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Biodegradation of 2,4,5-Trichlorophenol Using Cell-Free Culture Broths of *Phanerochaete chrysosporium*

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Abstract Cell-free culture broth of *Phanerochaete chrysosporium* has been adopted to biologically degrade 2,4,5-trichlorophenol. Two different medium compositions of nitrogen-sufficient and nitrogen-limited were compared for their distribution of isozymes, activity of lignin peroxidase, and production of oxalate. The two different culture broths were tested for their ability to degrade 2,4,5-trichlorophenol, and the biodegradation efficiency was estimated in terms of the disappearance of 2,4,5-trichlorophenol. The degradation efficiency for the nitrogen-limited culture broth was higher than that of the nitrogen-sufficient culture broth, since the nitrogen-limited culture broth induced lignin peroxidases (LiPs) and manganese peroxidases (MnPs), and contained sufficient oxalate for producing necessary radicals. Finally, the possible mechanism of 2,4,5-CP degradation using the nitrogen-limited culture broth was proposed.

Key words: *Phanerochaete chrysosporium*, 2,4,5-trichlorophenol, lignin peroxidase, cell-free culture broth, oxalate

Many studies on the degradation of recalcitrant chemicals using biological methods have been performed [6, 14, 18, 26]. The most widely used strain is white rot fungus, *Phanerochaete chrysosporium*. An aerobic white rot fungus, *P. chrysosporium*, is able to degrade lignin, which is known to be one of the main components of wood when other substrates are exhausted or limited [13, 25, 27, 30]. Through degradation of lignin, they can utilize the cellulose component of wood as an energy source. Cells placed in a substrate-limited or -exhausted environment secrete a few secondary metabolites for their survival under severe conditions [4, 23]. These secondary metabolites secreted from *P. chrysosporium* cells, such as extracellular peroxidase isozymes (lignin peroxidases and manganese peroxidases), hydrogen peroxide, veratryl alcohol, oxalate, etc., form a lignin biodegradation system to obtain an energy source [15, 22]. Moreover, this biodegradation system, including extracellular peroxidases, is very nonspecific towards the substrates so that it can attack some recalcitrant chemicals, including organo-pollutants, which have a structural similarity to lignin [1, 3, 9, 11, 12, 20].

However, it has been shown that purified lignin peroxidases (LiPs) cannot completely mineralize recalcitrant chemicals, including pentachlorophenol (PCP) and polyaromatic hydrocarbons, but that they are involved only in the early stage of oxidation [3, 9]. Therefore, complete mineralization has been thoroughly studied using whole cell cultures. In these cases, cells produce radicals such as $O_2^{\cdot-}$ and $OH^{\cdot}$ as well as extracellular peroxidases, and the degradation efficiency is enhanced as well. In addition, veratryl alcohol (VA) and oxalate, which are produced as secondary metabolites, are known to be positive regulators of the enzyme activities [2, 16, 17, 19, 29]. Oxalate has also been reported to be involved in the production of radicals in the presence of LiP, VA, and H_2O_2, which enhances biodegradation efficiency [2, 17]. In addition, veratryl alcohol can activate LiPs deactivated by hydrogen peroxide and it results in the formation of a veratryl alcohol radical, which is also a positive effector for the biodegradation of recalcitrants [9, 29].

Although using whole cell cultures as a biodegradation system has many advantages, as mentioned above, some disadvantages such as difficulty in reuse of the cells adsorbed with recalcitrants and partial or complete inhibition of biodegradation due to toxicity of the recalcitrants can also be seen [20, 31]. These disadvantages have led us to search for a new system for recalcitrant degradation using a cell-free culture broth. In fact, separation of the cells and products, including enzymes, radicals, and other key components such as veratryl alcohol, hydrogen peroxide, and oxalate, may solve the difficulties arising from the use of whole cell

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culture. In addition, the development of many culturing methods, which reuse the cells for effective production of secondary metabolites [5, 21], give hope for use of this new biodegradation system in real applications.

