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ARTICLE

Ecotoxicity by the Biodegradation of Alkylphenol Polyethoxylates Depends on the Effect of Trace Elements

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The bacteria *Sphingomonas* sp. strain BSN22, isolated from bean fields, degraded octylphenol polyethoxylates ($OPEO_n$) to octylphenol (OP) under aerobic conditions. This biodegradation mechanism proceeded by the following two-step degradation process: (1) degradation of $OPEO_n$ to octylphenol triethoxylate ($OPEO_3$), (2) degradation from $OPEO_3$ to OP via octylphenoxy acetic acid ($OPEC_1$). The chemical structure of $OPEC_1$ was confirmed by analysis using ^{18}O-labeled water. Quantitative studies revealed that magnesium (Mg^{2+}) and calcium (Ca^{2+}) ions were essential for the biodegradation of $OPEO_n$. Furthermore, the rate of biodegradation was especially accelerated by ferric ions (Fe^{3+}), and the accumulated amounts of endocrine active chemicals, such as OP, $OPEO_1$, and $OPEC_1$, significantly increased to the concentration of 22.8, 221.7, and 961.1 μM in the presence of 37.0 μM Fe^{3+}, respectively. This suggests that environmental elements significantly influence the resultant ecotoxicity as well as the rate of their biodegradation in the environment. This study on the mechanism of $OPEO_n$ biodegradation may play an important role in understanding and managing environmental safety, including drinking water safety.

KEYWORDS: alkylphenol polyethoxylate; octylphenol; nonylphenol; ferric ion; endocrine disruptors; ecotoxicity

INTRODUCTION

Nonionic surfactant alkylphenol polyethoxylates ($APEO_n$), such as octylphenol polyethoxylate ($OPEO_n$), and nonylphenol polyethoxylate ($NPEO_n$), are widely used as detergents, emulsifiers, and adjuvants of pesticides. The hydrophilic ethylene oxide (EO) chains of $APEO_n$ are easily degraded to alkylphenols (AP), alkylphenol monoethoxylate ($APEO_1$), alkylphenol diethoxylate ($APEO_2$), and the corresponding alkylphenol carboxylates ($APEC_{1,2}$) in the environment; therefore, many studies have focused on the development of analysis and monitoring methods (*1*−*3*). In addition, accumulated knowledge of $APEO_n$ biodegradation has revealed that their biodegradation mechanism proceeds via exotype shortening of the EO chain (*4*) accompanied by either oxidation of the EO moiety (oxidative biodegradation) (*5*−*9*) or a nonoxidative hydroxy-shift (*10*, *11*).

These degraded compounds, especially AP, $APEO_1$, and $APEO_2$, have accumulated in the environment because of their relatively high hydrophobicity and act as endocrine disruptors (*4*, *12*, *13*). The estrogenic activities of $APEO_n$ and the corresponding alkylphenol carboxylates ($APEC_{1,2}$) increased as the EO chain shortened. In particular, octylphenol (OP) had higher estrogenic activity than nonylphenol (NP) (*14*). In our previous studies, OP, octylphenol monoethoxylate ($OPEO_1$) and octylphenol diethoxylate ($OPEO_2$) also had antiandrogenic activity, and as the EO chain length decreased, $OPEO_n$ increased its antiandrogenic activities (*13*). Although ecotoxicity depended on chemical structures with or without EO chain length, there is little evidence concerning their accurate biodegradation mechanism to clarify their ecotoxicity derived from $APEO_n$ in the environment. Our recent evidence showed that bacteria which can degrade $APEO_n$ to estrogenic and antiandrogenic metabolites exist fairly universally in paddy fields in Japan (*15*, *16*). Gram-negative rod-shaped saprotrophic soil bacteria *Pseudomonas putida* strain S5, isolated from rice paddy fields, can degrade $OPEO_n$ to $OPEO_2$ via oxidative biodegradation (*9*, *15*), which was confirmed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) analysis and biodegradation tests using ^{18}O-labeled water (*17*). Moreover, this proposed biodegradation mechanism was confirmed by recombinant alcohol dehydrogenase 1 (ADH1), which has both alcohol and aldehyde dehydrogenase activity (*18*, *19*).

