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短 報（Note）

# Selection of *Pleurotus pulmonarius* from domestic basidiomycetous fungi for biodegradation of chlorinated dioxins as environmentally persistent organopollutants

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**Abstract**

Screening of 174 strains of 20 native Japanese white-rot basidiomycetous fungi was carried out based on the decolorization rate of Remazol brilliant blue R, a persistent aromatic dye compound. A strain of *Pleurotus pulmonarius* was chosen on the basis of its decolorization rate. Since *P. pulmonarius* is cultivated in a scale of domestic outputs 250 tons in 2005 as an edible mushroom, the mushroom bed wastes appear to have the possibility of practical application of biologically treating dioxins on farmland in Japan. It was ascertained that the mycelium of *P. pulmonarius* spreads easily from the industrial culture bed wastes into unsterilized soils. Therefore, we assayed the fungal degradability of the following chlorinated dibenzo-*p*-dioxins: 2-monochloro-; 2,7-dichloro-; 1,2,4-trichloro-; 2,3,7,8-tetrachloro-; 1,2,3,7,8-pentachloro-; 1,2,3,4,7,8-hexachloro-; 1,2,3,4,6,7,8-heptachloro-; and octachloro-dibenzo-*p*-dioxins. *Pleurotus pulmonarius* degraded mono-, di-, and trichloro-dibenzo-*p*-dioxins to some extent (> 25%) and tetra-, penta-, hexa-, and hepta-dibenzo-*p*-dioxins to moderate extent (< 10%), whereas octachloro-dibenzo-p-dioxin was not degraded at all. The fungal metabolism of 2-monochloro-dibenzo-*p*-dioxin appeared to be initiated by biotransformation to hydroxylated monochloro-dibenzo-p-dioxin. These results suggest that the degradation of polychlorinated dibenzo-*p*-dioxins by *P. pulmonarius* is influenced by the chlorination pattern of the dibenzo-*p*-dioxins.

**Key words :** basidiomycetous fungi, bioremediation, polychlorinated dibenzo-*p*-dioxins

## Introduction

Together with cellulose and hemicellulose, lignin forms the cell walls of woody plants. Lignin is a three-dimensional phenylpropanoid polymer linked by different carbon-to-carbon and ether linkages between monomeric phenylpropane units, most of which are not considerably resistant to microbial degaradation. Thus, lignin is highly resistant to microbial degradation in comparison with polysaccharides and other natural biopolymers. Nevertheless, the white-rot basidiomycetous fungi have been found as typical lignin-degrading microorganisms (Higuchi, 1990).

In addition, some white-rot basidiomycetous fungi have been shown to degrade polychlorinated dibenzo-*p*-dioxins (Tachibana et al., 1996). Dioxins are serious organic environmental contaminants because they are persistent, highly toxic, and bioaccumulative, and have been noted to cause teratogenesis and thymic atrophy and to promote tumors (Rappe, 1980). Numerous studies have shown that white-rot basidiomycetous fungi have the ability to biologically remedy soils contaminated with dioxins and other pollutants (Bunz and Cook, 1993; Schreiner et al., 1997; Hong et al., 2002; Kamei et al., 2005).

The purpose of the present study was to select native basidiomycetous fungi for bioremediation, based on dye decolorization tests, and to analyze their ability to degrade polychlorinated dibenzo-*p*-dioxins. As a method of dealing with small amounts of dioxins broadly dispersed on farmland in Japan, biological treatments should be more cost-effective and benign than physical or chemical treatments. In addition, if culture bed wastes from the domestic mushroom industry can be used for the biological treatment, the microbial method will have the possibility of practical application. For bioremediation, using native organisms is absolutely essential to prevent ecological disturbance. Therefore, the most intensively studied white-rot fungus, *Phanerochaete chrysosporium*, which is an exotic in Japan, was omitted from this study.

## Materials and Methods

### Fungi

We obtained 174 strains of 20 species of edible white-rot basidiomycetous fungi that are native to Japan from the culture

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stocks of the Forestry and Forest Products Research Institute. The 20 fungal species are listed in Figure 1.

### Screening of fungi

The Remazol brilliant blue R (RBBR, Sigma Chemical Co., St. Louis, MO, USA) decolorization test (Pasti and Crawford, 1991; Tachibana *et al*., 1996; Sato *et al*., 2002) was used to screen the 174 strains of basidiomycetous fungi. Each strain was grown in a petri plate with a medium composed of 1% potato dextrose agar (Nissui Seiyaku, Japan) and 1.0% RBBR. The inoculated plates were incubated at 28°C for 14 days, and the level of decolorization was assessed. The strain showing the highest level of decolorization was chosen for the dioxin-degradation analyses.

