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Biodegradation of 2,7-Dibenzo-*p*-Dixoin and 2,4,8-Trichlorodibenzofuran in Soil by Fungi Screened from Nature

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Abstract: Microbial degradation of 2,7-Dichlorodibenzo-*p*-Dioxin (2,7-DCDD) and 2,4,8-Trichlorodibenzofuran (2,4,8-TCDF) with four fungi (PL1, PL2, PC and 267) screened from nature was conducted to select fungi for the bioremediation of dioxins in soil. The four fungi degraded 63 to 96% of 2,7-DCDD and 24 to 78% of 2,4,8-TCDF, respectively. Maximal degradation (96 and 78%) was obtained with PL1 when the incubation was conducted for 30 days after addition of 0.25 mM of 2,7-DCDD and 2,4,8-TCDF to the culture medium, respectively. Two fungi (PL1 and 267) were selected for bioremediation experiments. In 15 and 30 days, 267 degraded 37 to 63% of 2,7-DCDD and 18 to 58% of 2,4,8-TCDF, present at 1 and 10 ppm in soil. Maximal degradation (63 and 58%) was obtained with 267 when the bioremediation was conducted for 30 days after addition of 10 ppm of 2,7-DCDD and 2,4,8-TCDF to the soil, respectively. Furthermore, the degradation of 2,7-DCDD was enhanced using a solid medium. Also, 2,7-DCDD and 2,4,8-TCDF were indeed degraded by the fungi (267 and PL1), because 4-Chlorocatechol and 3,5-Dichlorosalicylic acid and 5-Chlorosalicylic acid, considered to be the intermediates in the bioremediation of 2,7-DCDD and 2,4,8-TCDF, respectively, were detected among the reaction products.

Key words: 2,7-Dichlorodibenzo-*p*-Dioxin, 2,4,8-Trichlorodibenzofuran, microbial degradation, bioremediation by fungi

INTRODUCTION

Pollution caused by environmental hormones such as dioxins discharged from incinerators has become a major social problem[1-3]. Measures have been taken to reduce the amount of dioxins discharged from incinerators and so on and to newly determine the TDI (Tolerance Daily Intake)[4,5]. However, toxic effects on the environment have been reported[3]. Furthermore, several methods of dealing with environmental pollution caused by dioxins have been proposed[6-8], including the use of ultraviolet light[6], chemical reagents like hydrogen peroxide[7] and super critical water[8] and so forth. All of these methods apply to the pollution concentrated in a small area, but are expensive because the contaminated soils must be sent to institutions capable of treating dioxins. However, bioremediation with microorganisms does not require polluted soils to be sent to institutions capable of degrading dioxins[9]. So, bioremediation is considered a most effective method of dealing with widespread pollution involving a low concentration of pollutant[10,11]. For efficient bioremediation, it is necessary to obtain microorganisms with greater ability to degrade dioxins. Previously, we described a method of screening for fungi able to degrade dioxins and the biodegradation of 2,8-Dichlorodibenzo-*p*-Dioxin (2,8-DCDD) with fungi screened from nature[12].

The present report describes the biodegradation of 2,7-DCDD and 2,4,8-TCDF, two kinds of dioxins, for the selection of fungi suitable for bioremediation of dioxins in soil. Furthermore, the bioremediation of 2, 7-DCDD and 2,4,8-TCDF in soil with the screened fungi was tried.

MATERIALS AND METHODS

This research project was carried out in the Faculty of Agriculture, Ehime University, Japan during 2000-2003 in an effort to degrade dioxins for the bioremediation of contaminated soil using fungi.

Fungi used for testing: Two fungi (PL2 and PC) screened in the present report and two fungi (PL1 and 267) already screened by Tachibana *et al.*[13] were used as test fungi.

