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Bioremediation of 2,3,7,8-Tetrachlorodibenzo-*p*-Dixoin in Soil by Fungi Screened from Nature

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Abstract: To purify dioxin-contaminated soil by bioremediation with the fungi screened from nature, microbial degradation of 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin (2,3,7,8-TCDD) was conducted with two fungi (PL1 and 267) screened from nature. The two fungi degraded 22 to 62% of 2,3,7,8-TCDD. Maximum degradation (62%) was obtained when PL1 was incubated for 30 days at 1 ng. Furthermore, bioremediation of 2,3,7,8-TCDD in soil with PL1 was conducted. The PL1 degraded 27 to 51% of the dioxin at 1 and 10 ng g^{-1} soil in 15 and 30 days, respectively. Maximum degradation (51%) was obtained when PL1 was incubated for 30 days at 1 ng g^{-1} soil. In addition, 2,3,7,8-TCDD was indeed degraded by the fungi, because 4,5-Dichlorocatechol considered to be an intermediate was detected among the reaction products.

Key words: 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin, microbial degradation, bioremediation by fungi

INTRODUCTION

Environmental pollution caused by endocrine disrupting chemicals (environmental hormones) such as dioxins discharged from incinerators has become a major social problem (Onabe, 1991; Kearney *et al*., 1973 and Zoller and Ballschmiter, 1986). Measures to reduce levels of dioxins in discharge from incinerators as well as to newly determine the TDI (tolerance daily intake) (Takeuchi *et al*., 1999; Yanagibashi, 1999), have greatly reduced the amount of dioxins discharged into the environment. However, toxic effects on the environment have been reported (Hanson, 1991; Neubert, 1991; Sako, 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) 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. Previously, we described a screening method for fungi able to degrade dioxins and the biodegradation of 2,8- Dichlorodibenzo-*p*-Dioxin (2,8- DCDD), 2,7-Dichlorodibenzo-*p*-Dioxin (2,7-DCDD) and 2,4,8-Trichlordibenzofuran (2,48-TCDF), three kinds of dioxins, with fungi screened from nature (Tachibana *et al*., 1996; Miyoshi *et al*., 2005). Furthermore, we described the bioremediation of 2, 7-DCDD and 2,4,8-TCDF in soil with fungi screened from nature (Tachibana *et al*., 2005).

In the present report, biodegradation of 2,3,7,8,-Tetrachlorodibenzo-*p*-Dioxin (2,3,7,8,-TCDD) with fungi screened from nature and the bioremediation of 2,3,7,8-TCDD in soil were examined for the purification of dioxin-contaminated soil.

MATERIALS AND METHODS

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

Reagents: ^{12}C- and ^{13}C-2,3,7,8-TCDD were purchased from Wako Pure Chemical Industry Co., Ltd. 4,5-Dichlorocatechol was purchased from Aldrich Co., Ltd.

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Degradation of 2,3,7,8-TCDD by fungi: Test fungi: PL1 and 267 screened from nature by Tachibana *et al.* (2005) 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 (Kirk *et al.*, 1978). 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 it was sterilized with an autoclave for 20 min at 121°C (Miyoshi *et al.*, 2005). After cooling on a clean bench, each of the two test fungi mentioned above was inoculated in the solution and pre-incubated standing for 7 days at 25°C in the dark.

Addition of 2,3,7,8-TCDD: 2,3,7,8-TCDD (1 ng and 10 ng) dissolved in 200μL of N, N- dimethylformamide (DMF) and 200 μL of Tween 80 (10% solution) was added to the Elenmeyer flask which had been pre-incubated in advance (Tachibana *et al.*, 2005). 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: Extraction of metabolites was conducted by reference to the method of Takata *et al.* (1996). After a fixed period of culture, to recover the TCDD adsorbed to the mycelia and to dissolve the mycelia thoroughly, concentrated H_2SO_4 (20 mL) was added to the culture medium. After the addition of 1 ng of ^{13}C-2,3,7,8-TCDD dissolved in 5 mL of *n*-hexane to the culture medium, the culture medium was extracted twice with 15 mL and 20 mL of *n*-hexane, respectively. Further, the residue in the flask was extracted with acetone (5 mL), *n*-hexane (5 mL) and finally *n*-hexane (10 mL) again. The extracted solution was combined and then concentrated under reduced pressure to obtain extracts.