Furthermore, the characterization of $APEO_n$ degradation *P. putida* S5 revealed that the final degradation products and their quantity depended on the presence of environmental elements such as calcium and magnesium ions (*13*). This demonstrated that ecotoxicity by the accumulated $APEO_n$ degradation products was influenced by environmental mineral conditions. Since we had a clue to solve why various $APEO_n$ degradation products were detected in the environment, our research has focused on the effect of environmental elements on the biodegradation mechanism.

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In this study, the isolated bacteria, named *Sphingomonas* sp. strain BSN22, was elucidated as follows; a novel biodegradation mechanism of $APEO_n$ to AP under aerobic conditions by strain BSN22 proceeded by a two-step process and depended on mineral conditions in the environment. Our findings will give useful evidence to fully understand the environmental problems arising from the $APEO_n$ issue.

MATERIALS AND METHODS

Chemicals. Octylphenol (OP) and octylphenol polyethoxylates, which has the commercial name Triton X-100, were purchased from Wako (Kyoto, Japan) and Aldrich Chemical Co. (USA), respectively. $OPEO_1$, $OPEO_2$, and $OPEO_6$ were synthesized based on our previous study (*15*, *20*). All other chemicals were obtained from Wako. The water was prepared by Milli-Q (Millipore, USA).

Culture Media. The liquid basal salt medium with 0.1% (w/v) of TX-100 as a sole carbon source, named TX-A medium, was described in our previous study (*9*). Briefly, the composition of 1 L of TX-A medium contains TX-100 (1.0 g), NaCl (2.5 g), Na_2HPO_4 (3.7 g), KH_2PO_4 (0.9 g), $(NH_4)_2SO_4$ (2.0 g), $MgSO_4 \cdot 7H_2O$ (0.3 g), $CaCl_2$ (0.1 g), 1.0 mL of minimal inorganic salt solution, and 2.0 mL of vitamin solution. The composition of 100 mL of minimal inorganic salt solution contains $MnCl_2 \cdot 4H_2O$ (20.0 mg), $CoCl_2 \cdot 7H_2O$ (4.0 mg), $Na_2MoO_4 \cdot 2H_2O$ (26.0 mg), and $FeCl_3 \cdot 6H_2O$ (15.0 mg). The composition of 100 mL of vitamin solution contains pyridoxine hydrochloride (10.0 mg), riboflavin (5.0 mg), thiamin hydrochloride (5.0 mg), nicotinic acid (5.0 mg), lipoic acid (5.0 mg), *p*-aminobenzoic acid (5.0 mg), folic acid (2.0 mg), biotin (2.0 mg), and vitamin B12 (0.1 mg). The composition of 1 L of TX-B medium contains sucrose (1.0 g), polypepton (0.5 g), yeast extract (0.3 g), $NaNO_3$ (0.2 g), Na_2HPO_4 (0.1 g), $MgSO_4 \cdot 7H_2O$ (0.05 g), KCl (0.05 g), and $FeSO_4 \cdot 7H_2O$ (1 mg) in 1 L of TX-A medium. TX-A and TX-B media containing 0.02% (w/v) $OPEO_6$ as the sole carbon source instead of 0.1% (w/v) TX-100 were named 6EO-A and 6EO-B media, respectively. The basal salt medium with 100 mM glucose instead of 0.1% (w/v) TX-100 was prepared as preincubation medium of strain BSN22.

Analysis. Gas chromatography (GC) analysis was performed under the same conditions as described previously (*13*) except for the oven temperature program. An aliquot of 1 μL of the sample was subjected to capillary gas chromatography using a GC-17A with a flame ionizing detector (Shimadzu, Japan). The GC was operated using a stainless capillary column (15 m × 0.25 mm I.D., Ultra ALLOY$^+$-1 HT; Frontier Lab, Japan) and programmed as follows: injector temperature, 360 °C; detector temperature, 390 °C; split ratio, 1/40; helium carrier flow rate, 100 kPa; oven temperature program, (1) 100 °C up to 235 °C at a rate of 20 °C/min, (2) 235 °C up to 270 °C at a rate of 10 °C/min, (3) 270 °C up to 350 °C at a rate of 7.5 °C/min, and (4) maintained at 350 °C for 6 min.