Screening of fungi capable of degrading dioxins: Thirty samples of decayed wood and mushroom were collected

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from forests near Matsuyama city and Ozu city, Ehime Prefecture, Japan. About 1 g of sample was suspended with 10 mL of sterilized water by vigorous shaking for 20 sec. After standing for 30 min, the supernatant (1 mL) was diluted to 10^3 to 10^4 by addition of sterilized water. One milliliter of the solution was spread on the surface of an agar medium containing RBBR prepared by the method of Tachibana *et al.*[13]

Reagents: 2,4,8-TCDF was purchased from Wako Pure Chemical Industry Co., Ltd. 3,5-Dichlorobenzoic acid, 3,5-Dichlorosalicylic acid, 5-Chlorosalicylic acid, 2-Bromo-4-chlorophenol, Bis (2-ethoxyethyl) ether, Ethylenediacetate and 4-Chlorocatechol were purchased from Tokyo Kasei Kogyo Co., Ltd.

Synthesis of 2,7-DCDD: 2,7-DCDD was synthesized from 2-Bromophenol (25 mM) (5.19g) by the method of Aniline[14] in a yield of 39%. (3.18g, white crystal, mp 208-210°C) (Lit mp 209-210°C)[14]. MS (m/z): 256 (M +4), 254(M^++2), 252(M^+) (base), 217, 189, 126.

Degradation of 2,7-DCDD and 2,4,8-TCDF by screened fungi

Test fungi: Four kinds of fungi (PL1, PL2, PC and 267) screened from nature in the present report and by Tachibana *et al.*[13] were used as test fungi.

Liquid culture: Sodium succinate (20 mM), glucose (2%) and ammonium tartrate (1.2 mM) as a nitrogen source were added to Kirk's basal solution[15]. After the basal solution was adjusted to a pH of 4.5, 20 mL of the culture medium was added to an Erlenmeyer flask (100 mL) and was sterilized with an autoclave for 20 min at 121°C[13]. After cooling on a clean bench, the solution was inoculated with each of the four test fungi mentioned above and pre-incubated standing for 6 days at 25°C in the dark.

Addition of 2,7-DCDD and 2,4,8-TCDF: 2,7-DCDD [1.26mg (0.25 mM)] and 2,4,8-TCDF [1.4mg (0.25 mM)] dissolved in 200 μL of N, N- dimethylformamide (DMF) and 200 μL of Tween 80 (10% solution) were added to a flask which had been pre-incubated in advance. After addition of the dioxins, incubation was conducted for 15 and 30 days at 25°C in the dark, with oxygen purged every day.

Extraction of metabolites: After a fixed period of culture, HCl (1N, 5 mL) was added. The extraction of metabolites was conducted as described previously[12].

Analysis of the extracts: The extracts were analyzed using gas chromatography and mass spectrometry (GC-MS)[9]. A Shimadzu GC-MS system (QP-5050A) equipped with a capillary column, TC-1 (internal diameter 0.25 mm, length 30 m) was used. The conditions for the analysis were as follows: Temperature; after 1 min at 100°C, raised to 260°C at 10°C min^{-1} and then maintained at 260°C for 10 min. Detector and injection temperature: 250°C, carrier gas: He, split less; MS condition: ionized voltage: 70 eV.

Identification of intermediates in the biodegradation of 2,7-DCDD and 2,4,8-TCDF

Liquid culture: The culture medium[15] was prepared as described above. After inoculation of the medium with four fungi, the culture was pre-incubated by standing for 7 days at 25°C in the dark.

Addition of 2,7-DCDD and 2,4,8-TCDF: 2,7-DCDD [1.26 mg (0.25 mM)] and 2,4,8-TCDF [1.4 mg (0.25 mM)] dissolved in 200 μL of N, N-dimethylformamide (DMF) and 200 μL of Tween 80 (10% solution)[13] were added to each culture medium as described above. The incubation was conducted for 15 and 30 days at 25°C in the dark.

Extraction and purification of the extracts: The extracts were purified using silica gel column chromatography by successive elution with *n*-hexanes, *n*-hexane and dichloromethane (1:1 v/v) and ethanol and then *n*-hexane and dichloromethane solubles and ethanol solubles were obtained as mentioned in a previous report[9].