### Analysis of degradation of chlorinated dibenzo-*p*-dioxins

The chosen strain (*Pleurotus pulmonarius*) was grown on a potato dextrose agar plate (9-cm diameter) at 28°C for 1 week. A small piece of the grown mycelium was transferred into a 100-ml Erlenmeyer flask containing 50 ml Kirk-LN liquid culture medium (low-nitrogen basal III medium [pH 4.5], composed of 1.0% glucose, 1.2 mM ammonium tartrate, and 20 mM sodium acetate; Tien and Kirk, 1998) and the culture medium containing inoculum was homogenized for 1 min. One milliliter of the homogenate was inoculated into a 50-ml Erlenmeyer flask containing 10 ml Kirk-LN liquid culture medium. The fungal culture was preincubated statically at 28°C in the ambient atmosphere for a week. After preincubation, a substrate in DMSO solution (100 µl) containing 2000 pg of chlorinated dibenzo-*p*-dioxin (either of the following ones: 2-monochloro-, 2,7-dichloro-, 1,2,4-trichloro-, 2,3,7,8-tetrachloro-, 1,2,3,7,8-pentachloro-, 1,2,3,4,7,8-hexachloro-, 1,2,3,4,6,7,8-heptachloro-, or octachloro-dibenzo-*p*-dioxin; Cambridge Isotope Laboratories, Inc., Cambridge, MA, USA) was added. The headspace of the flask was flushed with oxygen, and the flask was sealed with a glass stopper and sealing-tape and then incubated statically at 28°C for 1 month. After incubation, cultivation was stopped by adding 0.2 g of sodium azide. The cultivation in control was stopped by adding 0.2 g of sodium azide immediately after 1 week of preincubation.

After incubation, the culture medium was homogenized with 20 ml of acetone. After spiking with 17 species of $^{13}C_{12}$-labeled 2,3,7,8-substituted tetra-, penta-, hexa-, hepta-, and octa-chlorinated dibenzo-*p*-dioxin, the homogenized sample was mixed with 15 ml of ethanol and the mixture was sonicated for 15 min. For the first extraction cycle, the sonicated sample was mixed with 100 ml water and 50 ml dichloromethane and shaken to partition the polychlorinated dibenzo-*p*-dioxins into the dichloromethane layer. From this procedure, about 50 ml of dichloromethane extract was separated and stored in a clean flask. For the second and third cycles, 50 ml of dichloromethane was added to the crude layer of extract and the above procedure was repeated. The three dichloromethane extracts were combined and washed with water. The remaining water in the dichloromethane extracts was then removed using a $Na_2SO_4$ column, and the water-free extract was concentrated to a 20-ml volume using an evaporator. Some portion of extract sample was evaporated and dissolved in hexane. The residual extract was further subjected to column chromatographic procedures. The lipid was removed through multilayer silica gel column purification.