Therefore, in this study, the optimum culture and production conditions for the biodegradation of 2,4,5-trichlorophenol as a model recalcitrant have been studied, by using a cell-free culture broth. Isozyme distribution of LiPs and MnPs and production of oxalate have been investigated in respect to different nitrogen concentrations. Finally, a possible mechanism for the degradation of 2,4,5-trichlorophenol by a cell-free culture broth has been proposed.

MATERIALS AND METHODS

Strain and Medium Compositions

P. chrysosporium ATCC 24725 was maintained at 39°C on malt-agar plates, and spores were harvested and filtered through glass wool. The spore concentration was determined by measuring the absorbance at 650 nm (an absorbance of 1.0 cm^{-1} is approximately 5×10^6 spores/ml). The growth medium had the same composition as used by Tien and Kirk [28], except that veratryl alcohol and Tween 80 were added after two days of growth. The nitrogen concentration ratio was modified as described in the Results and Discussion.

Cell Immobilization on Polyurethane Foam

The supporting matrix for immobilization was the polyurethane foam, which was synthesized in the Department of New Materials Science and Engineering at the Kwangju Institute of Science and Technology (K-JIST) [7, 8]. A total amount of 12 g of foam sliced into cubes (15 mm×15 mm×5 mm) were placed in each of 250-ml Erlenmeyer flasks and autoclaved at 121°C for 20 min. After inoculation of 200 ml of the medium with 1.2×10^8 spores, the cultures were flushed with pure oxygen once a day at the rate of 250 cc/min for 5 min.

Biodegradation of 2,4,5-Trichlorophenols using Cell-Free Culture Broths

2,4,5-Trichlorophenol (2,4,5-CP) was purchased from Sigma Culture broth harvested from the nitrogen-limited and nitrogen-sufficient cultures containing secondary metabolites were used to degrade 2,4,5-trichlorophenol. Each sample contained 0.5 ml of the cell-free culture broth, and 0.45 ml of sodium tartarate buffer containing 3.33 mM of veratryl alcohol (pH 2.5) and 0.05 ml of 10 mM H_2O_2. 2,4,5-Trichlorophenol to a final concentration of 0.2 mM (39.5 ppm) was added to the reaction mixture. In addition, a control sample containing fresh medium was constantly tested to compare the effect of medium components on the biodegradation of 2,4,5-trichlorophenol.

Measurements of Enzyme Activities and SDS-PAGE

Lignin peroxidase activity was measured with veratryl alcohol by the method described previously [28]. Equal volumes of extracellular fluid were concentrated 25-fold using membrane filters (Microcon-30 Amicon Co., U.S.A.), and the concentrates were subjected to sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 3% of stacking gel and 12% of running gel. Proteins were visualized by staining with Coomassie Brilliant blue.

Chemical Analysis

2,4,5-Trichlorophenol degradation was analyzed by HPLC (WATERS™, U.S.A.) with SUPELCOSIL™LC-8 column. A gradient between 1% of acetic acid in methanol and 1% of acetic acid in water (35:65 to 100:0 for 20 min, return to 35:65 for 5 min) with a flow rate of 1.2 ml/min was used to elute the compounds. The concentration of oxalate was measured by HPLC (WATERS™, U.S.A.) on a SUPELCOGEL™C-610H column with 1% phosphoric acid at a flow rate of 0.5 ml/min.

RESULTS AND DISCUSSION

Our previous studies [7, 8] showed that production of lignin peroxidase (LiP) was enhanced more than 10 times by immobilizing *P. chrysosporium* on polyurethane foams. This result provided us with the information to have a high concentration of LiP required in order to make an attempt to use cell-free culture broth for the degradation of chlorophenols. Therefore, all the culture broths used in this study were obtained from immobilized cell culture on polyurethane foams.