For gas chromatography mass spectrometry (GC-MS) analysis, an aliquot of 1 μL of the sample was subjected to capillary gas chromatography using a GC-17A and QP5050A instrument with a quadrupole mass spectrometry detector (Shimadzu, Japan). Mass spectra were obtained using the electron impact ionization (EI) mode at 70 eV. The GC-MS was operated using a glass capillary column (30 m × 0.25 mm I.D., DB-5MS; J&W Scientific, USA) and programmed as follows: injection temperature, 300 °C; detector temperature, 280 °C; injection system split ratio, 1/71; helium carrier flow rate, 0.7 mL/min; oven temperature program, (1) 50 °C for 2 min, (2) 50 °C up to 180 °C at a rate of 7 °C/min, (3) 180 °C up to 300 °C at a rate of 12 °C/min, and (4) maintained at 300 °C for 30 min.

For nuclear magnetic resonance (^{1}H NMR) analysis, the sample in $CDCl_3$ was subjected to a JNM-EX270 (JEOL, Japan) using tetramethylsilane (Cambridge Isotope Laboratories Inc.) as an internal standard.

Isolation of $OPEO_n$-Utilizing Bacteria. Bean plantation soil was collected in Gifu, Japan. The isolation procedures of $OPEO_n$-degrading bacteria were the same as described previously (*15*). Briefly, 0.5 g of soil was added to 5 mL of TX-A medium in a test tube. After shaking at 30 °C for 2 weeks, 100 μL of the sample suspension solution was transferred to 5 mL of newly prepared TX-A medium. This procedure was performed three times. Finally, 100 μL of the enriched sample solution was spread on the basal salt medium agar plate with 1% (w/v) TX-100. After one-week incubation, single colonies were selected and subjected to further studies.

Identification of Bacteria. The isolated bacteria were identified based on the sequence homology of the 16S rRNA gene (*21*). Chromosomal DNAs from the isolated bacteria were extracted using the Isoplant II kit (Nippon Gene, Japan). PCR amplification was performed using the general bacterial primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′). PCR amplification conditions of the 16S rRNA gene were as follows: (1) 5 min at 95 °C, (2) 30 cycles of 30 s at 95 °C, 30 s at 50 °C, and 1.5 min at 72 °C, (3) 5 min at 72 °C. The sequencing reaction was carried out using a DYEnamic ET Terminator Cycle Sequencing kit (GE Healthcare) and an ABI PRISM 310 Genetic analyzer (Applied Biosystems) according to the manufacturer's instructions. The Basic Lical Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/) program was used for homology analysis. Phylogenetic trees based on the 16S rRNA gene sequences were constructed with the Clustal X program (*22*), using the neighbor-joining (NJ) method (*23*).

Calibration Curve. Calibration curves for OP, $OPEO_1$, $OPEO_2$, $OPEO_3$, $OPEO_6$, and intermediate product ($OPEC_1$) were obtained using *n*-eicosane as the internal standard (IS). Two milligrams of these substrates were dissolved in 10 mL of ethyl acetate with 25 mg/L *n*-eicosane (IS-solvent), respectively, and further diluted to the designated concentrations with IS solvent.

The *x*-axis and *y*-axis indicate the ratio of the substrate area to the IS area and the ratio of substrate to IS concentration, respectively. The equations obtained as calibration curves were as follows: OP; $y = 0.9117x$ ($r^2 = 0.998$), $OPEO_1$; $y = 0.7176x$ ($r^2 = 0.998$), $OPEO_2$; $y = 0.7248x$ ($r^2 = 0.986$), $OPEO_3$; $y = 0.4584x$ ($r^2 = 0.982$), $OPEO_6$; $y = 0.4441x - 0.283$ ($r^2 = 0.999$), $OPEC_1$; $y = 0.4032x - 0.3276$ ($r^2 = 0.996$). These calibration curves were subjected to further study. Three separate experiments were performed simultaneously, and then this entire process was performed three times for a total of nine separate experiments.

Bacterial Biodegradation Test. The experimental procedures of all biodegradation tests were as described previously (*9*), except for the different treated medium and incubation time. Extraction procedures of degradation metabolites were as described previously (*17*) except for the use of IS solvent. Data are expressed as the means ± SD resulting from three separate experiments, with the entire process performed three times for a total of nine separate experiments. The extracts were analyzed by GC and GC-MS.

To confirm the biodegradation profile of TX-100, the isolated bacteria were incubated in 5 mL of TX-A and TX-B media for 14 days.

