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Bioremediation of Dioxin-Contaminated Soil by Fungi Screened from Nature

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Abstract: To degrade dioxins in contaminated soil, bioremediation was conducted with two fungi (PL1 and 267) screened from nature. A comparison of the concentration of dioxins (Toxicity equivalent quantity) before and after the bioremediation revealed 20 to 90% of dioxins in the soil to be degraded in 15 and 30 days, respectively. Maximum degradation (90%) was obtained with PL1 after 30 days in the presence of 0.1% surfactant.

Key words: Dioxin-contaminated soil, bioremediation by fungi, purification of contaminated soil

INTRODUCTION

Environmental pollution caused by endocrine disrupting chemicals (environmental hormones) such as dioxins discharged from incinerators and polychlorinated biphenyls (PCBs) leaked from transformers has become a major social problem (Onabe, 1991; Kearney *et al*., 1973; Hanson, 1991). The amount of dioxins discharged into the environment has been greatly reduced by measures to reduce levels of dioxins in discharge from incinerators as well as to newly determine the TDI (Tolerance Daily Intake) (Zoller and Ballschmiter, 1986; Neubert, 1991). However, toxic effects on the environment have been reported (Yanagibashi, 1999; Takeuchi *et al*., 1999). Therefore, several methods of dealing with environmental pollution caused by dioxins discharged from incinerators and so on have been proposed (Corbet *et al*., 1983; Watts *et al*., 1991; Sako, 1999), including the use of ultraviolet light (Corbet *et al*., 1983), chemical reagents like hydrogen peroxide (Watts *et al*., 1991) and super critical water (Sako, 1999). But, all these methods apply only to pollution concentrated in a small area. Further, they are expensive because the contaminated soils must be sent to institutions capable of treating dioxins.

However, bioremediation with microorganisms does not require that polluted soils are sent to institutions capable of degrading dioxins (Alexander, 1994). So, bioremediation is considered a most effective method of dealing with widespread pollution involving a low concentration of pollutant (Tachibana, 1999; Tachibana *et al*., 2003). For efficient bioremediation, it is necessary to isolate microorganisms with greater ability to degrade dioxins. A screening method was described earlier for fungi able to degrade dioxins and the biodegradation of three kinds of dioxins, 2, 7-Dichlorodibenzo-*p*-Dioxin (2, 7- DCDD) (Tachibana *et al*., 1996), 2,8-Dichlorodibenzo-*p*-Dioxin (2,8-DCDD) (Miyoshi *et al*., 2005), 2,4,8-Trichlordibenzofuran (2,4,8-TCDF) and 2,7-DCDD (Tachibana *et al*., 2005), with fungi screened from nature. Furthermore, the present study described the bioremediation of 2,7-DCDD and 2,4,8-TCDF (Tachibana *et al*., 2005) and 2,3,7,8-TCDD (Tachibana *et al*., 2006) in soil with fungi. Furthermore, we clarified that 2,7-DCDD, 2,4,8-TCDF and 2,3,7,8-TCDD were indeed degraded by confirming the presence of intermediates (Tachibana *et al*., 2005, 2006).

In the present study, the purification of dioxin-contaminated soil by bioremediation with fungi screened from nature was conducted.

MATERIALS AND METHODS

Reagents: A mixture of ^{12}C- and ^{13}C-containing polychlorodibenzo-*p*-dioxins (PCDDs), 2,3,7,8-T4CDD, 1,2,3,7,8-P5CDD,1,2,3,4,6,7,8-H6CDD,1,2,3,6,7,8-H6CDD, 1,2,3,7,8,9-H6CDD, 1,2,3,4,6,7,8-H7CDD, 1,2,3,4,6,7,8,9-O8CDD and ten polychlorodibenzofurans (PCDFs), 2,3,7,8-T4CDF, 1,2,3,7,8-P5CDF, 2,3,4,7,8-P5CDF, 1,2,3,4,7,8-H6CDF,1,2,3,6,7,8-H6CDF,2,3,4,6,7,8-H6CDF 1,2,3,4,6,7,8-H7CDF, 1,2,3,4,6,7,8-H7CDF, 1,2,3,4,7,8,9-H7CDF and 1,2,3,4,6,7,8,9-O8CDF, were purchased from Wako Pure Chemical Industry Co., Ltd.

