average of experimental values for the low PCP concentration range (<12 μmol/L). With no extracellular enzymes, cell mass mineralized about 1.2% of the added PCP to CO_2 over 48 h. By increasing extracellular enzyme concentrations from 1.29 to 9.3 mg/L, the amount of PCP mineralized was increased

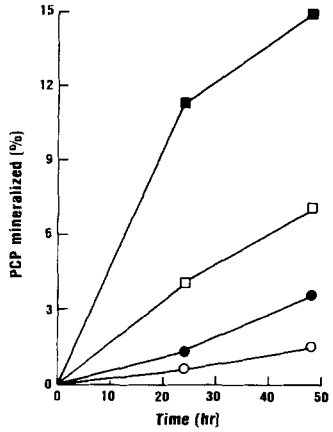

Figure 6. PCP mineralization by using different extracellular enzyme concentrations for a given cell mass concentration (C = 0.57 g/L) at the low PCP concentration range. ■, P = 9.30 mg/L; □, P = 2.76 mg/L; ●, P = 1.29 mg/L; ○, no enzymes.

about fivefold. The influence of the enzyme concentration was more significant in the lower enzyme concentration range than in the higher enzyme concentration range.

For a given enzyme concentration, the effect of varying cell mass concentrations on PCP mineralization is shown in Figure 7. Again, the data are from experiments with initial PCP concentrations less than 12 μmol/L. The rate of PCP mineralization was enhanced by increasing cell mass concentrations for a fixed enzyme concentration. For a period of about 170 h, the amount of PCP mineralized was increased approximately 3 times by increasing cell mass concentration from 0.27 to 1.86 g/L.

Modeling of Degradation Kinetics

Estimation of Model Parameters and Verification of the Model

The above experimental results at the low initial PCP concentration range (<12 μmol/L) can be used to estimate a number of the parameters in the proposed kinetic model.

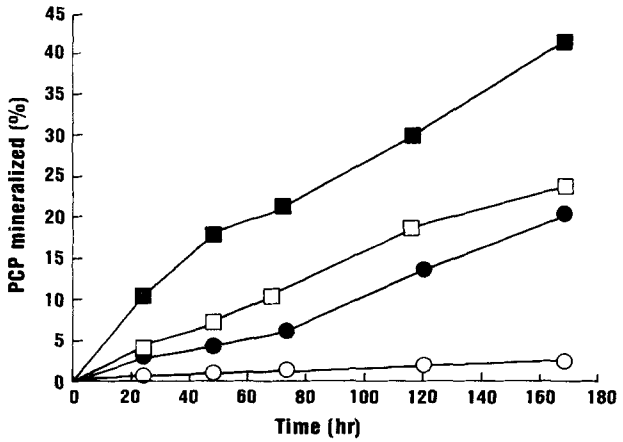

Figure 7. PCP mineralization by using different cell mass concentrations for a given extracellular enzyme concentration (P = 2.76 mg/L) at the low PCP concentration range. ○, No cell mass; ●, C = 0.27 g/L; □, C = 0.57 g/L; ■, C = 1.86 g/L.

Using the data from Figures 4 (only the data for the low PCP concentration) and 5 and Equation (9), rate constants k_1 and k_2 were determined by linear regression. Constants k_3 and k_4 were determined using the data in Figures 6 and 7 in conjunction with the nonlinear regression method. Marquardt's numeric method was used for the nonlinear regression program.

The rate constants were estimated as follows:

$$k_1 = 0.0196 \text{ L/mg protein-h}$$

$$k_2 = 0.0479 \text{ L/g cell-h}$$

$$k_3 = 0.00467 \text{ L/g cell-h}$$

$$k_4 = 0.000517 \text{ L/g cell-h}$$

Using these estimated rate constants, time profiles for PCP disappearance and mineralization were simulated. The results of independent experiments, rather than the data used for estimation of the model parameters, were then used to verify the simulation data. Figure 8 shows the comparison between experimental and simulated results at the low PCP concentration range using different concentrations of extracellular enzymes and cell mass. The model adequately describes both the disappearance and the mineralization of PCP.

Optimization of Degradation Based upon Simulation Results

One of the purposes of the kinetic study is to explore the criteria for effective use of extracellular enzymes and cell mass. However, the interaction between the extracellular enzymes and the cell mass is complicated, as shown in the above experimental data. To solve this problem, simulations were conducted to establish the relationship between PCP mineralization and the three independent variables: degradation time, cell mass concentration, and ratio of extracellular enzymes to cell mass (P/C).