To characterize the biodegradation, the isolated bacteria were incubated in 200 μL of 6EO-A and 6EO-B media for 1−7, 10, and 14 days using polyethylene film. Throughout the experiments, polyethylene film was used as a sealant to prevent water loss and as a result no water loss was detected.

To elucidate the effect of inorganic ions (Mg^{2+}, Ca^{2+}, Fe^{3+}) on the biodegradation of TX-100, the isolated bacteria were incubated in 5 mL of TX-A medium with or without $MgSO_4 \cdot 7H_2O$ (0.3 g/L), $CaCl_2$ (0.1 g/L), and $FeCl_3 \cdot 6H_2O$ (10 mg/L), and in 5 mL of TX-B medium with or without $FeCl_3 \cdot 6H_2O$ (10 mg/L) instead of $FeSO_4 \cdot 7H_2O$. The cell growth of the isolated bacteria was followed spectrophotometrically at 660 nm (UV-1600; Shimadzu) after dilution with an equivalent volume of methanol, and the measured figure of optical density was doubled.

Isolation and Identification of the Intermediate Product. The intermediate product was purified from the biodegradation medium using silica gel column chromatography with eluting solvent (ethyl acetate/acetone = 3/2 vol %, and/or ethyl acetate/acetone/water = 60/35/5 vol %). About 0.2 g of extract dissolved in 1 mL of an eluting solvent was applied to an open column (44 mm I.D. × 730 mm long) packed with 300 g of Silica gel 60 (Merck). Fractions of about 10 mL were collected, and the components of each fraction were checked by GC. To determine the chemical structure, the purified sample was analyzed by ^{1}H NMR and GC-MS.

Confirmation of the Intermediate Product. Two hundred microliters of 6EO-B medium prepared with purified water (normal water) in test tubes was dried using a vacuum dryer ADP-21 (Yamato, Japan). The same amount of ^{18}O-labeled water (Cambridge Isotope Laboratories Inc., ca. 95−98% purity) was added to each corresponding test tube. An aliquot of 4 μL of the diluted culture with an optical density of 0.2 at 660 nm was inoculated into the test tubes. As the inoculum was prepared with normal water, the final concentration of ^{18}O-labeled water in the culture medium

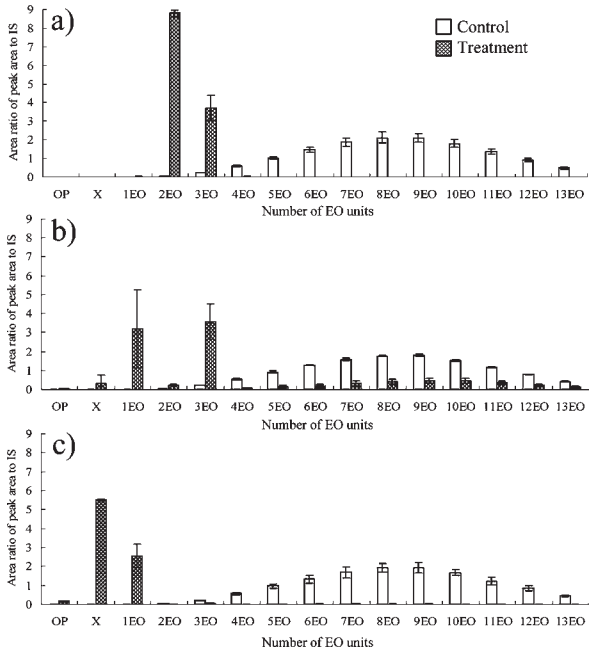

Figure 1. Biodegradation of TX-100 after incubation for 14 days. (a) Biodegradation by *P. putida* strain S5 in TX-A medium; (b) biodegradation by *Sphingomonas* sp. strain BSN22 in TX-A medium; (c) biodegradation by *Sphingomonas* sp. strain BSN22 in TX-B medium. Compound X: Intermediate product.

was slightly diluted to about 90−95%. The media were incubated with 100 rpm constant shaking at 30 °C for 14 days. As a control, media without bacteria were also incubated under the same conditions. After the designated time, the medium was extracted with 200 μL of IS solvent and the extracts were analyzed by GC and GC-MS.

