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Effect of Nutrient Nitrogen on the Degradation of Pentachlorophenol by White Rot Fungus, *Phanerochaete chrysosporium*

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Abstract The effect of nutrient nitrogen on the degradation of pentachlorophenol (PCP) by *Phanerochaete chrysosporium* in a liquid culture was investigated. PCP disappeared at almost the same rate in both nutrient nitrogen-sufficient (NS) and -limited (NL) stationary cultures. However, more pentachloroanisole (PCA) was accumulated in the NS culture than in the NL culture. The effect of nitrogen on the degradation of PCA was also tested in both cultures. PCA disappeared faster in the NL culture than in the NS culture, indicating that the lower accumulation of PCA during the degradation of PCP in the NL culture was due to the faster degradation of PCA in the NL culture than in the NS culture. In another experiment, PCA was added to shaking cultures rather than stationary cultures to search for any other metabolite(s). While no other metabolite but PCA was found in the NS stationary culture, 2,4,5,6-tetrachloro-2,5-cyclohexadiene-1,4-dione (TCHD) was found as the only identifiable product in the NL shaking culture. Thus, PCP would appear to be metabolized to TCHD via PCA or directly oxidized to TCHD by lignin peroxidase. Since all the above results indicate that no innocuous metabolite was formed during the degradation of PCP by the fungus, it is quite feasible to use the fungus in the biotreatment of PCP.

Key words: White rot fungus, pentachlorophenol, pentachloroanisole, degradation fate, nutrient nitrogen

White rot fungi can efficiently degrade and mineralize lignin, which is the most abundant aromatic polymer on earth [11]. Although lignin has a highly irregular three-dimensional structure that gives plants a barrier against most microbial attacks, the fungi have a lignin degradative (ligninolytic) system that appears to be nonspecific and extracelluar [11]. As such, the ligninolytic system of the fungi has been a focus of extensive research for the last decade [2, 4, 14, 18, 19]. *Phanerochaete chrysosporium*, a white rot fungus, has been used to study the physiological requirements and enzymatic system for the lignin degradation [2, 4, 11]. Because of the nonspecific nature of the ligninolytic system from *P. chrysosporium*, the fungus has been tested for the degradation and mineralization of many environmental organopollutants, including pentachlorophenol (PCP), lindane, polychlorinated biphenyls (PCBs), 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane (DDT), and polycyclic aromatic hydrocarbons (PAH) [2, 4, 10].

Under ligninolytic conditions, which are induced in response to low levels of key sources of carbon, nitrogen, or sulfur nutrients, *P. chrysosporium* produces several important enzymes and secondary metabolites, including lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), oxalate, and veratryl alcohol (VA) [2, 4, 18, 19]. The production of extracellular enzymes and other metabolites explain, at least in part, the ability of the fungus to degrade lignin and recalcitrant organopollutants [2, 4].

PCP has been used ubiquitously as a fungicide, especially as a wood preservative, because PCP is an inhibitor of oxidative phosphorylation; in other words, PCP acts as an ATPase uncoupler [7, 8]. However, its toxicity and extensive use has placed it among the worst environmental pollutants [7, 9]. Several studies have shown that *P. chrysosporium* has an ability to degrade PCP to CO_2 in both soil and water [6, 12, 13]. Yet, before the fungus can be considered for the use in bioremediation, it is also necessary to ascertain the ability of the fungus to degrade organopollutants to innocuous products (i.e., H_2O, CO_2, and Cl^-) without the

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formation of any toxic intermediates. Chung and Aust [6] and Lamar *et al.* [13] showed that, during bioremediation, some PCP in soil was transformed to pentachloroanisole (PCA) by *P. chrysosporium*. Mileski *et al.* [12] showed that PCP in water was mineralized by *P. chrysosporium*. In the present study, we showed that the degradation rate of PCP and its metabolite PCA in water by *P. chrysosporium* was significantly affected by the concentration of nutrient nitrogen. We also observed that, under certain condition, only a small amount of 2,4,5,6-tetrachloro-2,5-cyclohexadiene-1,4-dione (TCHD) was formed during the degradation of PCA in water by *P. chrysosporium*, indicating that no toxic intermediate was formed.