Analysis: After vacuum drying of the *n*-hexane and dichloromethane solubles and ethanol solubles (100 μL) in a vial, N, O- bis-trimethylsilyl acetamide (40 μL), trimethylchlorosilane (20 μL) and pyridine (40 μL) were added, respectively. Trimethylsilylation of the solubles was conducted for 10 min at 80°C without contact with moisture[9,13]. The trimethylsilyl (TMS) derivatives of the extracts were analyzed by the method described above. GC/MS was done under the conditions described above.

Bioremediation of 2,7-DCDD and 2,4,8-TCDF in soil

Test soil: Soil was collected from the Ehime University Experimental Forest. The characteristics of the soil[16] were as follows: pH: 5.30; water content: 38.5%; organic substance quantity: 9.4%.

Test fungi: Two kinds of fungi (PL1 and 267) screened from nature were used as test fungi.

Incubation: Six hundred grams of soil (dry weight) collected from the experimental forest and wood meal (12 g, dry weight) (size: 40 mesh) prepared from Buna

(*Fagus crenata*) wood were added to a plastic box with a lid and mixed[9]. Afterwards, the glucose agar medium[13] in which each fungus grew well in a plastic petri dish (diameter 9 cm) was removed from the dish and put on the surface of the soils every week. This process was repeated for 3 weeks in order to incubate the two fungi in the soil. After that, 2,7-DCDD (2.38 and 23.8 mM) and 2,4,8-TCDF (2.22 and 22.2 mM) dissolved in DMF (2 mL) and 50 mL of Tween 80 (1% solution) were uniformly added to the soil. After addition of the dioxins, incubation was carried out for 15 and 30 days at 25°C in the dark. Samples of soil with no dioxins added or with dioxins but no fungi were used as controls.

Extraction and purification of the extracts: Ten grams of the soil (dry weight) was extracted for 16 h with toluene using a Soxhlet extractor. The extracts were then dissolved in *n*-hexane (60 mL) and transferred to a separatory funnel and the *n*-hexane solution was washed several times with concentrated sulfuric acid (20 mL). The *n*-hexane layer was concentrated under reduced pressure and the extracts were subjected to chromatography on an alumina column. After elution of the column with 60 mL of *n*-hexane, the fraction eluted with 40 mL of a *n*-hexane and dichloromethane mixture (1:1) was collected to obtain the extracts[9].

Analysis: The extracts were analyzed by GC/MS and 2,7-DCDD and 2,4,8-TCDF was determined quantitatively[9].

Extraction of metabolites: Ten grams of the soil (dry weight) was extracted for 16 h with ethanol using the Soxhlet extractor[9]. The extracts were the subjected to chromatography on a silica gel column. After elution of the column with 60 mL of *n*-hexane, the fractions eluted with 40 mL of a *n*-hexane and dichloromethane mixture (1:1) and 40 mL of ethanol were collected to obtain solubles[9]. Each soluble was trimethylsilylated as described above. The derivatives of each soluble were analyzed by GC/MS. As intermediate compounds derived from 2,4,8-TCDF, 3,5-Dichlorobenzoic acid, 2,5-Dihydroxybenzoic acid, 3,5-Dichlorosalicylic acid and 5-Dichlorosalicylic acid were identified based on comparisons of mass spectrum and retention time with those of authentic samples. In the case of 2,7-DCDD, 4-Chlorocatechol and 1,2,4-Trihydroxybenzene considered as intermediates were identified in the same way.

Bioremediation of 2, 7-DCDD in soil by the fungus PL1 using a solid medium

Test soil: The soil described above section was used.

Test fungus: PL1 was used as a test fungus.

Preparation of a solid medium: Eight percent of a nutrient mixture suitable for growth of the fungus[9] was added to a mixture of two different sizes of oak woodchip (1:1) (10 mesh size greater than 10 and less than 40) and the water content was adjusted to 65%[9]. After sterilization of the medium with an autoclave for 120 min at 121°C, PL1 fungus was inoculated into the medium and a seal was made using a sealer. The medium was incubated for 20 days at 25°C in the dark.