Effects of Nitrogen Concentrations on Batch Cultures

The growth rate of *P. chrysosporium*, which is indirectly estimated from the glucose consumption rate, is much faster in the nitrogen-sufficient medium (NS medium, 24 mM ammonium tartarate) than in the nitrogen-limited medium (NL medium, 0.2 mM ammonium tartarate) (Fig. 1a). The trends of LiP production as a secondary metabolite were quite similar in both cases with an exception for the delayed production in the NL medium (Fig. 1b). LiP activity in the NS medium appeared when glucose was exhausted and sharply decreased, probably due to protease activity, which also increased in the absence of glucose [27]. On the other hand, LiP was produced in the NL medium, due to the limitation of nitrogen, even in the presence of glucose, so LiP would be protected from protease activity and a relatively prolonged decay was observed (Fig. 1b). The average maximum production level (basis of volume) in NS medium (283 units/liter) was a little higher than that in NL medium (275 units/liter). The difference of the maximum LiP production between the

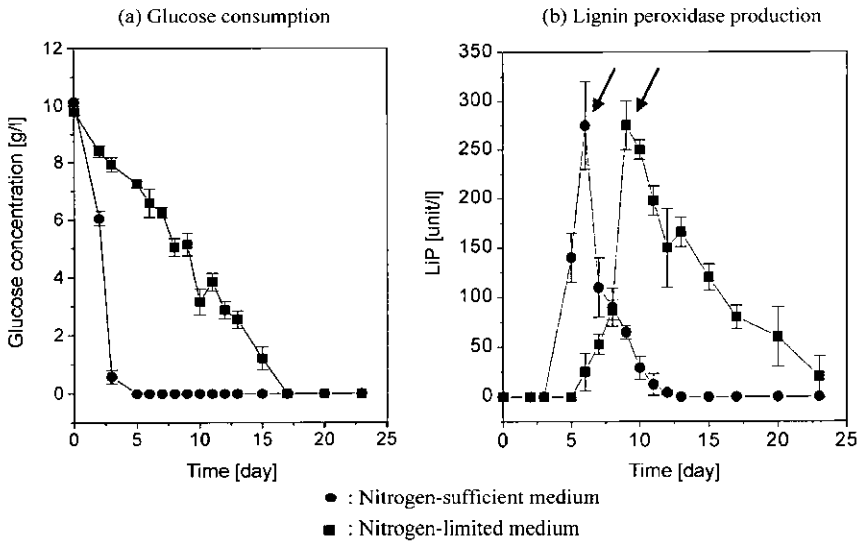

Fig. 1. The time course of cell growth represented by the glucose consumption and the production of lignin peroxidase in medium containing different nitrogen concentrations.

two cases did not seem to be very significant (within error range). This means that the maximum production of LiP was not affected by the concentration of nitrogen in the culture media.

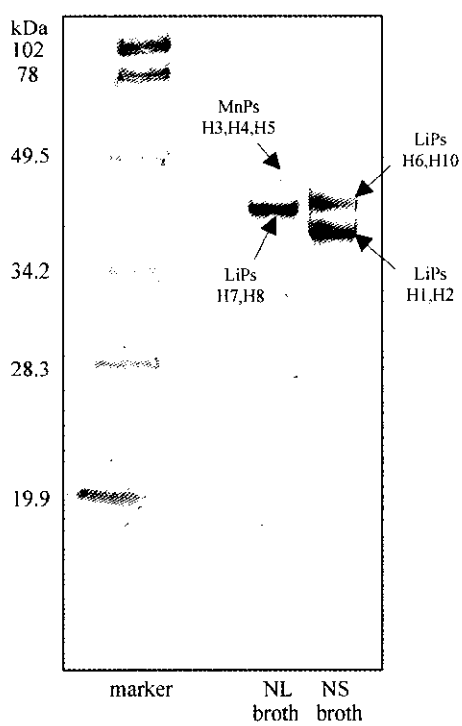

Fig. 2. SDS-PAGE showing the isozyme distribution.
Isozyme distribution of the samples taken from nitrogen-limited and nitrogen-sufficient culture medium at their maximum lignin peroxidase activity. Extracellular fluids (0.5 ml) were concentrated using membrane filters and subjected to SDS-PAGE using a 3% stacking gel and 12% running gel.