RESULTS

Biodegradation Characterization and Identification of $OPEO_n$-Degrading Bacteria. To address the effect of trace elements on $OPEO_n$ biodegradation, successive enrichment culture was performed using $OPEO_n$ as the sole carbon and energy source. Strain BSN22 isolated from bean plantation soil had the highest homology with *Sphingomonas* sp. EM0332 (100%) based on the BLAST program by using the 16S rRNA gene sequence; therefore, the isolated bacteria were identified as *Sphingomonas* sp. BSN22.

The main degrading products were determined by GC-MS (data not shown) and quantified by GC based on their calibration curves. While *Pseudomonas putida* S5, isolated from the rice paddy field, produced $OPEO_2$ as the final product (**Figure 1a**) (*9*, *15*), strain BSN22 produced $OPEO_1$ (111.4 ppm, 445 μM) and $OPEO_3$ (194.8 ppm, 576 μM), but did not accumulate $OPEO_2$ in nutrient-poor TX-A medium after 14 days of incubation. Furthermore, OP and an intermediate product with a retention time of 4.9 min were detected by GC at concentrations of 0.8 ppm (4 μM) and 40.2 ppm (152 μM), respectively (**Figure 1b**). In nutrient-rich TX-B medium, strain BSN22 produced mainly $OPEO_1$ (89.5 ppm, 357 μM) and an intermediate product (362.0 ppm, 1369 μM) after 14 days of incubation. Additionally, 4.4 ppm (21 μM) of OP was also detected (**Figure 1c**), whose concentration was about 5-fold higher than that of OP produced in TX-A medium. This means that strain BSN22 has a different characterization from *P. putida* S5 and its biodegradation pathway depends on the constituents in media.

P. putida S5 and *Sphingomonas* sp. BSN22 have accession numbers AB512773 and AB512774, respectively, in the DDBJ/EMBL/GenBank.

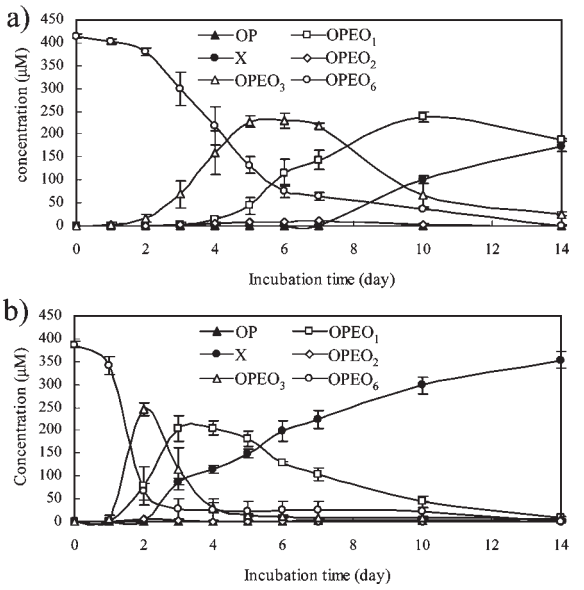

Figure 2. Quantitative analysis of $OPEO_6$ biodegradation after incubation in 6EO-A medium (a) and 6EO-B medium (b) for 14 days.

Quantitative Analysis of $OPEO_6$-Degrading Activity by Strain BSN22. Quantitative biodegradation analysis using $OPEO_6$ with a hexamer of EO units was performed to verify the difference between their biodegradation profiles caused by the treated medium (**Figure 2**). In 6EO-A medium, as the concentration of $OPEO_6$ decreased by 82.6 μM/day, and that of $OPEO_3$ increased by 72.2 μM/day and reached a maximum concentration of 77.7 ppm (230 μM) after 5 days of incubation. The concentration of $OPEO_1$ began to increase by 37.8 μM/day after 3 days of incubation, and reached a maximum concentration of 59.7 ppm (238 μM) after 10 days of incubation as the concentration of $OPEO_3$ decreased. The concentration of the intermediate product began to increase by 24.5 μM/day after 7 days of incubation, and reached a maximum concentration of 46.0 ppm (173 μM) after 14 days of incubation (**Figure 2a**).