Incubation: The solid medium (100 g) (dry weight) prepared above was added to the soil (300 g) (dry weight) in a plastic box, mixed and pre-incubated for 10 days at 25°C in the dark[9]. After addition of the substrates [2,7-DCDD and 2,4,8-TCDF (1 ppm and 10 ppm)], incubation was carried out for 15 and 30 days at 25°C in the dark[9]. In addition, samples with no dioxins added and dioxins in soil containing no solid medium were used as controls.

Extraction: The treated soil (30 g) (dry weight) was put in a 300 mL conical beaker, an ethanolic KOH solution (2N, 60 mL) was added and the mixture was agitated vigorously for 1 h at room temperature as described in a previous paper[9]. After standing for overnight in the dark, filtration was done to separate residue and filtrate. The residue was extracted for 16 h with toluene using the Soxhlet extractor[9]. The extracts were dissolved in *n*-hexane (60 mL). The filtrate was extracted thrice with *n*-hexane. The *n*-hexane solution from the filtrate and residue was combined and washed with *n*-hexane-saturated water until the water layer became acidic. After the drying of the solution with anhydrous sodium sulfate, the solution was concentrated under reduced pressure to obtain the extracts.

Analysis: The extracts were analyzed by GC/MS as described above.

RESULTS AND DISCUSSION

Screening of fungi capable of degrading dioxins: From the 30 samples of decayed wood and mushroom, 14 fungi were obtained by the screening method described in a previous paper[13]. Two of the fungi, PL2 and PC, from decayed wood and mushroom, respectively, were found to be highly capable of degrading dioxins. According to microscopic observations, PL2 and PC seemed to be Basidiomycetes because they have a clamp connection in their mycelium[17]. PC obtained from mushroom was judged

to be *Pyncoporus coccineus* based on morphological characteristics of the mushroom[18]. However, PL2 could not be identified because no fruiting body formed.

Itoh *et al.*[19] reported a linear relationship between the ability to degrade dioxins and the activities of lignin-degrading enzymes such as lignin peroxidase (Lip) and manganese peroxidase (MnP). A comparison of the growth rates of PL2 and PC on the agar medium containing RBBR with those of PL1 and 267 already screened from nature by Tachibana *et al.*[13] revealed that the two fungi screened in the present report have a similar ability to degrade dioxin. The results obtained above, four fungi (PL1, PL2, PC and 267) capable of degrading dioxins were screened from nature.

Degradation of 2,7-DCDD and 2,4,8-TCDF by the fungi screened from nature: PL1 degraded 69 and 96%, while PL2 degraded 63 and 89%, of 2,7-DCDD at 0.25 mM in 15 and 30 days, respectively (Table 1). PC degraded 69 and 82% , while 267 degraded 72 and 91%, of 2,7-DCDD at 0.25 mM in 15 and 30 days, respectively (Table 1). The maximal degradation rate was 72% when 267 was incubated for 15 days. The maximal degradation rate was 96% when PL1 was incubated for 30 days. Tachibana *et al.*[13] reported that six fungi (P.C, C.V, F.S, 563, V1 and V2) degraded 51 to 90% and 59 to 82% of 2,7-DCDD. The degradation rate obtained here was a little higher than the rates reported in that paper[13]. Joshi and Gold[20] found that *Phanerochaete chrysosporium*, a kind of Basidiomycete, degraded about 35% of 2,7-DCDD. Their report[20] also suggested that it became more difficult to degrade dioxins as the number of chlorine atom substituted for hydrogen atoms in the Dibenzo-*p*-Dioxin structure increased when the biodegradation was conducted in a liquid medium.