Isozyme Distribution Dependent on the Nitrogen Concentration

The isozyme distributions in both cases, however, were significantly different. Figure 2 shows the SDS-PAGE profile of isozymes distribution in the culture broths taken at the time when the maximum LiP activities were measured. Isozyme distributions (identified based upon protein sizes) for both cases seemed to be totally different: H1, H2, H6, and H10 (LiPs) appeared in NS medium but H3, H4, and H5 (MnPs) along with H7 and H8 (LiPs) were produced in NL medium (Fig. 2). It seemed that carbon limitation with NS medium caused the production of H1, H2, H6, and H10 (LiPs). On the other hand, nitrogen limitation with the NL medium stimulated the production of H3, H4, H5, (MnPs) as well as H7 and H8 (LiPs).

Comparison of Biodegradation Efficiency for 2,4,5-Trichlorophenol between NS and NL Broths

Degradation performances for both culture broths were compared with regards to their biodegradation of 2,4,5-trichlorophenol. Figure 3a shows the biodegradation kinetics of 2,4,5-trichlorophenol for both culture broths taken at the time of the maximum LiP activity shown on Fig. 1b. 2,4,5-Trichlorophenol was degraded only when the reaction mixture contained H_2O_2 and veratryl alcohol (data not shown), suggesting that 2,4,5-trichlorophenol degradation resulted from indirect VA^+ mediation in the presence of H_2O_2. The degradation efficiency, as represented by the disappearance of 2,4,5-trichlorophenol per unit culture volume applied, of NL culture broth was better than that of the NS broth, even though the LiP activity in the NL medium was slightly lower. One possible reason for this difference in efficiency

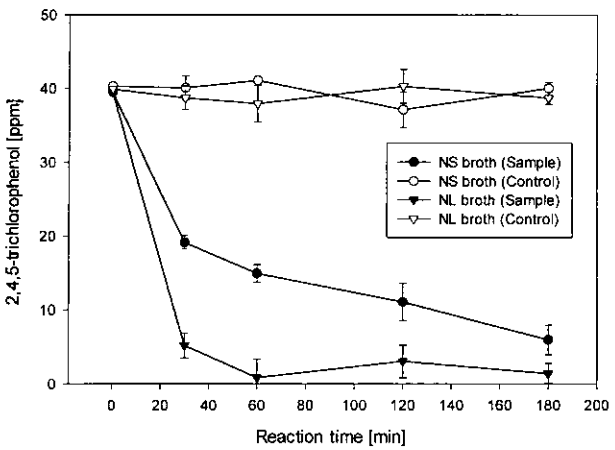

Fig. 3. The biodegradation kinetics of 2,4,5-trichlorophenol using nitrogen-limited and nitrogen-sufficient culture broths taken at the time of the maximum lignin peroxidase.

Each reaction mixture contained 0.5 ml culture broth taken from each culture condition (NS medium or NL medium), as well as 0.45 ml sodium tartarate buffer, containing 3.33 mM of veratryl alcohol (pH 2.5) and 0.05 ml of 10 mM H_2O_2. 2,4,5-Trichlorophenol was added to the reaction mixture to a final concentration of 0.2 mM. The reaction was initiated by adding H_2O_2. After set times, the reaction was stopped by adding acetonitrile and it was analyzed by HPLC. The disappearance rate of 2,4,5-chlorophenol was measured based upon the remaining percentage of 2,4,5-CP after set times.

might be due to the existence of MnP, because it was produced only in the NL medium as shown in Fig. 2. However, the MnP could not be a significant factor for the enhanced degradation, because the degradation conditions were adjusted for the optimum LiP activity (pH 2.5), but not for optimum MnP activity (pH 5) [10]. Therefore, this enhancement was most likely due to oxalate.