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Bioremediation of dioxin-contaminated soil with fungi screened from nature: Test fungi: PL1 and 267 screened from nature by Tachibana *et al.* (1996) were used as test fungi.

Test soil: Soil collected from a paddy filed in a suburb of Matsuyama city was used as a test soil. The characteristics of the soil were as follows: pH: 5.38; water content: 34.8%; organic content: 9.4%.

Preparation of a solid medium: The solid medium was prepared by the method described in a previous report (Tachibana *et al.*, 2003).

Incubation: The solid medium (75 g) (dry weight) was added to the soil (225 g) (dry weight) in a plastic box with a lid, mixed in and pre-incubated for 7 days at 25°C in the dark (Tachibana *et al.*, 2005). The incubation was conducted for 15 and 30 days at 25°C in the dark as described previously (Tachibana *et al.*, 2005; Tachibana *et al.*, 2006). Soil to which no solid medium had been added was used as a control.

Extraction: The extraction was conducted as described earlier (Tachibana *et al.*, 2005, 2006). The treated soil (30 g) (dry weight) put in a 300 mL conical beaker, to which was added an ethanolic KOH solution (2N, 60 mL), was agitated vigorously for 1 h at room temperature. After standing overnight in the dark, the mixture was filtrated to separate residue and filtrate. The residue was extracted for 16 h with toluene by a Soxhlet extractor. A few milliliters of toluene were obtained after the concentration. The solution was made to redissolve in *n*-hexane (60 mL). In contrast, the filtrate was extracted three times with *n*-hexane (60 mL). The two *n*-hexane solubles were combined and washed with *n*-hexane-saturated water until the water layer became acidic. After the addition of 10 ng of ^{13}C-PCDDs/PCDFs dissolved in 100 μL, the *n*-hexane solution was concentrated under reduced pressure to a small volume. The concentrated solution was applied to a multi-layer silica gel column (Lamparski *et al.*, 1979) and eluted with *n*-hexane (50 mL) followed by a mixture of *n*-hexane and dichloromethane (1:1) (40 mL). The eluate from *n*-hexane and dichloromethane (1:1) was purified by chromatography using a silica gel column with 100 mL of *n*-hexane. The eluate from *n*-hexane was concentrated and subjected to chromatography with an alumina column using *n*-hexane (40 mL) and a mixture of *n*-hexane and dichloromethane (1:1) (30 mL). The eluate from the mixture of *n*-hexane and dichloromethane (1:1) was concentrated to 4 or 10 mL to obtain a solution for analysis.

Analysis: The solution obtained above was analyzed by GC-MS using the method of JIS (Japanese Industrial Standards) (Hobo, 2001).

Effect of addition of fungi on bioremediation of dioxin-contaminated soil:
Test fungi: The two fungi (PL1 and 267) described above were used as test fungi.

Test soil: The soil described above was used as test soil.

Preparation of a solid medium: The solid medium was prepared by the method described in an earlier report (Tachibana *et al.*, 2003).

Incubation: Each solid medium (15 and 30 g) (dry weight) was added to each soil (285 and 270 g) (dry weight) and pre-incubated for 7 days at 25°C in the dark (Tachibana *et al.*, 2005, 2006). The incubation was conducted for 15 and 30 days at 25°C in the dark as described above. Soil to which no solid medium had been added was used as a control.

Extraction and analysis: The extraction was conducted as described above. The solution obtained by silica gel column chromatography was analyzed by GC-MS.