The extracts were purified using silica gel column chromatography (Lamparski *et al.*, 1979; Lamparski and Nestrick, 1980) by successive elution with *n*-hexanes (40 mL), *n*-hexane and dichlormethane (CH_2Cl_2) (1:1 v/v) (30 mL) and each soluble was obtained.

Analysis of the extracts: Each soluble was 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, HP-1 (internal diameter 0.25 mm, length 30 m). The conditions for the analysis (Ballschmiter and Bacher, 1996) were as follows: Temperature; after 1 min at 100°C, raised to 240°C at 5°C/min and then maintained at 240°C for 10 min. Detector and injection temperature: 250°C, carrier gas: He, splitless; MS condition: ionized voltage: 70 eV.

Identification of intermediates by degradation of 2,3,7,8-TCDD

Liquid culture: The culture medium was prepared as described above. After inoculation of the medium with fungi PL1 and 267, the culture was pre-incubated by standing for 7 days at 25°C in the dark as described in a previously report (Tachibana *et al.*, 2005).

Addition of 2,3,7,8-TCDD: 2,3,7,8-TCDD (100 ng) was added to each pre-incubated medium as described above. Then, the pre-incubated medium was incubated for 15 or 30 days at 25°C in the dark, while oxygen was purged every day during the incubation.

Extraction and purification of the extracts: The extracts obtained were purified using silica gel column chromatography as described above. *n*-Hexane and CH_2Cl_2 (1:1 v/v) and EtOH solubles were obtained.

Analysis of the extracts: After the vacuum drying of each solube (100 μL) obtained above 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 (Tachibana *et al.*, 2003). The trimethylsilyl (TMS) derivatives of the extracts were analyzed using GC-MS as described in a previous report (Miyoshi *et al.*, 2005). 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°C min. Detector and injection temperature: 250°C, carrier gas: He, splitless; MS condition: ionized voltage: 70 eV.

Bioremediation of 2,3,7,8-TCDD in soil by fungus PL1: Test soil: Soil collected from the Ehime University's Experimental Forest was used as a test soil as described in a previous report (Tachibana *et al.*, 1996). The characteristics of the soil (Yagi, 1974) were as follows: pH: 5.30; water content: 38.5%; organic content: 9.4%.

Test fungus: PL1 was used as a test fungus.

Preparation of a solid medium: The solid medium was prepared by the method described in a previous report (Tachibana *et al*., 2005).

Incubation: The solid medium (100 g) (dry weight) was added to the soil (300 g) (dry weight) in a plastic box with a lid, mixed and pre-incubated for 10 days at 25°C in the dark, (Tachibana *et al*., 2005). After addition of the substrate [2,3,7,8-TCDD (1 ng and 10 ng) by the method described in a previous report (Tachibana *et al*., 2005), the mixture was incubated for 15 and 30 days at 25°C in the dark. In addition, samples with no dioxins added and soil with dioxins but no solid medium were used as controls, respectively.

Extraction: The extraction was conducted as described in a previous report (Tachibana *et al*., 2005). The treated soil (30 g) (dry weight) was placed 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. After standing for overnight in the dark, the mixture was filtrated to separate the residue and filtrate. The residue was extracted for 16 h with toluene using a Soxhlet extractor. After concentration, extracts were obtained. The extracts were made to dissolve in *n*-hexane (60 mL). In contrast, the filtrate was extracted thrice with *n*-hexane (60 mL). The *n*-hexane solubles from the filtrate and the residue were combined and washed with *n*-hexane saturated water until the water layer became acidic. After addition of 1 ng of ^{13}C-2,3,7,8-TCDD dissolved in 5 mL of *n*-hexane to the *n*-hexane solution, the solution was concentrated under reduced pressure to obtain the extracts.

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

Identification of intermediates by degradation of 2,3,7,8-TCDD

Incubation: The culture was conducted as described above.

Addition of 2,3,7,8-TCDD: 2,3,7,8-TCDD (10 ng) was added to each medium as described above and incubated for 15 or 30 days at 25°C in the dark.

Extraction and purification of the extracts: The extracts obtained as described above were purified using silica gel column chromatography as described above. *n*-Hexane and CH_2Cl_2 (1:1 v/v) and EtOH solubles were obtained.