Effect of Nutrient Nitrogen on the Metabolism of Pentachlorophenol

Stationary-phase cultures of *Phanerochaete chrysosporium* BKM-F-1767 (ATCC 24725) were incubated at 39°C in 10 ml of a culture medium [12] containing 1.2 mM or 12 mM ammonium tartarate for nutrient nitrogen-limited (NL) or nutrient nitrogen-sufficient (NS) cultures, respectively. Cultures were inoculated with a spore suspension as described before [12], grown under ambient atmosphere for 3 days, and then flushed every 3 days with oxygen (99.9%). The experiments were initiated by adding PCP (Sigma Chemical Co., St. Louis, MO, U.S.A.) in a small amount of acetone or ethanol (100 µl or less) to cultures after 6 days of growth. After a period of incubation, PCP and PCA were extracted as described before [12]. Then, the extracts were analyzed for PCP and PCA by gas chromatography-electron capture detection. Gas chromatographic analyses of extracts were performed on a Varian 3700 gas chromatograph equipped with a ^{63}Ni electron capture detector, a model 3390A reporting integrator, and a splitless capillary column injection port. Operating temperatures were 220°C for the injector and 300°C for the detector. The carrier gas was helium and the make-up gas was N_2. The column was a DB-5.625 fused silica capillary column (30 m by 0.540 mm) with a film thickness of 1.5 µm (J&W Scientific, Folsom, CA, U.S.A). The temperature program was as follows: initial 100°C, 10°C/min for 21.4 min, hold at 250°C for 1 min.

The concentrations of nutrient nitrogen appeared to have no effect on the disappearance of PCP (Fig. 1). In other words, when PCP was added, it disappeared at almost the same rate in both cultures. However, PCA appeared differently in the NL and NS cultures. In the NL culture, the concentration of PCA increased up to ~0.44 µM after 6 h. In the NS culture, the concentration of PCA increased to ~1.88 µM after 12 h and decreased slightly thereafter. Mileski *et al.* [12] showed that the mineralization rate and extent of PCP are higher in an NL culture than in an NS culture. The present results suggest that the higher accumulation of PCA in the NS cultures may be a reason for the lower mineralization in the NS culture.