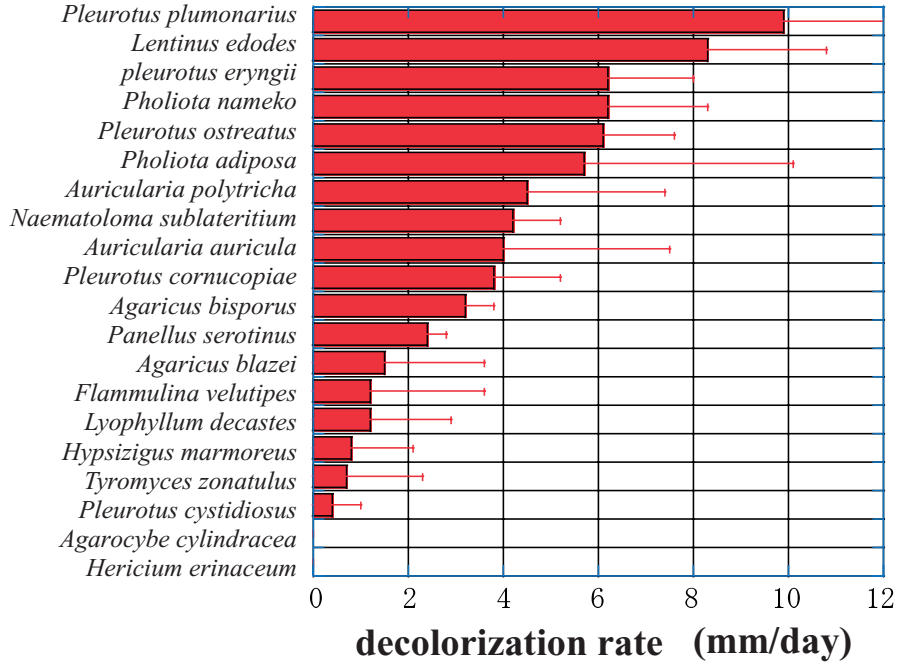


Fig. 1. Results of the RBBR decolorization tests for 20 species of basidiomycetous fungi

The purified sample was further concentrated to 100 µl. Prior to high-resolution gas chromatography/mass spectrometry (GC/MS) analysis, the sample was spiked with a syringe spike such as $^{13}$C-1,2,3,4-tetrachloro-dibenzo-*p*-dioxin for polychlorinated dibenzo-*p*-dioxins fractions.

Identification and quantification of polychlorinated dibenzo-*p*-dioxins were performed using a high-resolution GC (6890 Series, Hewlett Packard, ) coupled with a high-resolution MS (Micromass Autospec, Ultima; Micromass Ltd., Manchester, UK). Both an SP-2331 column (0.32 mm i.d. × 60 m length; SUPELCO,) and a DB-17HT column (0.32 mm i.d. × 30 m; J&W Scientific, Folsom, CA, USA) were used for separation. An autosampler (GC System Injector, Hewlett Packard) was employed for injection (2 µl, splitless). The temperature program was as follows: 160°C for 1 min, 6°C min$^{-1}$ to 220°C for 0 min, and 3°C min$^{-1}$ to 260°C for 30 min (SP-2331); 130°C for 1 min, 20°C min$^{-1}$ to 200°C for 0 min, 3°C min$^{-1}$ to 250°C for 0 min, and 5°C min$^{-1}$ to 300°C for 3 min (DB-17HT). For the quantification of mono-, di-, and trichloro-dibenzo-*p*-dioxins, the temperature program was as follows: 130°C for 1 min, 20°C min$^{-1}$ to 200°C for 0 min, 3°C min$^{-1}$ to 230°C for 0 min, and 10 °C min$^{-1}$ to 300°C for 3.5 min. The carrier gas was helium, and the electron impact ionization energy was 40 eV. The selected ion monitoring mode was used, and the resolution was kept higher than 10 000 (5% valley).

### Separation and identification of fungal metabolites

For the study of fungal metabolism of chlorinated dibenzo-*p*-dioxins, the fungal culture conditions were the same as described above, except the substrate concentration. After pre-incubation for 1 week, either of 2-monochloro-, 2,7-dichloro-, or 1,2,4-trichloro-dibenzo-*p*-dioxins (56, 64, and 73 µg, respectively) was added to the fungal culture in separate flasks, and each flask was incubated statically at 28°C for 2 weeks. After addition of acetone, the homogenate sample was separated into solid and fluid fractions by centrifugation. The fluid fraction was evaporated to remove the acetone under reduced pressure, acidified to pH 2.0 with 0.1 N HCl, and subjected to extraction three times with 40 ml ethyl acetate. The solid fraction was air-dried and then Soxhlet-extracted with ethyl acetate for 12 h. All the ethyl acetate extracts were combined, dried over anhydrous sodium sulfate, and evaporated to dryness. The concentrate of ethyl acetate extracts was separated by adsorption column on a silica gel cartridge (12 × 80 mm; Bond Elute Glass, GL Science, ). The silica gel column was eluted successively with n-hexane (10 ml), 20% ethyl acetate/80% n-hexane (10 ml), and 50% ethyl acetate/50% n-hexane (10 ml). The eluents with three solvents were collected separately and concentrated by evaporation of the solvents in a nitrogen atmosphere. Each eluted part was dissolved in n-hexane (50 µl) and subjected to analysis by GC/MS. For the methyl derivatization analysis of fungal metabolites (using diazo-methane) GC/MS was performed on an HP 6890 GC system-linked HP 5973 MS detector (Hewlett-Packard) and a 30-m fused DB-5 MS column (J&W Scientific). The oven temperature was programmed to increase from 80°C to 320°C at 20°C min$^{-1}$.

