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Biodegradation of 2, 8-Dichlorodibenzo-*p*-Dioxin by Fungi Screened from Nature

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Abstract: A screening of fungi capable of degrading dioxins was carried out using the dye Remazol brilliant blue R, as an indicator. Thirty four fungi were found by screening of 255 samples of decayed wood and soil collected from forests. Among them, R-60, R-67 and MG caused a greater decolorization of the dye than any other fungi separated by the screening. Microbial degradation of 2, 8-Dichlorodibenzo-***p***-Dioxin (2, 8-DCDD) was conducted with these three fungi and with two fungi (PL1 and 267) already screened. The five fungi degraded 50 to 90% of 2, 8-DCDD. Maximum degradation (90%) was obtained with 267 when the incubation was conducted for 30 days after addition of 0.05 mM of 2, 8-DCDD to the culture medium. The rate of degradation rose with the increase in the manganese peroxidase activity in an extracellular crude enzyme solution from each of the five fungi. The levels of enzymatic activity of the three fungi were also similar to those of the two previously screened fungi. The effect of co-cultivation of the screened fungi on degradation of the dioxin was also investigated. However, the degradation was less than that on the cultivation of each fungus alone. Furthermore, 2, 8-DCDD was indeed degraded by the fungi, because 4-Chlorocatechol considered to be an intermediate in the degradation of 2, 8-DCDD was detected among the reaction products.

Keywords: Screening, wood-rotting fungi, 2, 8-Dichlorodibenzo-*p*-Dioxin, microbial degradation, bioremediation by fungi

INTRODUCTION

Environmental pollution caused by dioxins discharged from incinerators and so forth has become a major social problem[1-3]. The amount of dioxins discharged from incinerators and so on has been greatly reduced, though toxic effects on the environment are reported[3]. In addition, several methods of dealing with environmental pollution caused by dioxins discharged from incinerators and so on have been proposed[4-6]. All these methods apply to pollution concentrated in a narrow area. However, bioremediation with the use of microorganisms is considered a most effective method for dealing with widespread pollution involving a low concentration of pollutant[7,8]. For efficient bioremediation, it is necessary to obtain microorganisms with good ability to degrade dioxins.

The present report describes the screening of fungi able to degrade dioxins[9] as well as the biodegradation of 2, 8-Dichlorodibenzo-*p*-Dioxin (2, 8-DCDD), a kind of dioxin, by the fungi. Furthermore, the co-cultivation of a fungus screened in the present report and a fungus already screened from nature[9] was tried to enhance the efficiency of the bioremediation.

MATERIALS AND METHODS

This research project was carried out in the Faculty of Agriculture, Ehime University, Japan during 2003-2005 in an effort to screen for fungi capable of degrading dioxins for the bioremediation of contaminated soils. Remazol brilliant blue R (RBBR), Benomyl and Tween 80 were purchased from Sigma Chemical Co., Ltd., Nihon Nohyaku Co. Ltd. and Wako Pure Chemical Industry Co., Ltd., respectively. 2 8-Dichlorodibenzo-*p*-Dioxin (2, 8-DCDD) was synthesized from 2, 4, 4'-Trichloro-2'-hydroxydiphenyl ether as described in a previous report[9].

Fungi used for testing: Three fungi (R-60, R-67 and MG) screened in the present report and two fungi (PL1 and 267) already screened by Tachibana *et al.*[9] were used as test fungi.

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Screening of fungi capable of degrading dioxins: Two hundred and fifty five samples of decayed wood and soil were collected from forests near Matsuyama city, Ehime Prefecture, Japan and Ehime University Agricultural Experimental Forest, respectively. 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.*[9]

Enzymatic activities of fungi used for testing

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[10]. After the basal solution was adjusted to a pH of 4.5, it was sterilized with an autoclave for 20 minutes at 12°C. After cooling on a clean bench, each of the five test fungi mentioned above was inoculated in the solution and incubated standing for a fixed period (4, 7, 15, 22, 30 and 37 days) at 25°C in the dark. Furthermore, PL1 and MG, or 267 and MG were inoculated into the solution and stood for a fixed period (4, 7, 15, 22, 30 and 37 days) at 25°C in the dark.