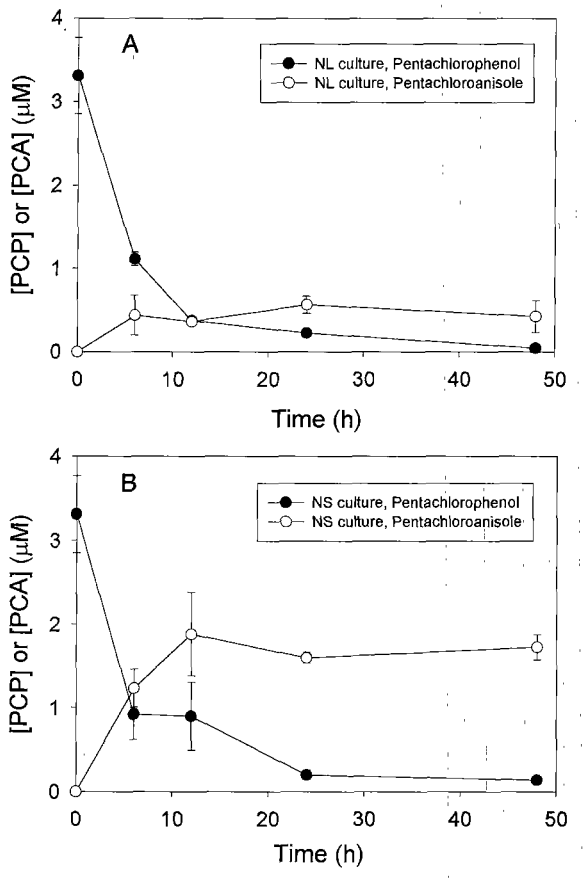

Fig. 1. Effect of nutrient nitrogen on the metabolism of pentachlorophenol by *P. chrysosporium*: nitrogen-limited (A), nitrogen-sufficient cultures (B).
The fungus was grown for 6 days before the addition of pentachlorophenol, as indicated in Methods and Materials. The values represent the mean ± the standard deviation of triplicate measurements.

Effect of Nutrient Nitrogen on the Metabolism of Pentachloroanisole

The results in Fig. 1 suggest that the methylation rate of PCP into PCA might have been the same, yet the degradation rate of PCA was different in each culture. To test this hypothesis, PCA (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) was added to both the NL and NS cultures and the disappearance of PCA was examined. The methods for the detection of PCA were the same as above. As shown in Fig. 2, PCA disappeared more rapidly in the NL culture than in the NS culture. This clearly showed that PCA was metabolized more easily in the NL culture than in the NS culture. That is, the difference in the disappearance rate of PCP in Fig. 1 could be explained by the difference in the disappearance rates of PCA.

Chung and Aust [6] and Lamar *et al.* [13] showed that, when PCP is added to soil along with *P. chrysosporium* grown on pulp wood chip or corncob, the PCP is transformed into PCA and the newly formed PCA was mineralized rapidly. When considering the paucity of

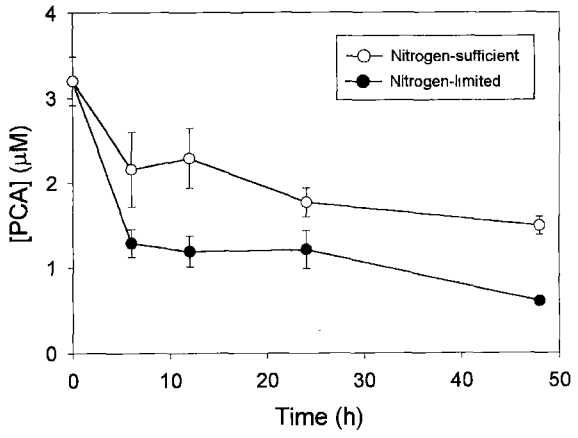

Fig. 2. Effect of nutrient nitrogen on the disappearance of pentachloroanisole of *P. chrysosporium*.
The fungus was grown for 6 days before the addition of pentachloroanisole, as indicted in Methods and Materials. The values represent the mean ± the standard deviation of triplicate measurements.

nutrient nitrogen in the employed substrate, the fungus may have provided NL culture-type conditions, thereby explaining the mechanism for successful bioremediation of PCP in soil. Overall, the above result indicates that the amount of nutrient nitrogen in the media for fungal growth is critical for the fast removal of PCP in both water and soil.

The Formation of an Intermediate during the Degradation of PCA in a Liquid Culture

It is important to know whether any harmful intermediate(s) is/are formed during the degradation of an organopollutant, since the generation of such intermediate(s) will be detrimental to the application of the fungus to bioremediation technology. Accordingly, an attempt was made to detect any intermediate(s) from the PCP or PCA degradation during the experiments mentioned above, however, we failed to detect any.