Analysis: The trimethylsilyl (TMS) derivative of each soluble was analyzed as described above. GC-MS was performed under the conditions outlined above.

Table 1: Degree of degradation of 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin with fungi screened from nature

		Degree of degradation (%)	
Fungus	Concentration (ng)	15 days	30 days
PL1	1	32	62
PL1	10	22	48
267	1	37	56
267	10	31	47

Table 2: Degree of degradation of 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin in soil by bioremediation with PL1

		Degree of degradation (%)	
Fungus	Concentration (ng)	15 days	30 days
PL1	1	32	51
PL1	10	27	40

RESULTS AND DISCUSSION

Degradation of 2,3,7,8-TCDD with fungi screened from nature: PL1 degraded 32 to 62% of 2,3,7,8-TCDD at 1 ng in 15 and 30 days, respectively (Table 1). PL1 also degraded 22 to 48% of 2,3,7,8-TCDD at 10 ng in 15 and 30 days, respectively (Table 1). 267 degraded 37 to 56% of 2,3,7,8-TCDD at 1 ng in 15 and 30 days, respectively (Table 1). Two hundred and sixty seven also degraded 31 to 47% of 2,3,7,8-TCDD at 10 ng in 15 and 30 days, respectively (Table 1). Maximum degradation (62%) of 2,3,7,8-TCDD was obtained when PL1 was incubated for 30 days at 1 ng. Maximum degradation (56%) with 267 was obtained after 30 days at 1 ng.

Bumpus *et al.* (1985) reported that *Phanerochaete chrysosporium* degraded ^{14}C-2,3,7,8-TCDD in a liquid medium and produced $^{14}CO_2$. However, they did not describe the rate of degradation. Takata *et al.* (1996) reported that *Phanerochaete sordida* YK-624 and *Phanerochaete chrysosporium* degraded 22 and 37% of 2,3,7,8-TCDD for 14 days at 0.5 ng in a liquid medium, respectively. They added glucose to the medium at 0 and 7 days, respectively, to enhance the rate of degradation. The degradation by *P. sordida* was increased about 10 to 20% compared to that when no glucose was added. The results obtained here suggested that the two fungi had a greater ability to degrade higher concentrations of dioxins than the fungi reported by Takata *et al.* (1996).

From the results obtained in the present report and in previous reports (Tachibana *et al*., 1996; Tachibana *et al*., 2005), it was found that the two fungi screened from nature degraded dioxins (2,8-DCDD, 2,7-DCDD, 2,4,8,-TCDF and 2,3,7,8,-TCDD) at a higher rate. These results suggest that the degradation of dioxins in soil with fungi screened from nature is possible.

Identification of intermediates in the reaction of 2,3,7,8-TCDD with fungi: To confirm the degradation of 2,3,7,8-