In contrast, in 6EO-B medium, the concentration of $OPEO_3$ reached a maximum concentration of 83.7 ppm (247 μM) after 2 days of incubation (240.2 μM/day) as the concentration of $OPEO_6$ decreased by 276.0 μM/day and $OPEO_1$ reached a maximum concentration of 51.4 ppm (205 μM) after 4 days of incubation (101.7 μM/day). The intermediate product was detected after 3 days of incubation, and its maximum concentration, 93.4 ppm (353 μM), was 2-fold higher than that obtained in TX-A medium after 14 days of incubation, but the 24.7 μM/day of biodegradation rate was almost the same as that of TX-A medium. OP was detected after 7 days of incubation, and reached a maximum concentration of 1.0 ppm (5 μM) after 14 days of incubation, while no OP was detected in 6EO-A medium (**Figure 2b**). The biodegrading rate in 6EO-B medium was accelerated three times compared to in 6EO-A medium. This means that some constituents in 6EO-B medium accelerate the biodegradation rate of $OPEO_6$.

Identification of the Intermediate Product. Since identifying the intermediate product was a key to understanding the biodegradation mechanism of $OPEO_n$ by strain BSN22, the intermediate product with a retention time of 26.8 min was determined by using GC-MS. Although both the typical fragment ion peaks of 135 (*m*/*z*) and 193 (*m*/*z*) derived from OP proved that the intermediate product came from the biodegradation of $OPEO_n$ (**Figure 3a**), three candidate degrading products (i.e., *p*-*t*-octylphenoxy acetate ($OPEC_1$), *p*-*t*-octylphenyl-2-hydroxyacetate,

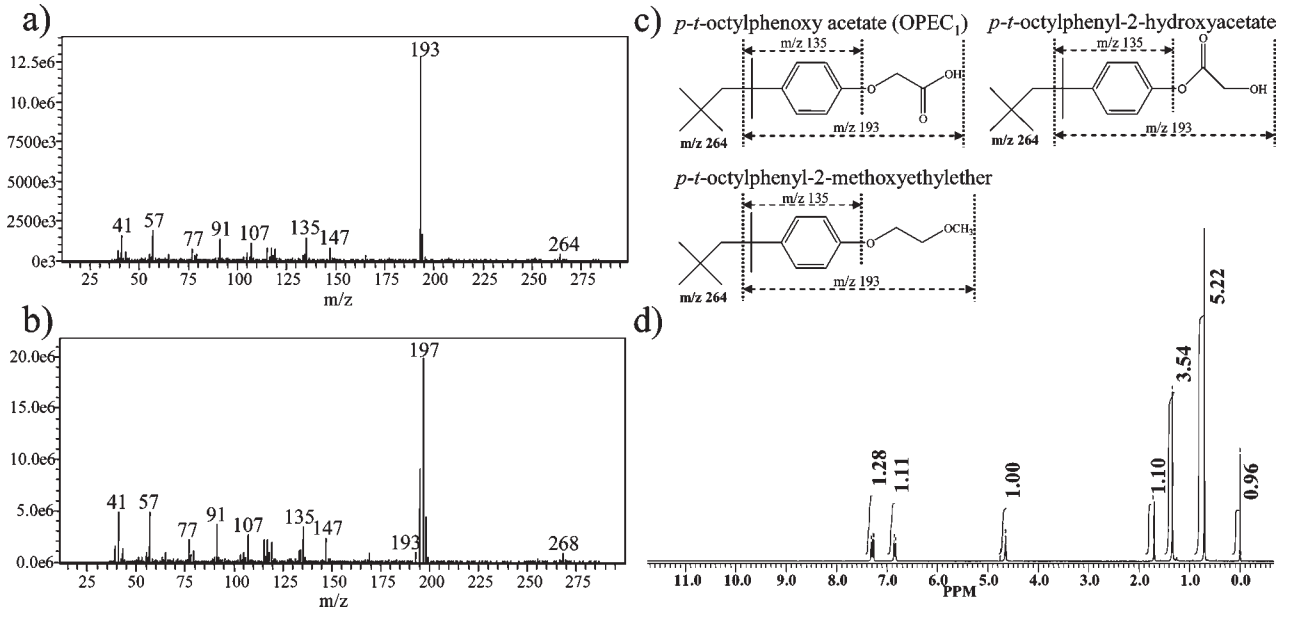

Figure 3. The identification of the intermediate product (X). Mass spectra of the $OPEO_6$ biodegradation product after incubation for 14 days in normal water (a) and that in ^{18}O-labeled water (b) by *Sphingomonas* sp. strain BSN22 were compared. Three possible chemical structures (c) were assigned by GC-MS analysis (a) and ^1H NMR spectra of the purified intermediate product (d).

and *p-t*-octylphenyl 2-methoxyethylether) were suggested based on its parent peak (*m*/*z* 264) (**Figure 3c**).