In another effort, a shaking (150 rpm) culture with a volume of 150 ml (in a 500-ml Erlenmeyer flask) containing 0.2 mM (NL) or 24 mM (NS) ammonium tartarate was employed, and 34.2 μM PCA or PCP was added to a 10-day-old culture to search for any possible intermediates. The culture medium was similar to that used by Tien and Kirk [19], except that 10 mM Na-acetate buffer (pH 4.5) was used in place of 2,2-dimethylsuccinate buffer and 5% Tween 80 plus 15 mM veratryl alcohol (Aldrich Chemical Co.) were included. The shaking cultures were inoculated with a spore suspension (1 ml; 0.5 to 0.7 absorbance units at 650 nm). After a period, the culture broth was extracted as described before [12]. Then, qualitative and quantitative analyses of metabolite(s) were performed by high performance liquid chromatography (HPLC) with an Econosphere (5 μm) C-18 reverse-phase column (4.6 by 250 mm) (Alltech Associates, Deerfield, IL, U.S.A.). The HPLC system

Table 1. Metabolism of pentachloroanisole by *P. chrysosporium* in nitrogen-limited shaking culture[a].

Time (h)	PCA (M)	TCHD (M)
0	34.2[b]	N.A.[c]
42	6.85	0.267
70	N.D.[d]	N.D.

[a]The culture contained 0.24 mM ammonium. Pentachloroanisole was added to the culture that had been grown for 10 days. Time indicates hours after the addition of pentachloroanisole.
[b]The numerical values are an average of duplicates.
[c]Not Applicable.
[d]Not Detected.

was a Beckman HPLC system Gold, consisting of a Model 166 detector module, a Model 110B solvent delivery module, a model 3390A reporting integrator, and a Model 507 autosampler. The mobile phase consisted of acetonitrile: H_2O:glacial acetic acid (75:25:0.125). The flow rate of the mobile phase was 1.0 ml/min, and the elution of metabolite(s) was monitored at 290 nm.

No metabolite other than PCA was observed in both NL and NS cultures after the addition of PCP. However, 2,4,5,6-tetrachloro-2,5-cyclohexadiene-1,4-dione (TCHD) was found as the only metabolite in the NL culture but not in the NS culture after the addition of PCA. As shown in Table 1, with the culture containing 0.2 mM ammonium tartarate, 0.267 μM TCHD was found 42 h after the addition of PCA and no TCHD 70 h after the addition of PCA, while the concentration of PCA decreased to 6.85 μM from 34.2 μM after 42 h and no PCA was found after 70 h. Figure 3 shows the HPLC chromatogram of the solvent extracts from the shaking culture of NL culture 42 h after the addition of PCA. TCHD (Aldrich Chemical Co.) and PCA were identified by comigration using authentic

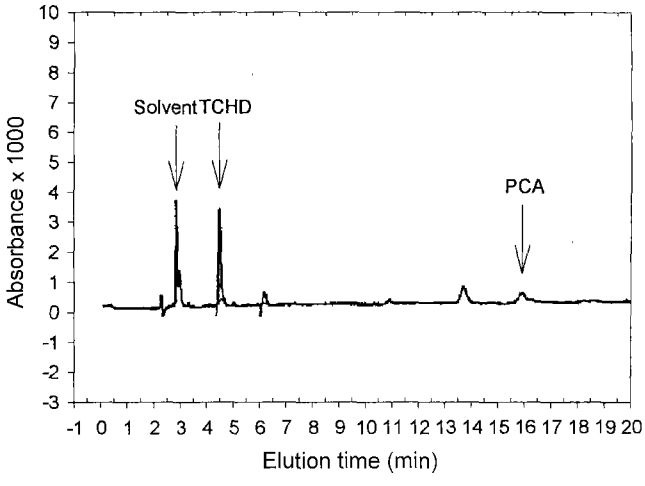

Fig. 3. HPLC elution profile of the solvent extracts from a nitrogen-limited shaking culture of *P. chrysosporium*.
The cultures were extracted with methylene chloride, 42 h after the addition of pentachloroanisole.

standards. The involvement of LiP in the transformation of PCA to TCHD in the shaking culture can be deduced from a previous study [3], where the LiP activity for a NS culture peaked at day 6 and then decreased quickly before day 10, at which point PCA was added in the present study. However, the LiP activity for the NL culture was at its peak by the time PCA was added to the culture, indicating that LiP might have been involved in the transformation. A similar LiP activity profile, which might be difficult to construct because of extremely low LiP activity, could be deduced from an another study on LiP activity during a stationary NL culture of *P. chrysosporium* [18, 19]. No LiP activity was observed in the stationary NS culture.

