食総研報（Rep. Nat'l. Food Res. Inst）No . 72, 83−87（2008）［報　文］

報　文

Biodegradation of Di−(2−ethylhexyl) phthalate by Fungi

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Abstract

The biological degradation of di−(2−ethylhexyl) phthalate (DEHP ; **1**) was studied with fungi. An initial DEHP concentration of 40 ppm (40 mg/L) in an aqueous solution was degraded using fungi in the dark for 7 days. Among the 14 strains tested, 9 strains degraded DEHP above 50 %. Further, 3 strains of *Fusarium* were more effective for degradation of DEHP.

Key word: DEHP ; Endocrine disrupter ; Fungi ; Biodegradation

Introduction

Di−(2−ethylhexyl) phthalate (DEHP, **1**) is generally used as a plasticizer for plastics in a broad range of products (as polyvinylchloride (PVC) resins, polyvinyl acetate, cellulosic, and polyurethanes) and contributes to environmental pollution. As the most common persistent phthalic acid ester (PAE) existing in wastewater, DEHP is less susceptible to degradation due to its long ester chain. Recently, there have been many reports on the toxicity of DEHP[1)~7)]. Elimination of DEHP by microorganism is considered to be one of the major routes of environmental degradation. Degradation of DEHP has been extensively studied[8)~14)], but such knowledge on isolated fungi[9) 14)] is limited. However, these biological methods to eliminate the pollutant in an aqueous solution cannot completely decompose the total organic carbon. In our previous work, we have reported that some filamentous fungi (such as *Fusarium sporotrichioides* NFRI−1012, *F. moniliforme* 2−2, *Aspergillus terreus* MT−13, and *Emericella nidulans* MT−78, etc.) were effective for degradation of BPA[15)]. In this paper, we would like to report the effectiveness of some fungi in the biodegradation of DEHP.

Materials and Methods

We investigated the degradation of DEHP by fungi. Fourteen isolates of fungi tested in this study are shown in Table 1. The fungi under NFRI numbers were the stock cultures of National Food Research Institute (Tsukuba, Japan). *A. ustus* MT−3 and *A. ustus* MT−

2007 年 11 月 20 日受付，2007 年 12 月 5 日受理

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23 were isolated from field soils sampled in Miyakojima, Okinawa, Japan. *Trichoderma viride* MT-6 was isolated from a soil sample of Taramajima, Okinawa, Japan. *F. moniliforme* 2-2 and *T. viride* PHF-2 were isolated from field soil collected in Tsukuba, Japan. *A. sydowii* KF-17 and *Curvularia lunata* FUTA-N were isolated from rice grains.

Polyoxyethylene (20) sorbitan monooleate (Tween 80) was purchased from Wako Pure Chemical Industries (Osaka, Japan). For the preparation of fungal inoculum, Czapek yeast-extract agar medium (CYA) was used. The CYA contained per liter of distilled water: 3.0 g of $NaNO_3$, 1.0 g of K_2HPO_4, 0.5 g of KCl, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.01 g of $ZnSO_4 \cdot 7H_2O$, 0.005 g of $CuSO_4 \cdot 5H_2O$, 5.0 g of yeast extract, 30.0 g of sucrose, 15 g of agar. For assay the ability of fungi to degrade DEHP, Czapek liquid medium (CZ) without the carbon source was used. The CZ contained per liter of distilled water: 3.0 g of $NaNO_3$, 1.0 g of K_2HPO_4, 0.5 g of KCl, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.01 g of $ZnSO_4 \cdot 7H_2O$, 0.005 g of $CuSO_4 \cdot 5H_2O$. Media were sterilized by autoclaving at 121℃ for 15 min.

To prepare the inoculum, the fungi were cultivated on CYA for 5 days at 25 ℃ in the dark. After cultivation, the fungal spores were suspended in 60 ml of sterile distilled water. Then, 2 ml of a spore suspension was inoculated to 50 ml of CZ liquid medium in 300 ml Erlenmeyer flasks supplemented with 2 mg of DEHP dissolved by 0.05 ml of Tween 80. The flasks were incubated on a reciprocal rotary shaker (120 rpm) at 30℃ in the dark.