Simulation results are presented in Figure 9 for percentage of PCP mineralization as a function of cell mass concentration and P/C ratio for a 300-h degradation period. Four characteristics can be seen from these simulation data. First, the maximum percentage of PCP mineralization depends upon cell mass concentration. This means that cell mass is the rate-limiting factor for complete mineralization. Second, after the value of P/C reaches about 0.01, any increase in extracellular enzyme concentration does not significantly enhance PCP mineralization. This P/C ratio, therefore, can be considered as the upper limit for increasing dosage of the extracellular enzymes. Third, at higher concentrations of cell mass, the effect of enhancing PCP mineralization by increasing cell mass diminishes rapidly. For instance, the effect of increasing cell mass concentration from 1.8 to 2.6 g/L is much less than from 0.4 to 1.0 g/L. This suggests there is an upper limit beyond which increasing cell mass does not significantly enhance PCP mineralization. Fourth, for a certain percentage of PCP mineralization, several combinations of extracellular

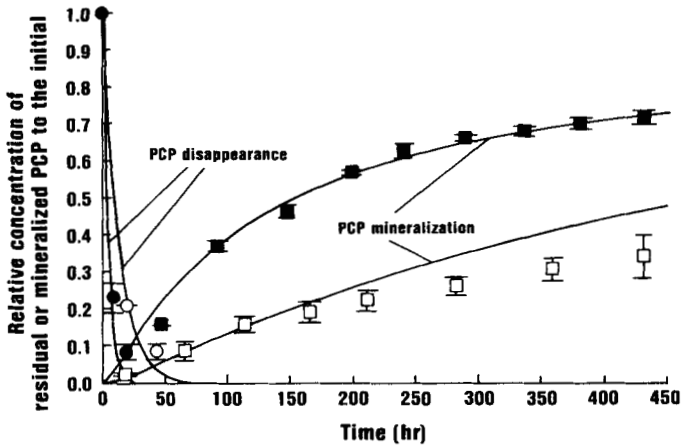

Figure 8. Comparison between experimental and simulated PCP degradation. Initial PCP conc. = 10.45 μmol/L. Smooth curves indicate the simulated results, experimental data are the average of duplicate samples. ○, P = 2.69 mg/L, C = 0.51 g/L: □, P = 2.69, C = 0.51; ●, P = 6.42, C = 2.21; ■, P = 6.42, C = 2.21.

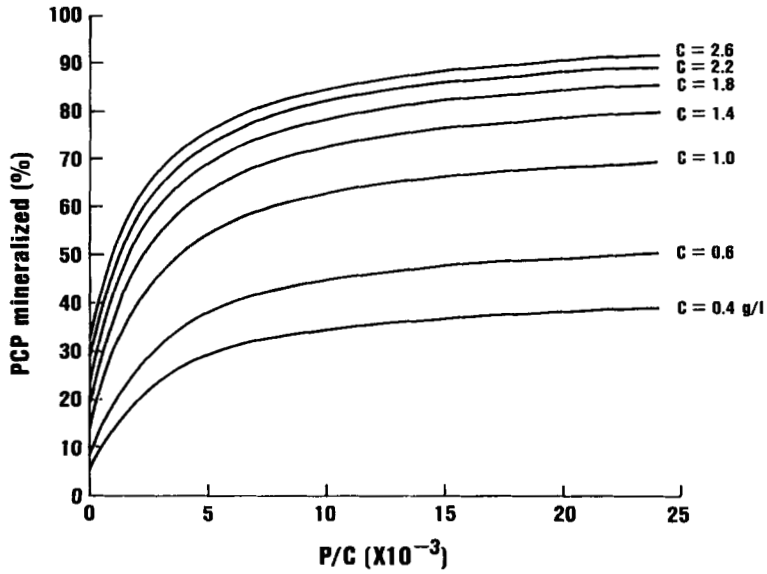

Figure 9. Relation between PCP mineralization and ratio of extracellular enzymes to cell mass with different cell mass concentrations; t = 300 hours, at the low PCP concentration range.

enzyme concentration and cell mass concentration can be chosen. The rational selection for dosages of extracellular enzymes and cell mass can be made based on the technical and economic evaluation.

DISCUSSION

At waste sites, the accessible concentration of toxic chemicals is usually very low. The cause for this is twofold. First, the solubility of many recalcitrant toxic organic chemicals, such as PCBs and PCP, is low. Second, the binding to various solid surfaces in the waste sites also reduces concentrations of toxic compounds. For this reason, many of the previous experiments were conducted with low concentrations of these pollutants.[2] In the present study, the work also focused on using a low PCP concentration range (<12 μmol/L).

TCHD was found to be a degradation intermediate of PCP when using the crude extracellular enzymes. It was also shown that an increase of extracellular enzyme concentration increased the disappearance and mineralization of PCP. These results provide both qualitative and quantitative data supporting the previous suggestion and confirm that the extracellular enzymes play an important role in the degradation process.