The Degradation Fate of PCP in the NL Culture

The results above suggest that PCP was transformed to PCA, which was then demethylated and dechlorinated to TCHD (Fig. 4). However, it should also be considered that PCP can be directly oxidized by LiP to TCHD [5, 12]. In a NL culture, PCP might be competitively oxidized to TCHD or methylated to PCA that can be converted to TCHD, as shown in the present study. The extent of the initial PCP conversion to PCA or TCHD will be dependent upon the concentration of nutrient nitrogen, such as the existence and extent of the ligninolytic system. Once TCHD is formed, it can easily be degraded further, making it difficult to detect any subsequent degradation products,

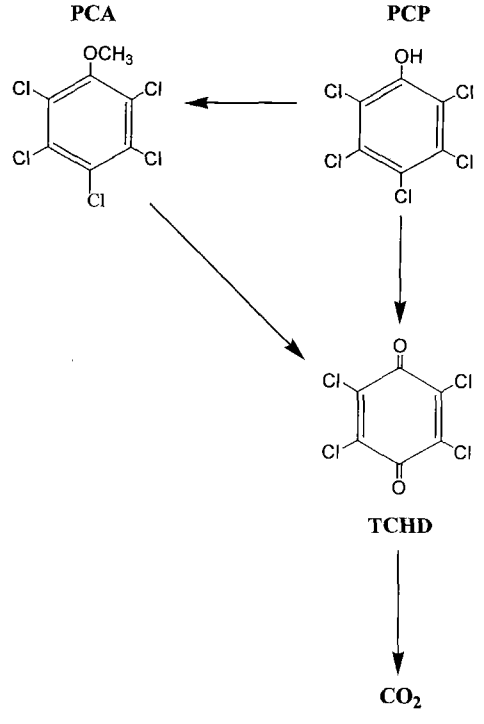

Fig. 4. Proposed mechanism for the degradation fate of pentachlorophenol by *P. chrysosporium* in the nitrogen-limited culture.

as proposed in Fig. 4. It has already been shown that PCA is less toxic than PCP to wood-rotting fungi, other microbes, and fish [8], and that it is a common metabolite of PCP as a result of soil microbial flora [16]. In addition, TCHD is detected in a small quantity only when PCA is the starting material. The proposed mechanism for PCP degradation (Fig. 4) also indicates that no harmful intermediate such as chlorinated dioxins, which are found with PCP degradation by some bacteria [7], was formed during the degradation of PCP by *P. chrysosporium*.

There are many reasons why *P. chrysosporium* is useful for PCP biodegradation. Firstly, *P. chrysosporium* is very resistant to the toxicity of PCP after initial growth [1, 12]. For example, *P. chrysosporium* can degrade 1,600 ppm of PCP in soil [6]. Secondly, the production of ligninolytic enzymes by *P. chrysosporium* is independent of the concentration of pollutants. Lignolytic enzymes are produced in response to nutrient limitation. Therefore, *P. chrysosporium* can degrade PCP to a near-zero concentration under nitrogen limitation, unlike bacteria that induce PCP-degrading enzymes and only degrade PCP up to a concentration that cannot induce PCP-degrading enzymes. Thirdly, many contaminated sites have mixtures of pollutants, therefore, only one chemical-degrading microorganism will not be adequate for such sites. *P. chrysosporium* can degrade pollutants mixtures that include PCP as a component [2, 4]. However, in spite of the extensive usefulness of *P. chrysosporium* for the removal of PCP in soil or water, further studies on fungal inocula for soil bioremediation [1, 15], bioreactor designs [17], and other technology factors are still necessary.

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