PL1 degraded 37 and 78%, while PL2 degraded 24 and 64%, of 2,4,8-TCDF at 0.25 mM in 15 and 30 days, respectively (Table 2). PC degraded 31 and 76%, while 267 degraded 40 and 69%, of 2,4,8-TCDF at 0.25 mM in 15 and 30 days, respectively (Table 2). The maximal degradation rate was 40% when fungus 267 was incubated for 15 days. The maximal degradation rate was 78% when PL1 was incubated for 30 days. Ohkawa *et al.*[21] reported that five fungi (P.C, C.V, 563, V1 and V2) screened from nature degraded 27 to 38% and 60 to 76% of 2,4,8-TCDF in 15 and 30 days at 0.25 mM in a liquid medium, respectively. The degradation rate obtained here was almost the same as those rates[21].

The four fungi (PL1, PL2, PC and 267) used here degraded 2,7-DCDD and 2,4,8-TCDF at a higher rate in the liquid medium. Judging from the results obtained here, PL1 and 267 have a similar ability to degrade dioxin.

Table 1: Degree of degradation of 2,7-Dichlorodibenzo-*p*-Dioxin (0.25 mM) with fungi screened from nature

	Degree of degradation (%)	
Fungus	15 days	30 days
PL1	69	96
PL2	63	89
PC	69	82
267	72	91

Table 2: Degree of degradation of 2,4,8-Trichlorodibenzofuran (0.25 mM) with fungi screened from nature

	Degree of degradation (%)	
Fungus	15 days	30 days
PL1	37	78
PL2	24	64
PC	31	76
267	40	69

Table 3: Degree of degradation of 2,7-Dichlorodibenzo-*p*-Dioxin in soil by bioremediation with fungus 267

	Degree of degradation (%)	
Concentration (ppm)	15 days	30 days
1	37	47
10	56	63

Table 4: Degree of degradation of 2,4,8-Trichlorodibenzofuran in soil by bioremediation with fungus 267

	Degree of degradation (%)	
Concentration (ppm)	15 days	30 days
1	18	48
10	22	58

Therefore, PL1 and 267 were used as test fungi for the bioremediation of dioxins in soil.

Bioremediation of 2,7-DCDD and 2,4,8-TCDF in soil using fungi screened from nature: 267 degraded 37 and 56% of 2,7-DCDD at 1 ppm and 47 and 63% at 10 ppm, respectively (Table 3). Similarly, 267 degraded 18 and 48% of 2,4,8-TCDF at 1 ppm and 22 and 58% at 10 ppm, respectively (Table 4). The maximal degradation rate for 2,7-DCDD was 63% when 267 was incubated for 30 days at 10 ppm. The maximal degradation rate for 2,4,8-TCDF was 58% when 267 was incubated for 30 days at 10 ppm. These results show that dioxins present in soil can be degraded by bioremediation with fungi screened from nature.

Identification of intermediates in the reaction of 2,7-DCDD and 2,4,8-TCDF with fungi: To confirm the degradation of 2,7-DCDD and 2,4,8-TCDF in soil with fungi, the identification of intermediates was conducted. Valli *et al.*[22] reported the pathway of degradation of 2,7-DCDD by *Phanerochaete chrysosporium* (Fig. 1). Ohkawa *et al.*[21] reported a proposed pathway for the