To confirm the chemical structure of the intermediate product, it was purified by silica gel column chromatography and analyzed by ^1H NMR (**Figure 3d**). Either $OPEC_1$ or *p-t*-octylphenyl-2-hydroxyacetate was assigned as possible chemical structures based on ^1H NMR spectra as follows: ^1H NMR δ ($CDCl_3$); 0.71 ($CH_3 \times 3$, s), 1.34 ($CH_3 \times 2$, s), 1.70 (CH_2, s), 4.65 (CH_2, s), 6.84 (2H, d), 7.28 (2H, d); however, there was no significant difference between them.

Determination of the Intermediate Product Using ^{18}O-Labeled Water. To determine the correct chemical structure of the intermediate product, mechanism-based biodegradation tests of $OPEO_6$ using ^{18}O-labeled water were performed. In the case of *p-t*-octylphenyl-2-hydroxyacetate, its *m*/*z* corresponding to the parent peak will shift to a 2 Da higher *m*/*z* peak due to its oxidation if oxygen is supplied from ^{18}O-labeled water. However, in the case of $OPEC_1$, the observed parent peak may shift to a 4 Da higher *m*/*z* peak based on our previous study because two ^{18}O atoms were included during the exo-oxidation degradation of $OPEO_6$ in ^{18}O-labeled water (*17*). The total ion chromatogram of the degraded $OPEO_6$ using both normal water and ^{18}O-labeled water showed a peak with a retention time of 26.8 min, whose spectra gave M^+ ions at 264 and 268 (**Figure 3a,b**), respectively, and the fragment ion peak of M-71 resulting from the loss of the 2,2-dimethyl-propyl group. The fact that the mass spectra corresponding to the intermediate product shifted to a 4 Da higher mass confirmed it as $OPEC_1$, suggesting that this degradation was exo-oxidized by alcohol dehydrogenase from strain BSN22.

Effect of Trace Elements on Biodegradation of $OPEO_n$. As our previous study showed that some inorganic cations influence the biodegradation of $OPEO_n$, the detailed effects of trace elements on $OPEO_n$ biodegradation were examined (**Table 1**, **Figure 4**). In TX-A medium, each ion, Mg^{2+}, Ca^{2+}, and Fe^{3+}, itself did not accelerate $OPEO_n$ biodegradation; however, whenever Mg^{2+} and Ca^{2+} coexisted in the medium, $OPEO_n$ biodegradation was confirmed. Furthermore, in TX-A medium with Mg^{2+}, Ca^{2+}, and Fe^{3+}, endocrine active substances such as OP, $OPEC_1$, and $OPEO_1$ were accumulated in medium at concentrations of 22.8, 221.7, and 961.1 μM, respectively, after 14 days of incubation, and were about 2.0, 2.6, and 18.8 times higher than that in TX-A medium with Mg^{2+} and Ca^{2+}, respectively. Since the concentrations of OP, $OPEO_1$, and $OPEC_1$ in the TX-B medium with Mg^{2+}, Ca^{2+}, and Fe^{3+} reached 32.8 μM, 416.4 μM, and 840.4 μM, respectively, after 14 days of incubation, it was about

Table 1. Effect of Trace Elements on Biodegradation of TX-100 by *Sphingomonas* sp. Strain BSN22 after Incubation in TX-A and TX-B Media for 14 Days[a]

trace element	OP	$OPEC_1$	$OPEO_1$	$OPEO_2$	$OPEO_3$
TX-A medium					
Mg^{2+}, Ca^{2+}, Fe^{3+}	22.8[b]	221.7	961.1	19.2	223.7
Mg^{2+}, Ca^{2+}	11.3	86.2	51.2	19.4	532.6
Mg^{2+}, Fe^{3+}	0.0	0.0	0.0	5.6	37.0
Ca^{2+}, Fe^{3+}	0.0	0.0	0.0	5.8	39.3
Mg^{2+}	0.0	0.0	0.0	5.3	36.5
Ca^{2+}	0.0	0.0	0.0	5.6	37.3
Fe^{3+}	0.0	0.0	0.0	8.5	41.3
—[a]	0.0	0.0	0.0	5.3	36.3
TX-B medium					
Mg^{2+}, Ca^{2+}, Fe^{3+}	32.8	416.4	840.4	11.0	14.0
Mg^{2+}, Ca^{2+}	10.7	138.2	761.2	25.1	390.7

[a] a: No trace elements, b: concentration (μM) of degraded compounds.