## Results

The results of the RBBR decolorization tests for the 20 fungal species are shown in Figure 1. White-rot fungi belonging to the genus *Pleurotus*, including *P. pulmonarius*, *P. eryngii*, *P. ostreatus*, and *P. cornucopiae*, were able to decolorize the RBBR to some degree. A strain of *P. pulmonarius* was the most active dye-decomposer among the 174 strains of native basidiomycetous fungi. For this reason, and because the mycelium of *P. pulmonarius* can spread easily into soils by inoculating a small amount of the culture bed wastes (Yamaguchi, 2002), we selected the strain of *P. pulmonarius* for the biological treatment of polychlorinated dibenzo-*p*-dioxins.

We investigated the fungal degradability of chlorinated dibenzo-*p*-dioxins by comparing the degradation rate of biologically treated substrates with that of the controls (Fig. 2). *Pleurotus pulmonarius* degraded 77% of 2-monochloro-dibenzo-*p*-dioxin, 29% of 2,7-dichloro-dibenzo-*p*-dioxin, and 27% of 1,2,4-trichloro-dibenzo-*p*-dioxin. The degradation rate of 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin, 1,2,3,7,8-pentachloro-dibenzo-*p*-dioxin, 1,2,3,4,7,8-hexachloro-dibenzo-*p*-dioxin, and 1,2,3,4,6,7,8-heptachloro-dibenzo-*p*-dioxin was less than 10%. Octachloro-dibenzo-*p*-dioxin was not degraded at all.

To investigate the fungal metabolic pathway, the ethyl acetate extract from the culture medium containing 2-monochloro-dibenzo-*p*-dioxin was analyzed by GC/MS. A molecular ion peak of 2-monochloro-dibenzo-*p*-dioxin was observed at m/z 218 (Fig. 3). After methylation of the ethyl acetate extract, a peak of monomethoxy-2-monochloro-dibenzo-*p*-dioxin at m/z 248 (molecular mass of 2-monochloro-dibenzo-*p*-dioxin [218] + 30 mass) and at m/z 233 (monomethoxy-2-monochloro-dibenzo-*p*-dioxin [248] - $CH_3$ [15]) were detected. These results suggest that 2-monochloro-dibenzo-*p*-dioxin was metabolized by *P. pulmonarius* to a compound having a hydroxyl residue at either site of 3, 7, or 8. In this condition, after methylation of ethyl acetate extracts from the culture medium containing 2,7-dichloro-dibenzo-*p*-dioxin or 1,2,4-trichloro-dibenzo-*p*-dioxin, however, no fragment ions corresponding to hydroxylation were detected.

## Discussion

Compared with other potential bioremediation systems, the extracellular, nonspecific, non-stereoselective lignin-degrading system has the advantage of being applicable to a variety of recalcitrant and toxic chemicals, and the unique lignolytic en-

zyme system of the basidiomycetous fungi is based on a highly reactive free-radical depolymerization mechanism. White-rot fungi have been reported to be ideal for the biodegradation of environmentally persistent organopollutants (Bumpus, 1985; Valli, 1992), and these studies have shown that the lignin peroxidases of white-rot fungi catalyze the oxidative cleavage of the aromatic ring of 2,7-dichloro-dibenzo-*p*-dioxin. Some lignin-degrading fungi decolorize RBBR dye (Pasti and Crawford, 1991; Tachibana *et al*., 1996), so we used the RBBR dye decolorization test for screening of fungi suitable to the biological treatment of polychlorinated dibenzo-*p*-dioxins.

The selected white-rot fungus, *P. pulmonarius*, was able to degrade 77% of 2-monochloro-dibenzo-*p*-dioxin, 29% of 2,7-dichloro-dibenzo-*p*-dioxin, and 27% of 1,2,4-trichloro-dibenzo-*p*-dioxin. The fungus could also degrade tetra-, penta-, hexa-, and hepta-dibenzo-*p*-dioxins to moderate extent (< 10%), but did not degrade octachloro-dibenzo-*p*-dioxin at all. These decrease less than 10% are regarded as absorption to fungal body or analysis errors. These results suggest that the lignin-degrading fungus does not degrade all polychlorinated dibenzo-*p*-dioxins in a similar manner: for instance, 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin was hardly degraded, perhaps because of the high degree of chlorine substitution and the deadly rank poison. Kamei and Kondo (2005) have reported that the degradation of polychlorinated dibenzo-*p*-dioxins depends on the chlorination pattern of the substrates. Likewise, the degradation of polychlorinated dibenzo-*p*-dioxins by *P. pulmonarius* also depended on the chlorination pattern.