After the indicated incubation period, the mycelial pellet was removed by filtration, and the filtrates were extracted with distilled ether. The amounts of residual DEHP were measured by high-performance liquid chromatography (HPLC: LC-9A, Shimadzu, Japan) using a reversed-phase column Shim-pack CLC-ODS (6.0 x 150 mm)(Shimadzu). Fluorescence detector (SPD-6A, Shimadzu) was set with excitation at 275 nm. The mobile phases consisted of acetonitrile were pumped at a flow rate of 1 ml/min under isocratic conditions. Column temperature was 25 ℃ and sample injection volumes were 20 μl. The chemical structures of the degradation products were determined by gas chromatography-mass spectrometer (GC-MS: GCMS-QP5050, Shimadzu). GC (GC-17A, Shimadzu) using DB-1 column (0.32 mm x 30 m) (J & W Scientific, USA) was programmed to raise the oven temperature from 50℃ to 250℃ at 18℃ /min, and MS was conducted at 70 eV (EI).

Results and Discussion

Among 14 fungal strains tested (shown in Table 1), 9 strains degraded DEHP more than 50 % and the decomposed products **2**-**4** were obtained (Scheme 1 and Fig. 1). *Fusarium* species were found to be effective in degradation: all the *Fusarium* species degraded more than 98 % of DEHP. GC-MS spectra of compounds **2**-**4** (Fig. 2) are as follows: compound **2**, *m/z*: 148 (27) $[M]^+$, 104 (96), 76 (100), 59 (3), and 50 (95); compound **3**, *m/z*: 112 (2) $[M]^+$, 98 (4), 83 (16), 70 (19), 57 (100), 43 (50), and 41 (60); compound **4**, *m/z*: 116 (10) $[M]^+$, 101 (12), 88 (83), 73 (100), 57 (36), 43 (32), and 41 (57). The spectra of **2** are in agreement with that of *o*-phthalic acid. Compounds **3** and **4** are identified to be 2-ethylhexanol and 2-ethylhexanoic acid by their GC-MS spectra. On the basis of these results, it seems that the biological degradation of DEHP using selected fungi proceeds by oxidative cleavage of the C-C bond.

The results of time-course mesurementsexperiments are shown in the Fig. 1. It can be seen that the final recovery ratios of DEHP become 2 % after 14 days of incubation, and *o*-phthalic acid (**2**, 20.8 %), 2-ethylhexanol (**3**, 11.4 %) and 2-ethylhexanoic acid (**4**, 13. 4 %) were obtained as products of biodegradation.

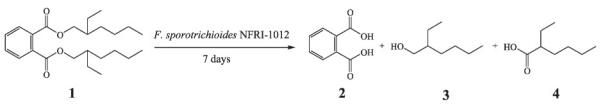
In this study, 14 fungi have been tested for degradation of DEHP. It was found that 9 strains of fungi had the ability to degrade DEHP (40 mg/L) in liquid CZ medium over 50 %. *F. graminearum* NFRI-1280, *F. moniliforme* 2-2 and *F. sporotrichioides* NFRI-1012 were more effective in the degradation of DEHP. Above all, *F. moniliforme* 2-2 and *F. sporotrichioides* NFRI-1012 were found to be the best biocatalysts for degradation of DEHP as well as bisphenol A[15].