Preparation of extracellular crude enzyme solutions: Culture solutions of each fungus were filtered through a membrane filter. The filtrate obtained was used as an extracellular crude enzyme solution. In addition, the amount of protein in the solution was measured by the method of Lowry[11].

Measurement of lignin peroxidase activity: Lignin peroxidase (LiP) activity was measured by the method of Tien and Kirk[12]. The extracellular crude enzyme solution (1 mL) was added to a solution of veratryl alcohol (0.4 mM), sodium tartrate buffer solution (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[13]. 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 activity: Laccase (Lac) activity was measured by the method of Leonowcz[14]. 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}).

Degradation of 2, 8-DCDD by screened fungi

Liquid culture: The liquid medium was prepared as described above PL1, 267, R-60, R-67 and MG as test fungi were inoculated into the liquid medium. The pre-incubation was carried out for 7 days at 25°C in the dark.

Addition of 2, 8-DCDD: 2, 8-DCDD (0.25 mM and 0.05 mM) dissolved in 200 μL of N, N- dimethylformamide (DMF) and 200 μL of Tween 80 (10% solution) was added to a flask which had been pre-incubated. After addition of the dioxin, the 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 (1 N, 5 mL) was added. Ethyl acetate (20 mL) was then added and the culture was agitated vigorously for 30 min with a stirrer. Then, the culture was stood overnight at room temperature. After that, it was filtrated through a buchuner funnel which was placed on filter paper weighed beforehand and divided into the fungal body and culture filtrate. The fungal body which remained on the filter paper was wrapped in aluminum foil and dried for one day at 65°C in an oven. The culture filtrate was transferred to a separatory funnel and extracted with ethyl acetate. The organic layer was removed and then ethyl acetate was further added to the water layer and extracted. The organic layer was combined and then dried over anhydrous sodium sulfate. After drying, the fungal body was cut into small pieces, put it in an Elenmeyer flask and then extracted with ethyl acetate overnight at room temperature. The organic layer was removed by decantation and then the extraction repeated one more time. The two organic layers were combined and dried over anhydrous sodium sulfate. The organic layer from the filtrate and fungal body were combined and then concentrated under reduced pressure to obtain extracts.

Analysis of the extracts: The extracts obtained above were analyzed using gas chromatography and mass spectrometry (GC-MS). The GC-MS analysis was conducted using a Shimadzu GC-MS system (QP-5050A) equipped with a capillary column, TC-1 (internal diameter 0.25 mm, length 30 m). The conditions for the analysis were as follows: Temperature; after 1 min at 100°C, raised to 260°C at 10°C/min and then maintained at 260°C for 10 min. Detector and injection temperature: 230°C, carrier gas: He, splitless; MS condition: ionized voltage: 70 eV.

Degradation of 2, 8-DCDD by dioxin-degrading fungi

Liquid culture: To the culture solution prepared as described above, the fungi PL1 and MG and 267 and MG were added. The co-culture was pre-incubated for 7 days at 25°C in the dark as described above.

Addition of 2, 8-DCDD: 2, 8-DCDD was added to each pre-incubated medium. Then, 0.25 mM or 0.05 mM of the dioxin was added to the pre-incubated medium and incubated for 15 or 30 days at 25°C in the dark, while oxygen was purged every day during the incubation.

Extraction and analysis of the extracts: The extracts obtained as described were analyzed with the Shimadzu GC/MS system (QP-5050A).

Identification of intermediates by biodegradation of 2, 8-DCDD

Liquid culture: The culture medium was prepared as described above. After inoculation of the medium with PL1, the culture was pre-incubated by standing for 7 days at 25°C in the dark as described above.

Addition of 2, 8-DCDD: 2, 8-DCDD (0.25 mM and 0.05 mM) dissolved in 200 μL of N, N-dimethylformamide (DMF) and 200 μL of Tween 80 (10% solution) were added to each culture medium in a flask which had been pre-cultivated in advance. The incubation was conducted for 15 and 30 days at 25°C in the dark.