In nature, the white rot fungus *P. chrysosporium* degrades lignin. The structural features and large size (600–1000 kilodaltons) of lignin make it difficult for it to enter the cells.[12] Therefore, the requirements for lignin degradation are that the initial step must be extracellular and

probably nonspecific. The same requirements are also true for degradation of toxic chemicals with a high molecular weight and a complex structure. However, in case of toxic chemicals of small molecule, such as PCP, entry of these toxic compounds into the cells also makes them accessible to cell-bound or intracellular degradative enzymes. Thus the degradation mechanism for these toxic compounds may be multiple rather than only being degraded by a sequence of reactions initialized by extracellular enzymes. Demonstration of the parallel mechanism in the present experiment provides some information about the characteristics of degradation of small-molecule toxic chemicals.

Chua et al.[26] observed binding of synthetic ^{14}C-labeled lignin to the mycelium of *P. chrysosporium*. They concluded that the binding was irreversible and was associated with degradation. Adsorption of PCP to the mycelium was also seen in the present experiment. We found that after a low dosage of the extracellular enzymes and cell mass was first used to degrade PCP for about 2 weeks to approximately 20% of PCP mineralization, addition of the same amounts of fresh extracellular enzymes and cell mass did not enhance the mineralization (data not shown). It is therefore suggested that the adsorption of PCP to the mycelium is irreversible. Thus, PCP disappearance from the solution is probably not an accurate measurement of the degradation. Since the rate constant of the adsorption is much higher than the rate constant of the degradation of the mycelium-bound PCP, PCP may accumulate in a mycelium-bound form for a certain period of time. Because the adsorption decreases the accessibility of PCP to the extracellular enzymes, the adsorption kinetics can also affect the utilization efficiency of the extracellular enzymes.

Under the culture conditions used in these experiments, the ratio of extracellular enzymes to cell mass in the culture broth was approximately 0.0026 for a cell mass concentration of 0.6 g/L. If these amounts of extracellular enzymes and cell mass are used for PCP degradation under the conditions of the present experiments, the following overall rate constants for this process can be calculated by using the estimated rate constants: $k_1P = 0.031\ h^{-1}$, $k_2C = 0.0287\ h^{-1}$, $k_3C = 0.0028\ h^{-1}$, and $k_4C = 0.0004\ h^{-1}$. It is clear that the mineralization steps mediated by the cell mass (with the rate constants k_3 and k_4, respectively) are rate limiting under the condition shown. Since the degrading ability of *P. chrysosporium* is not practically maintained for a very long period of time, the percentage of mineralization of toxic chemicals would be restricted by these slower steps. This suggests a possible reason why the mineralization of some toxic compounds by the growing *P. chrysosporium* culture was not satisfactorily complete.[2,3] For a given amount of toxic compounds, increasing the cell mass may be a means whereby increased mineralization can be realized.

The process of PCP degradation by *P. chrysosporium* is mediated by two separable factors: the extracellular enzymes and the cell mass, which can be obtained after pregrowth of the culture. Direct applications of the extracellular enzymes and both the enzymes and the cell mass for degradation of toxic compounds have been proposed.[5,10-13] In our laboratory, an investigation on using these enzymes and cell mass in a co-immobilized system is underway. The kinetic relationship presented by this study allows some insight into the interaction between the extracellular enzymes and the cell mass and assists in development of the degradation strategies.

CONCLUSIONS

When initial PCP concentration is less than 12 μmol/L, the combination of first-order reactions can describe the degradation process adequately. Increase in extracellular enzyme concentration or cell mass concentration significantly enhanced PCP disappearance and mineralization. By using higher concentrations of extracellular enzymes and cell mass, as high as 70% of added PCP could be mineralized. This demonstrates that the rational use of extracellular enzymes and cell mass is a key to reaching a higher percentage of PCP mineralization with concurrent lower levels of accumulation of PCP and intermediates in the cell mass and aqueous phase.

The degradation scheme, which was proposed based on experimental results, includes two mechanisms for PCP mineralization by *P. chrysosporium*. First, extracellular enzymes degrade PCP into some intermediate(s) with subsequent conversion of the intermediate(s) to CO_2 by the cell mass. Second, direct mineralization of PCP is also catalyzed by the cell mass. For the low PCP concentration range, the kinetic model of parallel-series reactions provides an appropriate basis to quantify the interactions between PCP degradation and dosages of extracellular enzymes and cell mass. Simulation based upon this kinetic model offers the criteria for rational use of the extracellular enzymes and the cell mass. These criteria can be used for development of bioremediation processes and optimization of the process economics.