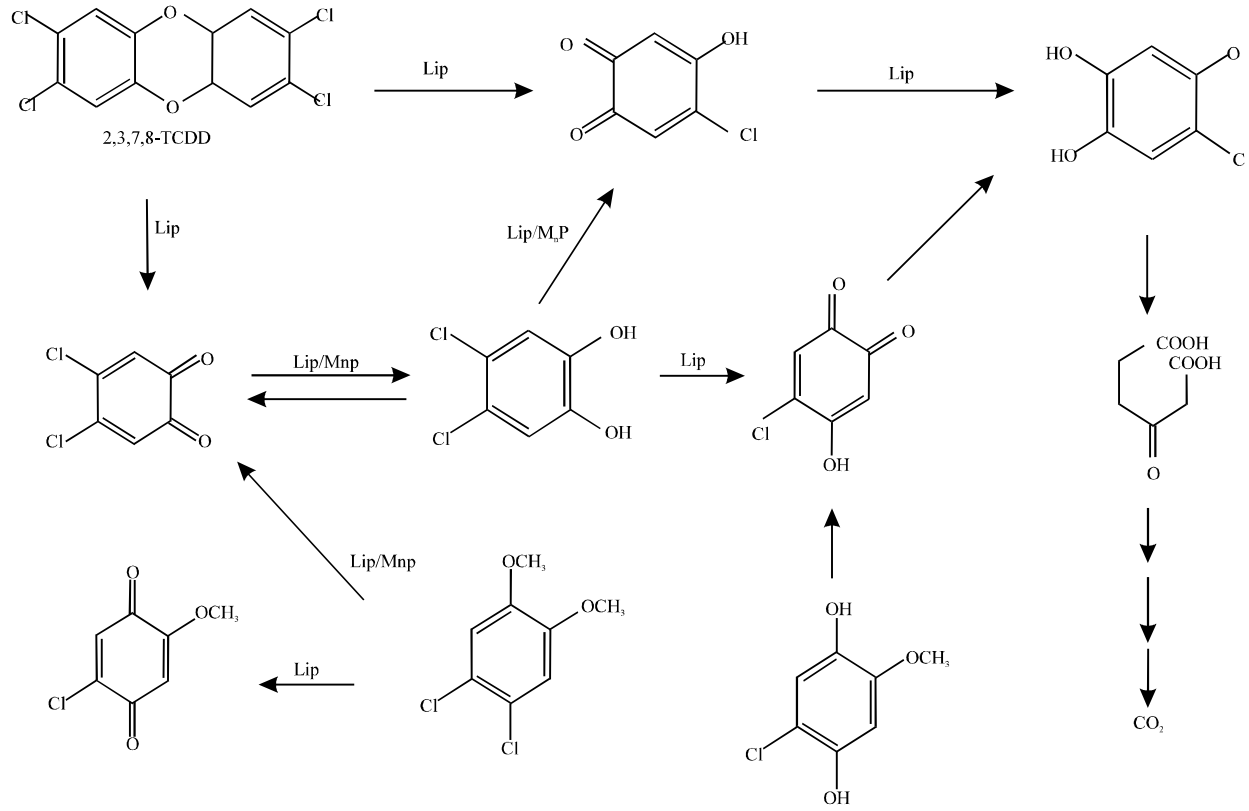

Fig. 1: A proposed pathway for the degradation of 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin (2,3,7,8-TCDD) by fungi screened from nature. LiP: Lignin peroxidase; MnP: Manganese peroxidase

TCDD with fungi, an attempt was made to identify the intermediates. Valli *et al.* (1992) reported the pathway by which 2,7-DCDD is degraded by *Phanerochaete chrysosporium* and Ohkawa *et al.* (1999) reported a proposed pathway for the degradation of 2, 4, 8-TCDF by fungi screened from nature. From the pathways for 2,7-DCDD and 2,4,8-TCDF, a degradation pathway for 2,3,7,8-TCDD was proposed. The proposed pathway is shown in Fig. 1. 4,5-Dichlorocatechol and 5-Chloro-1,2,4-trihydroxybenzene were considered to be major intermediates. These compounds were identified in the extracts using the method described in the experimental section. In the GC profile, a trimethylsilyl (TMS) ether of 4,5-Dichlorocatechol was recognized. The mass spectrum of the TMS ether of 4,5-Dichlorochatechol is shown in Fig. 2. It coincided with that of the authentic TMS ether of 4,5-Dichlorochatechol. The retention time of the TMS ether of the compound also coincided with that of the authentic TMS ether of 4,5-Dichlorochatechol. Therefore, 4,5-Dichlorocatechol was identified in the extracts, however, 5-Chloro-1,2,4-trihydroxybenzene could not be identified because no authentic sample was available. Takata *et al.* (1996) reported that the degradation of ten kinds of Polycholorodibenzo-*p*-Dioxins and Polychlorodibenzofurans (PCDDs/PCDFs) including 2,3,7,8-TCDD and Octachlorodibenzo-*p*-Dioxin (OCDD) by *Phanerochate sordida.* They also reported that 4,5-Dichlorocatechol and 3,4,5,6-Tetrachlorocatechol were intermediates in the reaction of 2,3,7,8-TCDD and OCDD with the fungus, respectively. The results obtained here and the results reported by Takata *et al.* (1996) indicated that 4,5-Dichlorocatechol is a major intermediate in the reaction of 2,3,7,8-TCDD with fungi. Therefore, it was confirmed that 2,3,7,8-TCDD was actually degraded by the fungi screened from nature because 4,5-Dichlorocatechol was detected as an intermediate in the reaction of 2 ,3,7,8-TCDD with the fungi.