White-rot fungi of the genus *Pleurotus* tended to decolorize the RBBR dye to some degree. Kamei and Kondo (2005) have proposed that the degradation of polychlorinated dibenzo-*p*-dioxins would be governed by the phylogenetic relationship of fungi, suggesting that all of *P. pulmonarius*, *P. eryngii*, *P. ostreatus*, and *P. cornucopiae* might have the ability to degrade polychlorinated dibenzo-*p*-dioxins. According to the Forestry Agency (2006), fruit bodies (250 tons) of *P. pulmonarius* were produced in Japan in 2005. This edible fungus is cultivated on mushroom beds consisting of wood meal and rice bran. The mushroom bed wastes, which may amount to 500 to 750 tons per year, are partly used for the production of compost. Based on our findings, and because the mycelium of *P. pulmonarius* can spread easily from industrial culture bed wastes into unster-

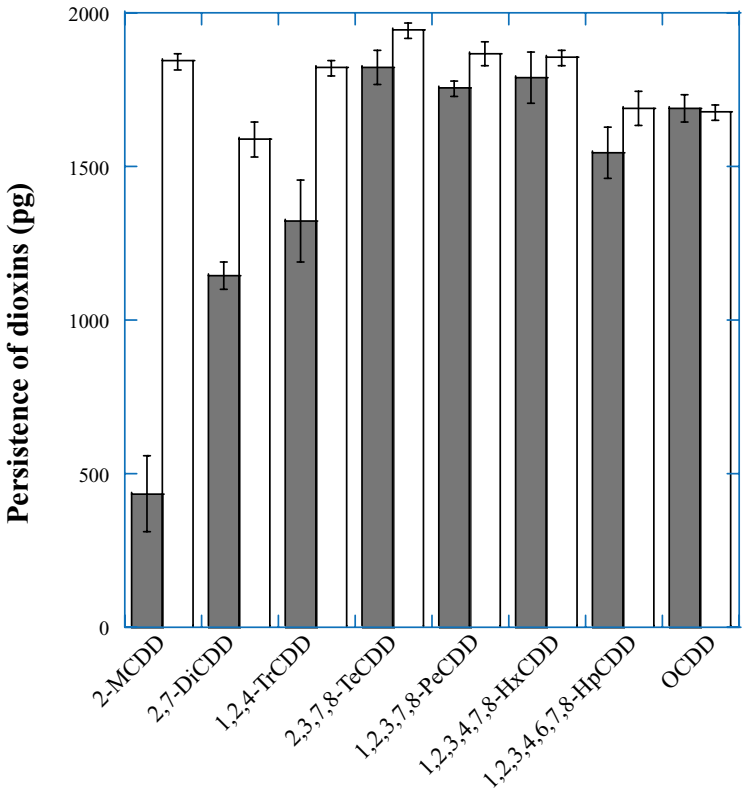


Fig. 2. Degradation of chlorinated dibenzo-*p*-dioxins by *Pleurotus pulmonarius*.
Persistence of dioxins after incubation with *P. pulmonarius* for 1 month (closed columns) versus that in the same medium without *P. pulmonarius* after 1 week of preincubation (open columns). MCDD, monochloro-dibenzo-*p*-dioxin; DiCDD, dichloro-dibenzo-*p*-dioxin; TrCDD, trichloro-dibenzo-*p*-dioxin; TeCDD, tetrachloro-dibenzo-*p*-dioxin; PeCDD, pentachloro-dibenzo-*p*-dioxin; HxCDD, hexachloro-dibenzo-*p*-dioxin; HpCDD, heptachloro-dibenzo-*p*-dioxin; OCDD, octachloro-dibenzo-*p*-dioxin.

ilized soils (Yamaguchi, 2002), the fungus may be helpful for the biological treatment of polychlorinated dibenzo-*p*-dioxins in polluted farmland. The development of DNA analysis to monitor the mycelium growth rate of *P. pulmonarius* in soils is under way.