Effect of addition of surfactant on bioremediation of dioxin-contaminated soil with fungi: Test soil, test fungi, solid medium: Soil, fungi and solid medium were used as described above.

Incubation: Fifteen milliliters of surfactant (Tween 80) (0, 0.05, 0.1 and 0.25% of soil) was added to the contaminated soil (270 g) (dry weight). Each solid medium (30 g) (dry weight) was added to each contaminated soil containing surfactant (0, 0.05, 0.1 and 0.25%). The pre-incubation and incubation were conducted as described above.

Extraction and analysis: The extraction was conducted as described above. The solution obtained by silica gel and alumina column chromatography was analyzed by GC-MS.

Enzyme activities during bioremediation with fungi:
Assay of protein content: Protein content was measured by the method of Lowry (Samejima, 1985).

Measurement of lignin peroxidase activity: Lignin peroxidase (LiP) activity was measured by the method of Tien and Kirk (1984). The extracellular crude enzyme solution (1 mL) was added to a solution of veratryl

alcohol (0.4 mM), sodium tartrate buffer (0.1 M, pH 3.0) and Tween 80 (0.1%). After the addition of hydrogen peroxide (0.15 mM) to the enzyme solution, the reaction was started. The reaction was carried out at 37°C and the absorbance at 310 nm was measured. Lip activity was calculated from the change in absorbance (Molar extinction coefficient ε: 9300 mol^{-1} cm^{-1}).

Measurement of manganese peroxidase activity: Manganese peroxidase (MnP) activity was measured by the method of Perie and Gold (1991). The crude enzyme solution (1 mL) was added to a solution of 2, 6-Dimethoxyphenol (1 mM), $MnSO_4$ (1 mM) and malonate buffer (50 mM, pH 4.5). After the addition of hydrogen peroxide (0.2 mM) to the solution, the reaction was started. The reaction was carried out at 28°C and the absorbance at 470 nm was measured. The MnP activity was calculated from the change in absorbance (Molar extinction coefficient ε: 49600 $mol^{-1}cm^{-1}$).

Measurement of laccase: Laccase (Lac) activity was measured by the method of Leonowcz and Grzywnowicz (1981). An ethanol solution of Syringaldazine (0.5 mM, 0.2 mL) and 1.5 mL of acetate buffer (0.1 M, pH 5.3) were mixed and then added to 1.8 mL of crude enzyme solution and the reaction was started. The reaction was carried out at 20°C and the absorbance at 525 nm was measured. The Lac activity was calculated from the change in absorbance (Molar extinction coefficient ε: 6500 $mol^{-1}cm^{-1}$).

RESULTS AND DISCUSSION

Biodegradation of dioxins in contaminated soil by bioremediation with fungi screened from nature: Each congener of dioxins in the contaminated soil could be degraded by bioremediation with 267 (Fig. 1). The concentration of dioxins in the soil used here was 61.5 pg-TEQ g^{-1} soil. Among the dioxins, 2,3,7,8-T4CDF was degraded the most effectively with 58% removed over 30 days (Fig. 1). For the other dioxin congeners, the rate of degradation was only 5 to 30%. Still, purification of the contaminated soil by bioremediation with fungi screened from nature was possible, though the rate of degradation was not so high, possibly because the amount of 267 added to the soil was small. To improve the rate of degradation, an increase in the amount of fungus added was considered necessary. So, effect of added amount of the fungus on degradation ratio of dioxins in the dioxin-contaminated soil was investigated.