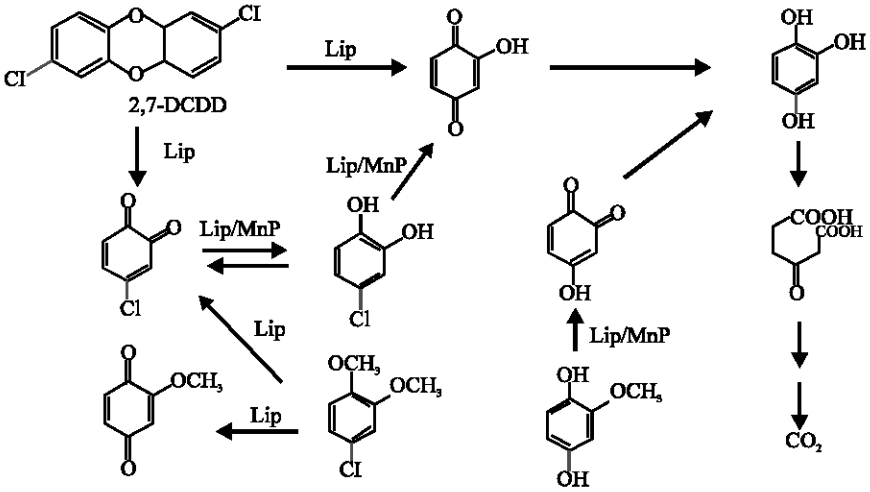

Fig. 1: Pathway of degradation of 2,7-Dichlorodibenzo-*p*-Dioxin (2,7-DCDD) by *Phanerochaete chrysoporium.*[*1]
Lip: Kignin peroxidase, MnP: Manganese peroxidase
Source: Valli *et al.*[22]

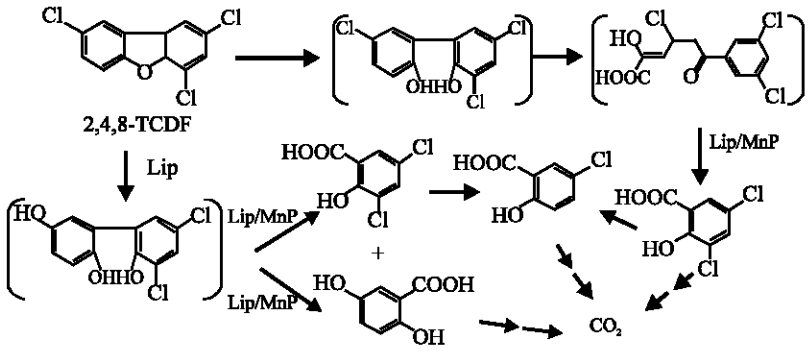

Fig. 2: A proposed pathway for the degradation of 2,4,8 Trichlorodibenzofuran by fungi screened from nature[*2]
Lip: Lignin peroxi dase; MnP: Manganese peroxidase
Source: Ohkawai *et al.*[21]

degradation of 2,4,8-TCDF by fungi screened from nature (Fig. 2). According to the degradation pathway for 2,7-DCDD, 4-Chlorocatechol and 1,2,4-Trihydroxybenzene were considered to be major intermediates (Fig. 1). Identification of these compounds in the extracts using the method described in the experimental section was conducted. In the GC profile, the trimethylsilyl (TMS) ether of 4-Chlorocatechol was recognized as described in a previous report[13]. The mass spectrum of the TMS ether of the compound coincided with that of the authentic TMS ether of 4-Chlorochatechol. The retention time also coincided with that of the authentic TMS ether of 4-Chlorochatechol. Therefore, 4-Chlorocatechol was identified in the extracts, however, 1,2,4-Trihydroxybenzene could not be identified. This compound may be easily metabolized to another compound by the fungi during the incubation in comparison with 4-Chlorocatechol as described in the biodegradation of 2,8-DCDD[12]. However, it was confirmed that 2,7-DCDD in soil was actually degraded by the fungi because 4-Chlorocatechol was detected as an intermediate.

In the pathway of degradation of 2,4,8-TCDF proposed by Ohkawa *et al.*[21], 3,5-Dichlorosalicylic acid, 5-Chlorosalicylic acid and 2,5-Dihydroxybenzoic acid are major intermediates as shown in Fig. 2. In the GC profile, trimethylsilyl (TMS) ether of 3,5-Dichlorosalicylic acid and 5-Chlorosalicylic acid were recognized. The mass spectrum of the TMS ether of 5-Chlorosalicylic acid is shown in Fig. 3. The mass spectra of TMS ethers of these compounds coincided with those of the authentic TMS ethers of 3, 5-Dichlorosalicylic acid and 5-Chlorosalicylic