Extraction and purification of the extracts: The extracts obtained were purified using silica gel column chromatography by successive elution with *n*-Hexanes, *n*-Hexane and CH_2Cl_2 (1:1 v/v) and EtOH and EtOH solubles were obtained.

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

RESULTS AND DISCUSSION

Screening of fungi capable of degrading dioxins: From the 255 samples of decayed wood and soil collected from forests near Matsuyama city, Japan, 171 fungi were obtained. Thirty four kinds of fungi having the ability to degrade dioxins were selected by the screening method[9]. Two of the fungi, R-60 and R-67, from the decayed wood and one fungus, MG from soil were found to be highly capable of degrading dioxins. According to microscopic observations, R-60 and R-67 seemed to be Basidiomycetes because they have a clamp connection in their mycelium[15]. In contrast, MG does not have a clamp connection in the mycelium. Fungus MG produce a chlamydospore, therefore, the fungus was seemed to be a kind of filamentous fungus belonging to genus *Fusarium*[16].

Tachibana *et al.*[9] 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 R-60, R-67 and MG on the agar medium containing RBBR with those of PL1 and 267 already screened from nature by Tachibana *et al.*[9] revealed that the three fungi screened in the present report have a similar ability to degrade dioxin. Judging from the results obtained in the present report, highly capable of degrading dioxins are very likely present in nature.

Enzymatic activity of the fungi screened from nature: The activity levels of three enzymes, manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase (Lac), related to the degradation of dioxins, were measured. The enzymatic activities of R-60 and R-67 in the initial stage of the cultures were slightly weaker than those of PL1 and 267(Table 1). However, the activities of the former in the late stage of the cultures were stronger than those of the latter. The MnP activity of MG was weaker than that of R-60 or R-67(Table 1).T he Lac activities of all five fungi were shown only in the initial stage of the cultures. However, the Lac activities of PL1 and 267 were greater than those of R-60, R-67 and MG in comparison with the MnP activity(Table 2). In addition, Lip activities could not be recognized in the five fungi. From the enzymatic activities of the fungi screened in the present report, it was considered that R-60 and R-67 were white-rot fungi which do not produce lignin peroxidase.

Table 1: Change in manganese peroxidase (MnP) activity during the incubation

	Incubation period (days)						
Fungi	0	4	7	15	22	30	37
PL1	0	0.59	11.28	5.11	4.29	3.65	6.39
	(0)[a)]	(0.59)	(11.87)	(16.98)	(21.27)	(24.92)	(31.31)
267	0	0.94	4.81	4.98	0	2.86	18.51
	(0)	(0.94)	(5.75)	(10.73)	(10.73)	(13.59)	(32.10)
R-60	0	2.20	3.96	2.42	0.16	29.83	26.01
	(0)	(2.20)	(6.16)	(8.58)	(8.74)	(38.57)	(64.58)
R-67	0	1.24	4.35	5.43	3.30	23.60	26.10
	(0)	(1.24)	(5.59)	(11.02)	(14.32)	(37.92)	(64.02)
MG	0	2.74	0.96	0.61	1.06	2.97	3.89
	(0)	(2.74)	(3.70)	(4.31)	(5.37)	(8.34)	(12.23)

a)Numbers in parentheses show cumulative MnP activity

Table 2: Change in laccase (Lac) activity during the incubation

	Incubation period (days)						
Fungi	0	4	7	15	22	30	37
PL1	0	10.48	0	0	0	0	0
	(0)[a)]	(10.48)	(10.48)	(10.48)	(10.48)	(10.48)	(10.48)
267	0	0	0.66	0	0	0	0
	(0)	(0)	(0.66)	(0.66)	(0.66)	(0.66)	(0.66)
R-60	0	9.34	0	0	0	0	0
	(0)	(9.34)	(9.34)	(9.34)	(9.34)	(9.34)	(9.34)
R-67	0	0	1.54	2.26	0	0	0
	(0)	(0)	(1.54)	(3.80)	(3.80)	(3.80)	(3.80)
MG	0	1.16	0	0	0	0	0)
	(0)	(1.16)	(1.16)	(1.16)	(1.16)	(1.16)	(1.16)

a) Numbers in parentheses show cumulative Lac activity

The relationship between degradation of 2, 7-DCDD and enzymatic activities of several fungi capable of degrading dioxins has been investigated by Itoh *et al.*[17]. They reported a positive correlation between cumulative enzymatic activity, especially, cumulative Lip and MnP activities and the degradation rate of dioxin. The cumulative MnP and Lac activities are also shown in Table 1 and 2, respectively. Judging from the results obtained[17]. and the present report, the rate of degradation of dioxins by R-60, R-67 and MG seemed to increase in the later stage of the cultures since the MnP activities of the two fungi increased in the late stage of the cultures.