Effect on the degradation of dioxins in contaminated soil of the amount of fungus used for bioremediation: The rate at which all seventeen kinds of dioxins were degraded increased as the amount of the fungus and length of the treatment time increased (Table 1). The degradation rates increased with the amount of fungus added (Fig. 2). Maximum degradation of dioxins (90%) was obtained when the bioremediation was conducted with PL1 for 30 days with the fungus composing 10% of the soil. It was shown that the amount of dioxins in the contaminated soil

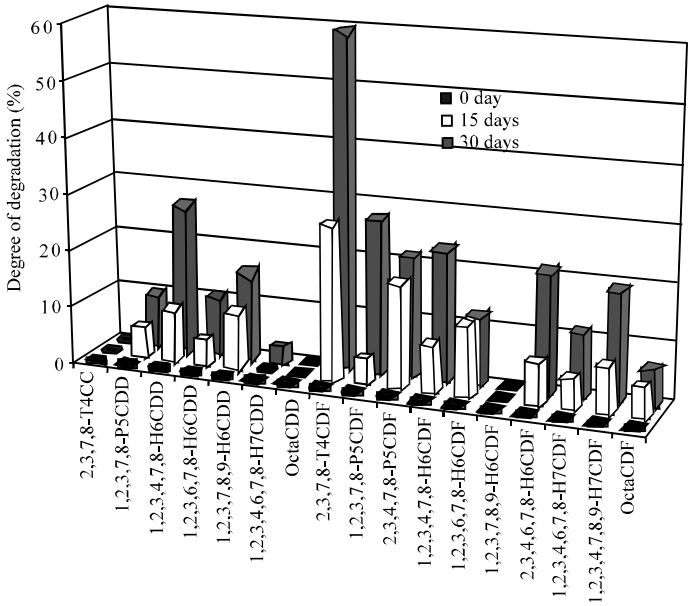

Fig. 1: Change in the rate of degradation of each congener of PCDD/Fs in dioxin-contaminated soil by bioremediation with fungus 267

Table 1: Rate of degradation of each congener of PCDD/Fs in dioxin-contaminated soil by bioremediation with PL1 for 15 and 30 days

	Added amount of fungus (% of the untreated soil)	
Dioxins	5	10
2,3,7,8-T4CDD	- (-)	- (-)
1,2,3,7,8-P5CDD	22.8 (38.2)	66.2 (76.3)
1,2,3,4,7,8-H6CDD	19.2 (38.3)	64.8 (75.6)
1,2,3,6,7,8-H6CDD	25.0 (37.5)	65.9 (79.4)
1,2,3,7,8,9-H6CDD	33.7 (37.7)	64.7 (79.3)
1,2,3,4,6,7,8-H7CDD	24.2 (38.2)	66.1 (79.0)
OctaCDD	25.0 (38.4)	66.0 (80.0)
2,3,7,8-T4CDF	- (-)	- (-)
1,2,3,7,8-P5CDF	- (-)	- (-)
2,3,4,7,8-P5CDF	- (-)	- (-)
1,2,3,4,7,8-H6CDF	23.3 (39.6)	70.2 (100)
1,2,3,6,7,8-H6CDF	29.6 (38.1)	57.2 (100)
1,2,3,7,8,9-H6CDF	- (-)	- (-)
2,3,4,6,7,8-H6CDF	26.8 (40.0)	65.7 (82.3)
1,2,3,4,6,7,8-H7CDF	24.6 (36.9)	65.0 (70.2)
1,2,3,4,7,8,9-H7CDF	25.0 (37.5)	100 (100)
OctaCDF	22.5 (38.8)	67.4 (67.4)

Note: The figure in parentheses shows the degradation rate for each congener of PCDD/Fs in dioxin-contaminated soil on bioremediation with PL1 for 30 days

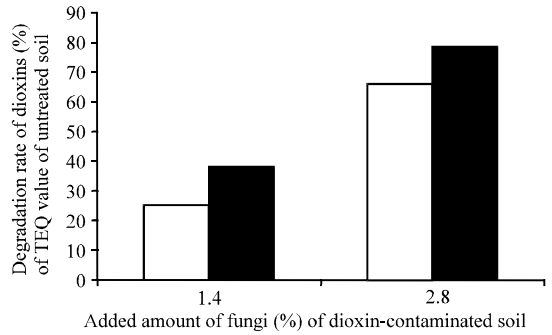

Fig. 2: Degradation of dioxins in contaminated soil by bioremediation with PL1 Notes: □: 15 days; ■: 30 days