Bioremediation of 2,3,7,8-TCDD in soils by the fungus screened from nature: PL1 degraded 32 to 51% of 2,3,7,8-TCDD through bioremediation at 1 ngg^{-1} soil in 15 and 30 days, respectively (Table 2). PL1 degraded 27 to 40% of 2,3,7,8-TCDD at 10 ngg^{-1} soil in 15 and 30 days, respectively (Table 2). Maximum degradation (51%) of 2,3,7,8-TCDD was obtained when PL1 was incubated for 30 days at 1 ngg^{-1} soil. These results show that dioxins

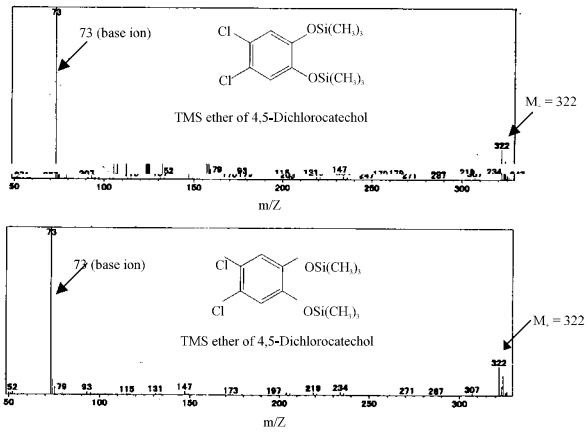

Fig. 2: Mass spectra of the trimethylsilyl (TMS) ether of 4, 5-Dicholocatechol in the TMS derivatives of the reaction product from bioremediation of 2, 3, 7, 8-Tetrachlorodibenzo-*p*-Dioxin(2,3,7,8-TCDD) with fungi screened from nature

Notes: Upper panel shows the mass spectrum of the TMS ether of authentic 2,3,7,8-TCDD; lower panel shows the mass spectrum of the TMS ether of reaction product (A) from the bioremediation of 2,3,7,8-TCDD with fungi screened from nature

present in soil can be degraded by bioremediation with fungi screened from nature and dioxin-contaminated soil can be purified by bioremediation.

Identification of intermediates in the reaction of 2,3,7,8-TCDD with fungus: To confirm the degradation of 2,3,7,8-TCDD in soils with fungi, the intermediates in the extracts were identified as described in the experimental section. In the GC profile, a trimethylsilyl (TMS) ether of 4,5-Dichlorocatechol was recognized as described above. The mass spectrum of the TMS ether of the compound coincided with that of the authentic TMS ether of 4,5-Dichlorochatechol. The retention time of the TMS ether of the compound also coincided with that of the authentic TMS ether of 4,5-Dichlorochatechol. Therefore, 4,5-Dichlorocatechol was identified in the extracts, however, 5-Chloro-1,2,4-trihydroxybenzene could not be identified as described above. However, it was confirmed that 2,3,7,8-TCDD in soils was actually degraded by the fungus screened from nature because 4,5-Dichlorocatechol was detected as an intermediate in the reaction products of 2,3,7,8-TCDD in the soil.

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REFERENCES

Alexander, M., 1994. Biodegradation and bioremediation, 2nd Edn., Academic press, New York, pp: 299-376.

Ballschmiter, K. and R. Bacher, 1996. Dioxine Chemie, Analytik, Vorkommen, Umweltverhalten und Toxikologie der halogenierten Dibenzo-*p*-dioxine und Dibenzofurane, Japanese version, NTS, Inc., Tokyo, pp: 185-217.

Bumpus, J.A., M. Tien, D. Wright and S.D. Aust, 1985. Oxidation of persistent environmental pollutants by a white rot fungus. Science, 228: 1434-1436.

Corbet, R.L., D.C.G. Muir and G.R.B. Webster, 1983. Photodecomposition of chlorinated dibenzo-p-dioxins. Chemosphere, 12: 523-527.

Hanson, D.J., 1991. Dioxin Toxicity: New Studies Prompt Debate, Regulatory Action. Chem. Eng. News, 69: 7-14.

Kearney, P.C., A.R. Isensee, C.S. Helling, E.A. Woolson and J.R. Plimmer, 1973, Environmental significance of chlorodioxins. Advan. Chem. Ser., 120: 105-111.