The time-course of MnP and Lac activities on co-cultivation of PL1 and MG and of 267 and MG was examined. The results are shown in (Table 3 and 4). Either MnP or Lac activity obtained by co-cultivation was weaker than that on the cultivation of PL1 or 267 (Table 1 and 2). This seemed to indicate that the growth of PL1 and 267 had been inhibited to some extent by the growth of MG when the co-cultivation was conducted. In addition, as in the cultivation of each fungus, Lip activity could not be recognized when co-cultivation was conducted (Table 3 and 4).

Degradation of 2, 8-DCDD by the fungi screened from nature: R-60 degraded 74% and 56% of 2, 8-DCDD at 0.05 mM and 0.25 mM in 30 days, respectively. R-67 degraded 83% and 77% of 2, 8-DCDD at 0.05 mM and 0.25 mM in 30 days, respectively. MG degraded 58% and 50% of 2, 8-DCDD at 0.05 and 0.25 mM in 30 days, respectively. PL1 and 267 degraded 89% and 90% of 2, 8-DCDD at 0.05 mM in 30 days, respectively, and 79% and 80% of 2, 8-DCDD at 0.25 mM in 30 days, respectively (Fig. 1 and 2).

R-60 has a similar growth rate and enzymatic activity to PL1 and 267 described above. However, the ability of R-60 to degrade dioxins was inferior to that of PL1 or 267. In contrast, the ability of R-67 to degrade dioxins has almost to that of PL1 and 267. But, the ability of R-67 did not exceed drastically that of PL1 or 267.

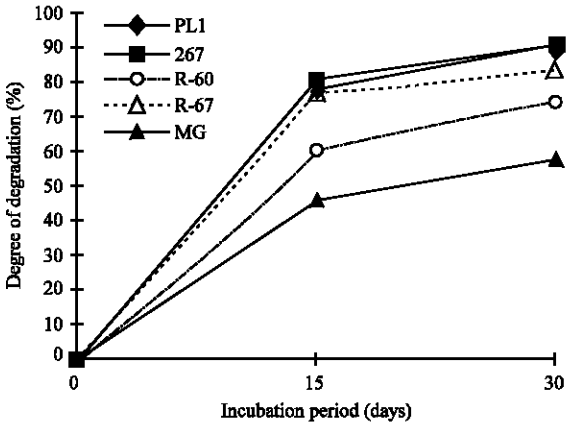

Fig. 1: Degree of degradation of 2,8-Dichlorodibenzo-*p*-Dioxin (2,8-DCDD) at a concentration of 0.05 mM with the fungi screened from nature

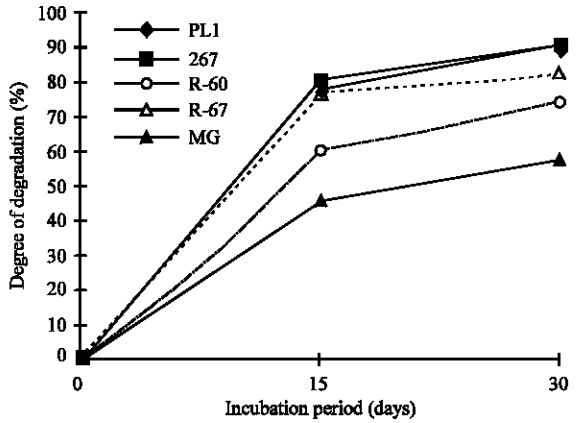

Fig. 2: Degree of degradation of 2,8-Dichlorodibenzo-*p*-Dioxin (2,8-DCDD) at a concentration of 0.25 mM with the fungi screened from nature