The Effect of Oxalate during Degradation

Oxalate, as mentioned briefly in the introduction, is thought to be another factor enhancing the biodegradation of phenolic compounds [2, 17]. Oxalate in the presence of

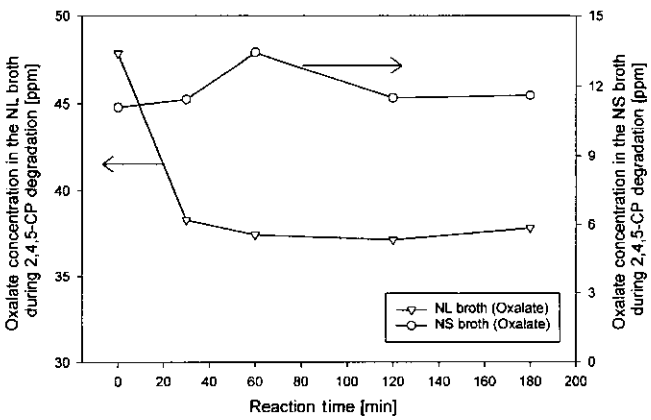

Fig. 4. The change in oxalate concentrations during the degradation reaction in NL broth and NS broth.

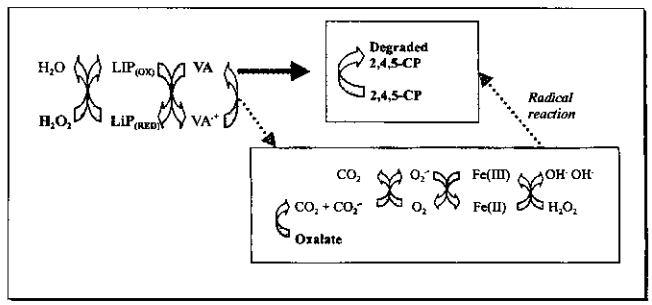

Fig. 5. The possible mechanism of 2,4,5-CP degradation using the cell-free culture broths [17].

LiP, VA, and H_2O_2 becomes a radical having a high biodegradation capability due to a relatively high redox potential [2]. This newly formed radical is involved in the degradation of the recalcitrants, which is not easily attacked directly by either LiP or MnP alone. The oxidation of oxalate to a radical was previously found to be severely inhibited during the biodegradation of phenol [24]. The reason for this inhibition is due to a higher substrate specificity of the veratryl alcohol cation radical, which prefers phenol to oxalate.

Interestingly enough, the initial levels of oxalate in both cases were significantly different; that is, about 4.5-fold higher in NL broth than in NS broth (Fig. 4). A decrease in the oxalate concentration during 2,4,5-CP degradation and the formation of some radicals from oxalate which perform some roles in the biodegradation were observed only in NL medium. Therefore, it would appear that a critical concentration existed where oxalate could be converted to radicals by VA^+ during 2,4,5-CP degradation. This would then explain why the oxalate concentration in the NS broth did not decrease (Fig. 4).

Possible Mechanism for 2,4,5-Trichlorophenol Degradation using Cell-Free Culture Broths

In the case of the NS broth, 2,4,5-trichlorophenol appeared to be degraded indirectly by VA^+ mediation without involvement of oxalate (see Fig. 5, solid arrow). It is proposed, however, that the NL broth can degrade 2,4,5-trichlorophenol through an oxalate-dependent radical reaction (see Fig. 5, solid arrow) as well as a VA^+-mediated indirect reaction (see Fig. 5, dotted arrow). Therefore, the NL broth is better in degrading 2,4,5-CP than the NS broth, since it contains LiPs and sufficient amount of oxalate necessary for producing radicals.

Acknowledgments

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