Jian-Er Lin gratefully acknowledges Michigan Biotechnology Institute for financial support through UM-MBI traineeship. The authors would like to extend thanks to Don Penner and Frank Roggenbuck of the Department of Plant and Soil Science, Michigan State University, for their assistance in the assay for ^{14}C residue in the mycelial material. We also thank Tom Naglak for his editorial assistance.

NOMENCLATURE

a	PCP concentration (μmol/L)
a_0	initial PCP concentration (μmol/L)
a_b	concentration of mycelium-bound PCP (μmol/L)
C	cell mass concentration (g/L)
f	CO_2 concentration (μmol/L)
k_1	rate constant for PCP disappearance by extracellular enzymes (1/mg protein h)
k_2	rate constant for PCP disappearance by cell mass (1/g cell h)
k_3	rate constant for PCP mineralization from the intermediate by cell mass (1/g cell h)
k_4	rate constant for PCP mineralization from the cell-bound PCP by cell mass (1/g cell h)

P	extracellular enzyme concentration (mg/L)
P/C	ratio of extracellular enzymes to cell mass (g/g)
t	time (h)
x	concentration of representative intermediate (μmol/L)

References

1. D. C. Eaton, *Enz. Microb. Technol.*, **7,** 194 (1985).
2. J. A. Bumpus, M. Tien, D. Wright, and S. D. Aust, *Science,* **228,** 1434 (1985).
3. G. J. Mileski, J. A. Bumpus, M. A. Jurek, and S. D. Aust, *Appl. Environ. Microbiol.,* **54,** 2885 (1988).
4. J. A. Bumpus and S. D. Aust, *Appl. Environ. Microbiol.*, **53,** 2001 (1987).
5. R. P. Schreiner, S. E. Stevens, Jr., and M. Tien, *Appl. Environ. Microbiol.,* **54,** 1858 (1988).
6. J. A. Bumpus, *Appl. Environ. Microbio.,* **55,** 154 (1989).
7. R. Blondeau, *Appl. Environ. Microbiol.,* **55,** 1282 (1989).
8. K. E. Hammel, B. Kalyanaraman, and T. K. Kirk, *J. Biol. Chem.,* **261,** 16948 (1986).
9. S. D. Haemmerli, M. S. A. Leisola, D. Sanglard, and A. Fiechter, *J. Biol. Chem.,* **261,** 6900 (1986).
10. R. T. Lamar, M. J. Larsen, T. K. Kirk, and J. A. Glaser, in *Hard Disposal, Remedial Action, Incineration and Treatment of Hazardous Waste,* Proceedings of the Thirteenth Annual Research Symposium, Cincinnati, OH, May 1987, p. 419.
11. P. S. Zurer, *C&EN,* September 14, 17 (1987).
12. J. A. Glaser and P. R. Sferra, *UNEP Ind. Environ.,* Oct/Nov/Dec, 20 (1987).
13. E. J. George and R. D. Neufeld, *Biotechnol. Bioeng.*, **33,** 1306 (1989).
14. J. A. Buswell and E. Odier, *Crit. Rev. Biotechnol.*, **6** (Issue 1), 1 (1987).
15. T. O. Peyton, *Enz. Microb. Technol.*, **6,** 146 (1984).
16. T. K. Kirk, E. Schultz, W. J. Connors, L. F. Lorenz, and J. G. Zeikus, *Arch. Microbiol.,* **117,** 277 (1978).
17. I. D. Reid, E. E. Chao, and P. S. S. Dawson, *Can. J. Microbiol.,* **31,** 88 (1985).
18. M. D. Mikesell and S. A. Boyd, *J. Environ. Qual.*, **14,** 337 (1987).
19. T. K. Kirk, W. J. Connors, R. D. Bleam, W. F. Hackett, and J. G. Zeikus, *Proc. Nat. Acad. Sci. USA,* **72,** 2515 (1975).
20. T. K. Kirk, S. Croan, and M. Tien, *Enz. Microb. Technol.,* **8,** 27 (1986).
21. R. L. Kelley and C. A. Reddy, *J. Bacteriol.*, **166,** 269 (1986).
22. P. J. Kersten and T. K. Kirk, *J. Bacteriol.*, **169,** 2195 (1987).
23. Y. Asada, M. Miyabe, M. Kikkawa, and M. Kuwahara, *Agric. Biol. Chem.,* **50,** 525 (1986).
24. A. Paszczynski, V. B. Huynh, and R. Crawford, *Arch. Biochem. Biophys.,* **244,** 750 (1986).
25. J. E. Bailey and D. F. Ollis, *Biochemical Engineering Fundamentals,* 2nd ed. (McGraw-Hill, New York, 1986).
26. M. G. S. Chua, S. Choi, and T. K. Kirk, *Holzforschung,* **37,** 55 (1983).