Kirk, T.K., E. Schulz, W.J. Connors, L.F. Lorenz and J.G. Zeikus, 1978. Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium.* Arch. Microbiol., 117: 277-285.

Lamparski, L.L., T.J. Nestrick and R.H. Stehl, 1979. Determination of parts-per-million levels of polychlorinated dibenzofurans and dioxins in fish. Anal. Chem., 51: 1453-1458.

Lamparski, L.L. and T.J. Nestrick, 1980. Determination of tetra-, hexa-, hepta and octachlorodibenzo-p-dioxin isomers in particulate samples at parts per trillion levels. Chemosphere, 19: 1165-1177.

Miyoshi, S., K. Kimura, R. Matsumoto, K. Itoh and S. Tachibana, 2005. Biodegradation of 2,8-Dichlorodibenzo-p-Dioxin by fungi screened from nature. Pak. J. Biol. Sci., 8: 1265-1271.

Neubert, D., 1991. Peculiarites of the toxicity of polyhalogenated dibenzo-*p*-dioxins and dibenzofurans in animal and man. Chemosphere, 25: 1869-1893.

Onabe, F., 1991. The dioxin issue in Japanese pulp and paper industry present status and future perspective. Japan Tappi, 45: 462-474.

Ohkawa, H., K. Itoh and S. Tachibana, 1999. Bioremediation by wood-rotting fungi (I). Degradation of 2,4,8-trichlorodibenzofuran and dibenzofuran by some fungi having ability for degradatiuon of dioxins. Japan Tappi, 53: 1054-1062.

Sako, T., 1999. Oxidation of dioxins with super critical water and application to the techniques for the treatment of dioxins. J. Water and Waste, 41: 38-42.

Tachibana, S., H. Ohkawa, K. Itoh and T. Oki, 1996. Bioremediation by wood-rotting fungi (I). Screening of fungi having ability for degradation of dioxins and degradation of 2,7-Dichlorodibenzo-p-Dioxin by some fungi separated by the screening and by some white-rotting fungi. Japan Tappi, 50: 1806-1815.

Tachibana, S., 1999. Biological treatment of dioxin-contaminated soil by wood-rotting fungi. J. Water and Waste, 41: 698-705.

Tachibana, S., K. Tokuoka and K. Itoh, 2003. Adsorption of dioxins by activated carbon and their degradation by a white-rot fungus. Mokuzai Gakkaishi, 49: 227-232.

Tachibana, S., Y. Kiyota and M. Koga, 2005. Biodegradation of 2,7-Dichlorodibenzo-p-Dioxin and 2,4,8-Trichlorodibenzofuran in soil by fungi screened from nature. Pak. J. Biol. Sci., 8: 1751-1757.

Takata, S., M. Nakamura, T. Matsuda, R. Kondo and K. Sakai, 1996. Degradation of polychlorinated Dibenzo-p-Dioxins and polychlorinated Dibenzofurans by the white rot fungus *Phanerochaete sordida* YK-624. Appl. Environ. Microbiol., 62: 4323-4328.

Takeuchi, M., H. Masunaga, T. Imagawa, N. Yamashita and M. Taga, 1999. Dioxins and environment, Sankyo publisher Co. Ltd., Tokyo, pp: 12-14.

Valli, K., H. Wariishi and M.H. Gold, 1992. Degradation of 2,7-dichlorodibenzo-p-dioxin by the lignin-degradaing basidiomycete *Phanerochaete chrysosporium.* J. Bacteriol., 174: 2131-2137.

Watts, R. J., B. R. Smith and G. C. Miller, 1991. Catalyzed hydrogen peroxide treatment of octachloro dibenzo-p-dioxin (OCDD) in surface soils. Chemospehere, 23: 949-955.

Yagi, H., 1974. Analysis of soil and experiment of fertilizer, Hakuyusha publisher Co. Ltd., Tokyo, pp: 21-22.

Yanagibashi, Y., 1999. Countermeasures of sources generated dioxins and future subjects. J. Water and Waste, 41: 661-673.

Zoller, W. and K. Ballschmiter, 1986. Formation of polychlorinated dibenzodioxins and dibenzofurans by heating chlorophenols and chlorophenates at various temperatures. Fresenius Z. Anal. Chem., 323: 19-23.