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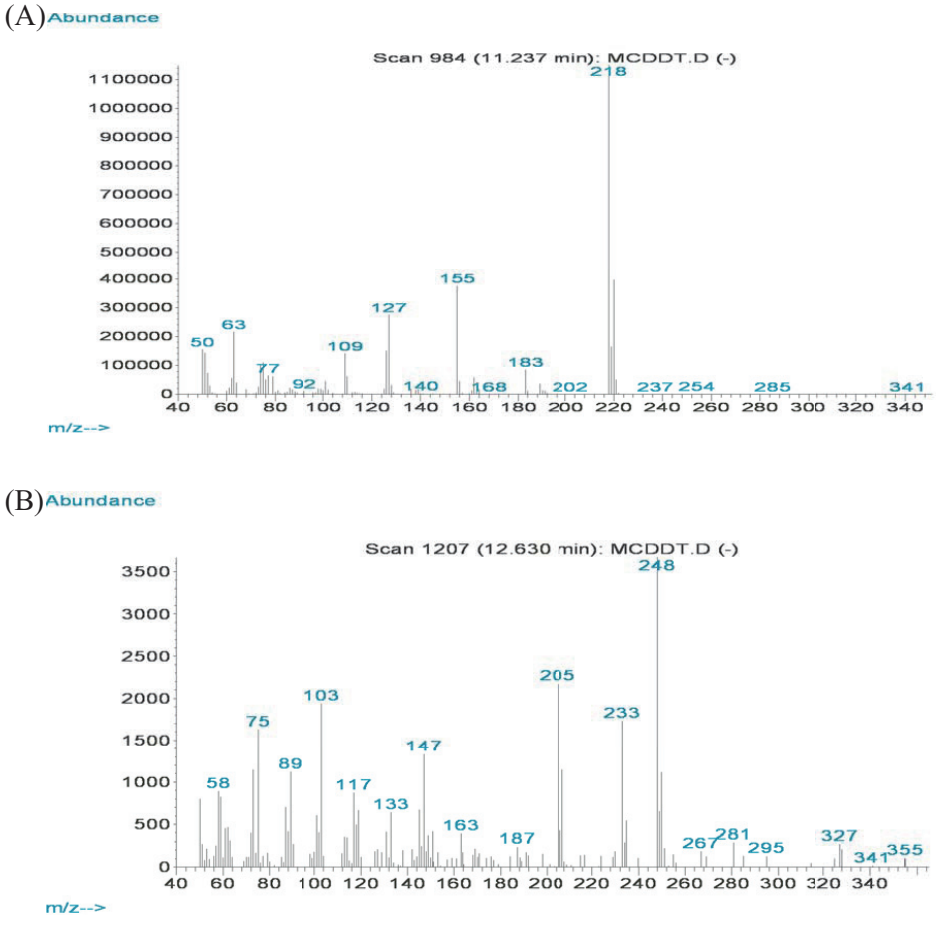


Fig. 3. (A) Mass spectra of the ethyl acetate extract from the culture medium containing 2-monochloro-dibenzo-*p*-dioxin. (B) Mass spectra of the methylation product of the ethyl acetate extract

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# 国内担子菌からの選抜による *Pleurotus pulmonarius*（ウスヒラタケ）のダイオキシン分解

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**要 旨**

国内の 20 種 174 菌株の選抜試験を、色素 RBBR の脱色の割合をもとに行った。脱色能の速さにより、*Pleurotus pulmonarius*　を選抜した。*P. pulmonarius* は食用きのことして 2005 年度には国内において 250t 生産され、日本の農地におけるダイオキシンの生物的処理の現実的な適用としては、きのこ生産過程で排出される廃菌床を利用することが有効であると考えられる。*P. pulmonarius* は、菌床を未滅菌土壌に混和することで容易に菌糸が蔓延することが確認されている。したがって、我々は *P. pulmonarius* の塩素化ダイオキシン（2-、2,7-、1,2,4-、2,3,7,8-、1,2,3,7,8-、1,2,3,4,7,8-、1,2,3,4,6,7,8-、オクタ塩素化ダイオキシン）の分解能を測定した。*P. pulmonarius* は 1 塩素化、２塩素化、３塩素化ダイオキシンを約 25% 以上分解し、4 塩素化、5 塩素化、6 塩素化、7 塩素化ダイオキシンを約 10% 以下であるが分解した。一方、８塩素化ダイオキシンは全く分解しなかった。2- 塩素置換ダイオキシンの代謝物を同定したところ、水酸化された 1 塩素化ダイオキシンが検出された。これらの結果より、*P. pulmonarius* による多塩素置換ダイオキシン分解はダイオキシンの塩素置換数によって影響されると考えられる。

**キーワード**：担子菌、バイオレメディエーション、多塩素置換ダイオキシン

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