Table 1. Degradation of DEHP using fungi

Run	Fungus	Degradation ratio (%)[a)]
1	*Aspergillus oryzae* NFRI-1571	65
2	*A. sydowii* KF-17	80
3	*A. ustus* MT-3	47
4	*A. ustus* MT-23	32
5	*Fusarium graminearum* NFRI-1280	100
6	*F. morniforme* 2-2	98
7	*F. sporotrichioides* NFRI-1012	98
8	*Penicillium citrinum* NFRI-1019	77
9	*P. expansum* NFRI-1021	83
10	*P. frequentans* NFRI-1022	31
11	*Curvularia lunata* FUTA-N	48
12	*Trichoderma viride* MT-6	89
13	*T. viride* PHF-2	39
14	*Rhizopus stlonifer* NFRI-1030	52

Reaction conditions: Di-(2-ethylhexyl) phthalate (DEHP, 2 mg), CZ culture medium (50 ml) and fungal spores were incubated for 7 days at 30℃ with shaking in the dark.

[a)] Degradation ratio was determined by HPLC peak area.



Scheme 1. Biodegradation of DEHP using *F. sporotrichioides* NFRI-1012.

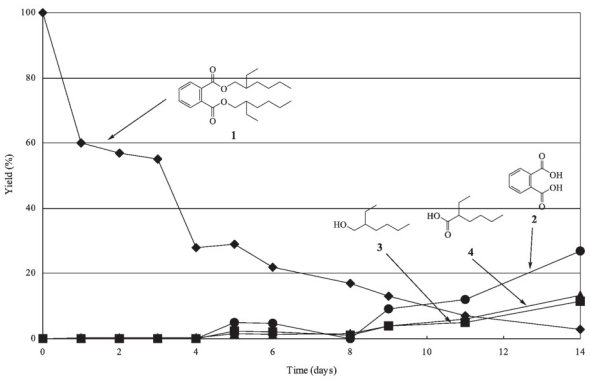


Fig 1. Biodegradation of DEHP using *F. sporotrichioides* NFRI-1012.

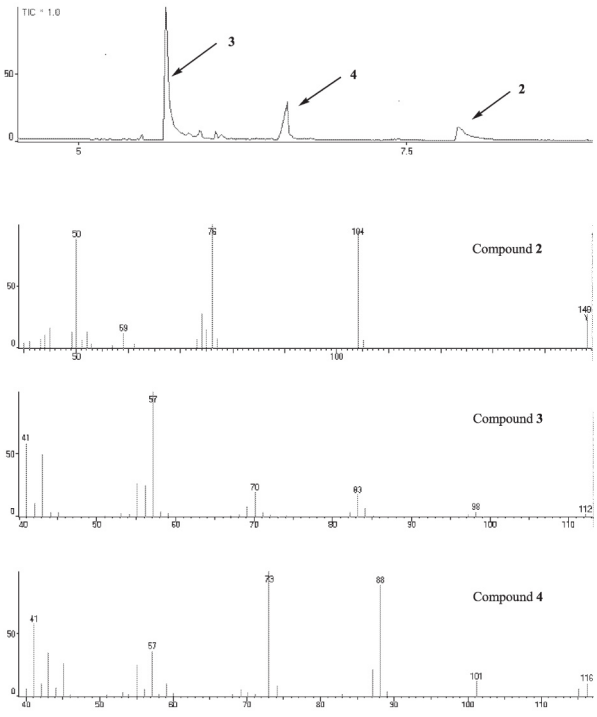


Fig 2. The spectrum of products (2-4) using fungi analyzed by GC-MS.

Acknowledgment

This work was partially supported by the Endocrine Disrupters Project of the Ministry of Agriculture, Forestry, and Fisheries, Japan, Frontier Project Adaptation and Evolution of Extremophile and a Grant-in-Aid for Scientific Research (no 18550142).

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Di-(2-ethylhexyl) phthalate の糸状菌による生分解

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Di-(2-ethylhexyl) phthalate（DEHP; **1**）の糸状菌による生分解を研究した。初期濃度 40 ppm（40 mg/L）の DEHP 溶液は糸状菌により，暗下，7 日間の培養で分解された．供試した 14 菌株のうち，9 菌株が DEHP を 50 %以上分解したが，そのうち の *Fusarium* 3 菌株がより効果的であった．