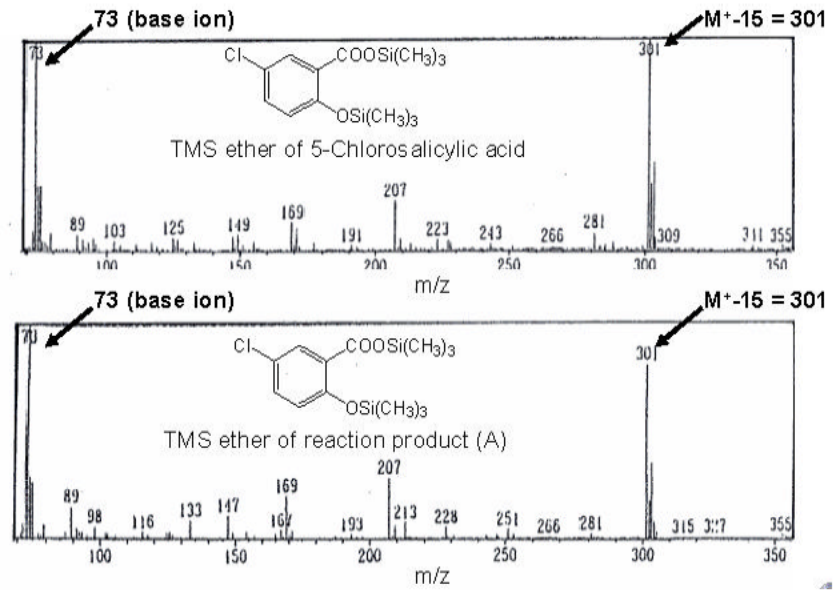

Fig. 3: Mass spectra of the trimethylsilyl (TMS) ether of 5-Chlorosalicylic acid in the TMS derivatives of the reaction product from bioremediation of 2,4,8-Trichlorodibenzofuran with fungi screened from nature
Upper spectrum is the mass spectrum of the TMS ether of authentic 5-Chlorosalicylic acid, lower spectrum is the mass spectrum of the TMS ether of reaction product (A) from bioremediation of 2,4,8-Trichlorodibenzofuran with fungi screened from nature

acid, respectively. The retention time of each TMS ether of these compounds also coincided with that of the authentic TMS ethers of 3,5-Dichlorosalicylic acid and 5-Chlorosalicylic acid, respectively. Therefore, 3, 5-Dichlorosalicylic acid and 5-Chlorosalicylic acid were identified in the extracts, however, 2,5-Drihydroxybenzoic acid could not be identified. Ohkawa *et al.*[21] reported that 3,5-Dichlorosalicylic acid and 2,5-Dihydroxybenzoic acid as intermediates in the biodegradation of 2,4,8-TCDF with fungi screened from nature in a liquid medium were identified by GC/MS analysis. In the present report, 5-Chlorosalicylic acid was newly identified in the extracts, however, 2,5-Dihydroxybenzoic acid found in the previous report[21] was not identified. 2,5-Dihydroxybenzoic acid may be easily metabolized to another compound by the fungi used here during the bioremediation in comparison with 3,5-Dichlorosalicylic acid as described for the biodegradation of 2,4,8-TCDF[9,21]. However, it was confirmed that 2,4,8-TCDF in soil was actually degraded by the fungi screened from nature because 3,5-Ddichlorosalicylic acid and 5-Chlorosalicylic acid were detected as intermediates.

Bioremediation of 2,7-DCDD in soil with fungi screened from nature using a solid medium: PL1 degraded 60 and 71% of 2,7-DCDD at 1 ppm in 15 and 30 days, respectively (Table 4). Furthermore, it degraded 61 and 70% of 2,7-DCDD at 10 ppm in 15 and 30 days, respectively (Table 4). The maximal degradation rate was 71% when PL1 was incubated for 30 days at 1 ppm. The results obtained here show that it is possible to degrade dioxins in soil by bioremediation with fungi screened from nature. It is said that the most important factor in the bioremediation of dioxin-contaminated soil with microorganisms is time[23]. From the results obtained here, however, it is possible to shorten the degradation period and to increase the degradation rate in bioremediation with fungi screened from nature by a solid medium.

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