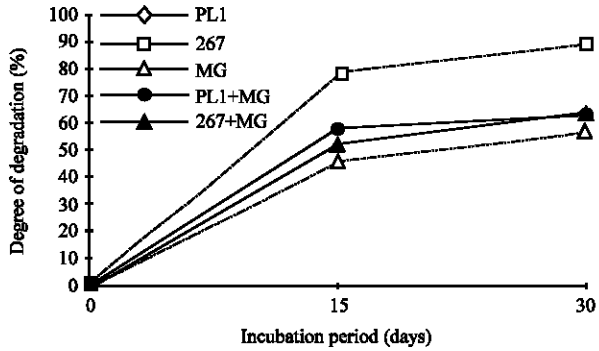

Fig. 3 : Degree of degradation of 2,8-Dichlorodibenzo-*p*-Dioxin (2,8-DCDD) at a concentration of 0.05 mM on the co-cultivation of the fungi screened from nature

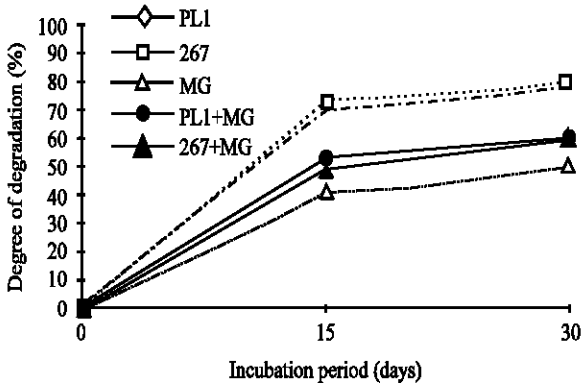

Fig. 4 : Degree of degradation of 2,8-Dichlorodibenzo-*p*-Dioxin (2,8-DCDD) at a concentration of 0.25 mM on the co-cultivation of the fungi screened from nature

Degradation of 2, 8-DCDD on co-cultivation of fungi having the ability to degrade dioxins: PL1 degraded 89% of 2, 8-DCDD when independently incubated for 30 days at 0.05 mM. In contrast, MG degraded 58% of 2,8-DCDD when incubated for 30 days at 0.05 mM. However, 63% of 2, 8-DCDD was degraded when the co-cultivation of fungi PL1 and MG was conducted for 30 days at 0.05 mM (Fig. 3) . Furthermore, PL1 degraded 79% of 2, 8-DCDD when incubated for 30 days at 0.25 mM. In contrast, MG degraded 50% of 2, 8-DCDD when independently incubated for 30 days at 0.25 mM. However, 60% of 2, 8-DCDD was degraded when the co-cultivation of PL1 and MG was conducted for 30 days at 0.25 mM (Fig. 4).

Ninety percent of 2, 8-DCDD was degraded when 267 was independently incubated for 30 days at 0.05 mM (Fig. 3). However, 64% of 2, 8-DCDD was degraded when the co-cultivation of 267 and MG was conducted for 30 days at 0.25 mM (Fig. 4). At 0.25 mM of 2, 8-DCDD, 80% was degraded when 267 was independently incubated for 30 days. But, 60% of 2, 8-DCDD was degraded when the co-cultivation of 267 and MG was conducted for 30 days at 0.25 mM. These results showed that the degradation rate of 2, 8-DCDD on the co-cultivation of PL1 and MG or of 267 and MG was lower than that on the cultivation of just PL1 or 267. No synergistic effect of the two fungi was recognized. It is considered that the growth inhibition of PL1 or 267 might have been caused to some extent by the growth of MG. However, the degradation rates of 2, 8-DCDD on co-cultivation of PL1 and MG, or of 267 and MG were increased in comparison with that on cultivation of MG alone.

Since the contribution to the degradation of 2, 8-DCDD by PL1 or 267 was greater than that by MG, the rate of degradation on co-cultivation of PL1 and MG or of 267 and MG did not seem to decrease remarkably, even though the growth of PL1 or 267 was inhibited to some extent by MG. From these results, PL1 and 267 may be useful for the bioremediation of dioxin-contaminated soils, though these fungi exerted some negative effects like growth inhibition of microorganisms present in the soil.