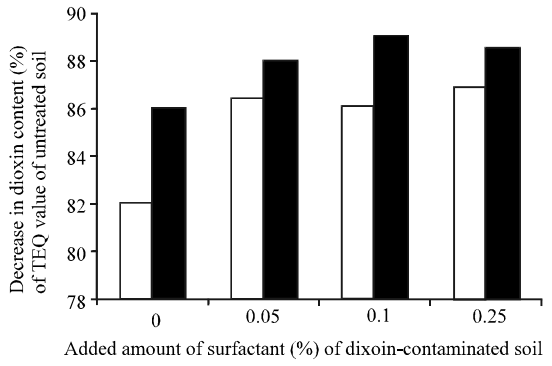

Fig. 3: Degradation of dioxins in contaminated soil by bioremediation with PL1 in the presence of surfactant Notes: □: 15 days; ■: 30 days

was markedly reduced by PL1 on the addition of the fungus (10%) to the soil. Further, a correlation between the increase in the rate of degradation of dioxins and increase in the amount of fungus added to the soil was recognized.

Bioremediation of dioxin-contaminated soils with *Terrabacter* sp. was conducted for 7 days at 35°C by Habe *et al.* (2002). The degradation rate for the congeners of dioxins (PCDD/Fs) was 0 to 15%. This is a relatively low rate in comparison with that obtained here. It is said that the ability of bacteria to break down dioxins is inferior to that of fungi like PL1 (Tachibana, 1999). Potential of bioremediation of xenobiotic compounds such as chlorinated organic compounds like as dioxins and PCBs (polychlorinated biphenyls), simple and polycyclic aromatic hydrocarbons and so on by using the white-rot fungus *Phanerochaete chrysosporium* were reported by Paszczynski and Crawford (1995).

From the results obtained here, it is clear that the purification of dioxin-contaminated soil by bioremediation with fungi screened from nature is possible.

Effect of surfactant on bioremediation of dioxin-contaminated soil with PL1: In comparison with the control (no addition of surfactant), an increase in the amount of surfactant added to the contaminated soil was found to increase the rate of degradation of dioxins (Fig. 3). Respectively, 82, 86, 86 and 87% of dioxins were break down when bioremediation using PL1 was conducted for 15 days with 0, 0.05, 0.1 and 0.25% surfactant added to the soil. Further, when the bioremediation was carried out for 30 days, these rates increased to 86, 88, 90 and 89%, respectively. The results showed that the rate of degradation of dioxins in the soil rose as the amount of surfactant added increased. Tween 80, the surfactant used here, reportedly functions as a kind of mediator in the biodegradation of lignin (Ehara *et al.*, 2000), especially the degradation of lignin by manganese peroxidase (MnP). The mechanism by which MnP breaks down lignins has been reported by Wariishi and Gold (1988) and Higuchi (1993). They showed the catalytic cycle of this enzyme. A proposed mechanism for the degradation of dioxins by MnP is shown in Fig. 4. The surfactant is considered to act as a mediator during the bioremediation of soil with PL1. Therefore, the rate of degradation was increased by addition of the surfactant. The degradation of dioxins reached a maximum at 0.1% surfactant and any further addition of the surfactant was ineffective. However, the rate of degradation on the addition of surfactant increased 5 to 10% in comparison with the control. Therefore, some compounds responsible for the degradation of dioxins with MnP functioning as a mediator, may be present in the soil.

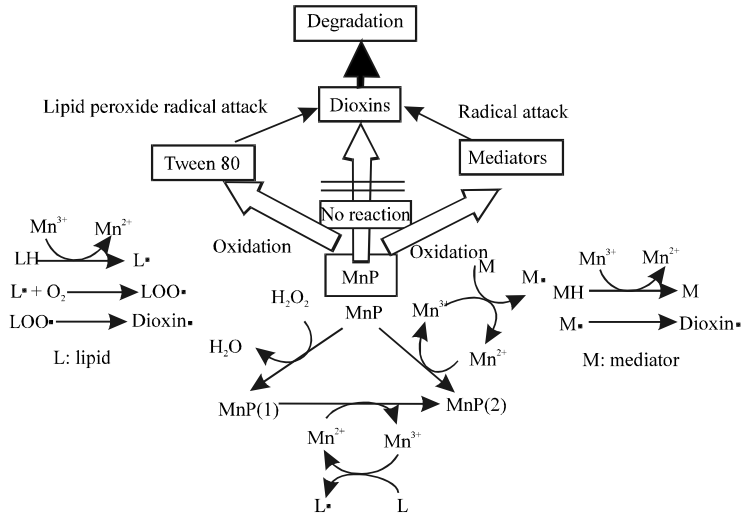

Fig. 4: A proposed mechanism for degradation of dioxins with MnP. Sources: Ehara *et al.* (2000) and Higuchi (1993)

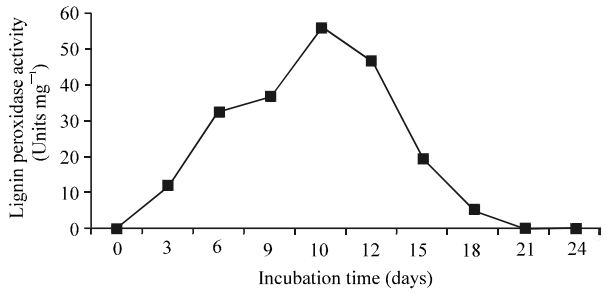

Fig. 5: Change in lignin peroxidase activity in dioxin-contaminated soil on bioremediation with PL1

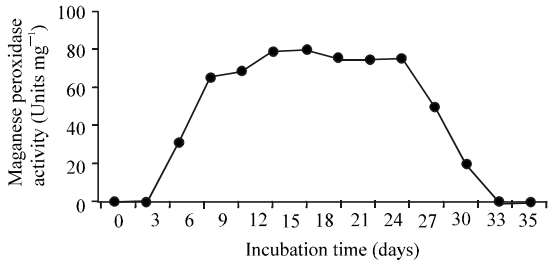

Fig. 6: Change in manganese peroxidase activity in dioxin-contaminated soil on bioremediation with PL1

Relationship between enzymatic activity during bioremediation with PL1 and rate of degradation: To examine the relationship between enzymatic activity and the rate at which dioxins are broke down during bioremediation, the activity of three kinds of enzymes, Lip, MnP and Lac, responsible for the degradation of dioxins (Higuchi, 1993; Tachibana, 1999) was measured. Lip activity appeared 3 days after the bioremediation started (Fig. 5). It reached a maximum in 12 days and decreased thereafter. No Lip activity was recognized after 24 days. In contrast, MnP activity began 6 days after the bioremediation started (Fig. 6) and continued for 21 days. However, it decreased after 27 days. Lac activity was not recognized during the bioremediation.

From the results obtained here, PL1 began to degrade dioxins in the soil in three days. Lip was responsible for the degradation in the initial stages of bioremediation. However, it was considered that MnP was mainly responsible for the degradation of dioxins.

It was shown that 60-90% of dioxins in contaminated soil were degraded by bioremediation with PL1 over 30 days. The maximal rate of degradation (90%) was obtained when the bioremediation was conducted for 30 days in the presence of 0.1% surfactant. The rate of degradation increased as the amount of fungus added increased. An effect of surfactant on the degradation of dioxins by PL1 was recognized. However, the maximal concentration of surfactant that could be added to the soil was 0.1%. Regarding the enzymatic activity in the soil during the bioremediation process, Lip activity was observed in the initial stages and MnP activity later on. MnP may therefore play an important role in the degradation of dioxins in soil.

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