Identification of intermediates in the reaction of 2, 8-DCDD with fungi: By comparison of the degradation pathway of 2, 7-DCDD[18], a plausible degradation pathway of 2, 8-DCDD is shown in Fig. 5. 4-Chlorocatechol and 1, 2, 4-Trihydroxybenzene were considered to be major intermediates. Identification of these compounds in the extracts by the method described in the experimental section was conducted. In the GS profile, compound (A) considered to be an intermediate

Table 3: Change in manganese peroxidase (MnP) activity during the co-cultivation.

	Incubation period (days)						
Fungi	0	4	7	15	22	30	37
PL1 + MG	0	1.04	0.25	5.39	3.01	3.94	4.97
	(0)[a)]	(1.04)	(1.29)	(6.68)	(9.69)	(13.63)	(18.60)
67 + MG	0	1.51	3.40	7.31	0.82	2.38	11.35
	(0)	(1.51)	(4.91)	(12.22)	(13.04)	(15.42)	(26.77)

a) Numbers in parentheses show cumulative MnP activity

Table 4: Change in laccase (Lac) activity during the co-cultivation.

	Incubation period (days)						
Fungi	0	4	7	15	22	30	37
PL1 + MG	0	0.88	0	0	0	0	0
	(0)[a)]	(0.88)	(0.88)	(0.88)	(0.88)	(0.88)	(0.88)
267 + MG	0	0.92	0.80	1.41	0.10	0	0
	(0)	(0.92)	(1.72)	(3.13)	(3.23)	(3.23)	(3.23)

a) Numbers in parentheses show cumulative Lac activity

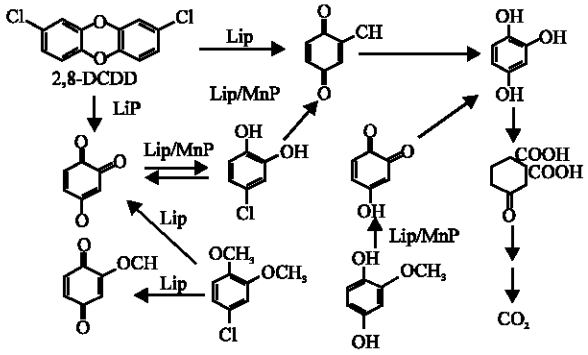

Fig. 5: A proposed pathway for the degradation of 2,8-Dichlorodibenzo-*p*-Dioxin (2, 8-DCCD) by fungi Legends: Lip: Lignin peroxidase; MnP: Managanse peroxidase; Source: Vali *et al.*, 1992. J. Bacteriol., 174: 2131-2137

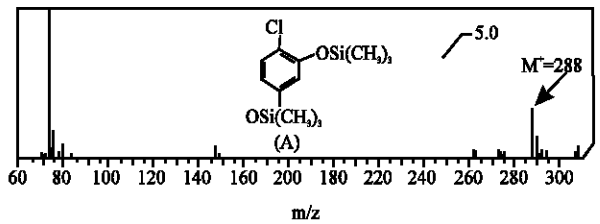

Fig. 6: Mass spectrum of the trimethylisilyl ether of 4-chlorcatechol (A) in the metabolites obtained from 2, 8-Dichlorodibenzo-*p*-Dioxin (2, 8-DCDD) with the fungi screened from nature

was recognized. In the spectrum, molecular ion and base ion peaks at 288 and 73, respectively were recognized. Since Compound (A) was suggested to be TMS ether of 4-Chlorocatechol by the analysis of the mass spectrum (Fig. 6). The mass spectrum of TMS ether of compound (A) coincided with that of the authentic TMS ether of 4-Chlorochatechol. The retention time of TMS ether of compound (A) also coincided with that of the authentic TMS ether of 4-Chlorochatechol. Therefore, compound (A) was identified as 4-Chlorocatechol. 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. However, it was confirmed that 2, 8-DCDD was actually degraded by the fungi screened in the present report because 4-Chlorocatechol was detected as an intermediate in the degradation of 2, 8-DCDD by the fungi screened in the present report.

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