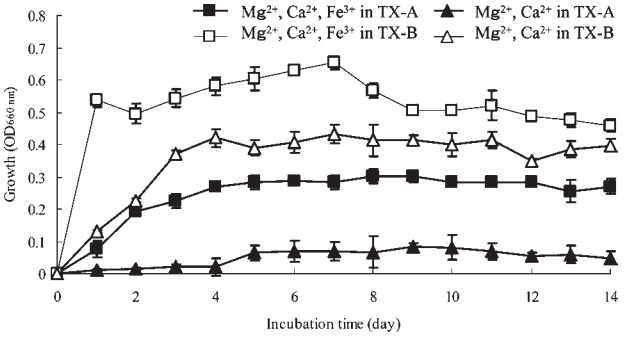

Figure 4. Growth curve of strain BSN22 on TX-A and TX-B media with different trace elements evaluated using optical density at 660 nm.

3.1, 3.0, and 1.1 times higher than in TX-B medium with Mg^{2+} and Ca^{2+}, respectively. No stimulation of $OPEO_n$ biodegradation activity by Fe^{3+} was observed in the absence of Mg^{2+} or Ca^{2+}. These results indicated that inorganic cations, Mg^{2+}, Ca^{2+}, and Fe^{3+}, in the environment were essential for biodegradation of $OPEO_n$ by strain BSN22 and the presence of Fe^{3+} significantly accelerated $OPEO_n$ biodegradation.

The cell growth of strain BSN22 that reached $OD_{660\ nm} = 0.27$ after 4 days of incubation in TX-A medium with Mg^{2+}, Ca^{2+} and Fe^{3+} was about 4 times higher than that of TX-A medium without Fe^{3+}, and its rate was $OD_{660\ nm} = 0.07$ /day. In TX-B medium, the cell growth of strain BSN22 in the coexistence of Mg^{2+} and Ca^{2+} reached $OD_{660\ nm} = 0.42$ after 4 days incubation and its rate was $OD_{660\ nm} = 0.11$ /day, while $OD_{660\ nm}$ in TX-B medium with Mg^{2+} and Ca^{2+} was higher than that in TX-A medium with Mg^{2+}, Ca^{2+}, and Fe^{3+}. In TX-B medium with Mg^{2+}, Ca^{2+}, and Fe^{3+}, since $OD_{660\ nm}$ reached 0.54 after 1 day of incubation, it was higher than in TX-B medium with Mg^{2+} and Ca^{2+}. These results suggested that the presence of Fe^{3+} and yeast extract in the medium increased the cell growth of strain BSN22.

DISCUSSION

Our previous study revealed that harmless $APEO_n$ was degraded to metabolic toxicants with diverse chemical structures like AP, $APEO_{1,2}$, and $APEC_{1,2}$ (*13*, *15*, *17*). The IC_{50} value of antiandrogenic activity of OP was 6.1×10^{-6} M (*13*, *24*), while the estorogenic effect of OP on MCF-7 cell growth was observed at a concentration of 1×10^{-5} M (*14*). Therefore, their antiandrogenic adverse effect may have more latent impact against wildlife because antiandrogenic activity was expressed by a lower concentration of OP than estrogenic activity; however, it is necessary for understanding ecotoxicity to clarify the detailed degradation mechanism by which diverse degraded compounds detected in the environment were produced. Although inorganic elements influenced the biodegradation of $APEO_n$ (*13*), there is little evidence of how environmental elements influenced $APEO_n$ degradation products. Since a zymogenic group is usually responsible for the biodegradation of man-made chemicals in the environment, diverse media which reflected copiotrophic and oligotrophic conditions were used to understand the various $APEO_n$-degrading activities in this study.

Liu reported AP formation from alkylphenol oligoethoxylates by isolated bacteria under aerobic conditions (*25*, *26*); however, no specific degradation mechanism degraded long EO-chained $APEO_n$ to AP under aerobic conditions by those bacteria. On the basis of our stoichiometrical study of $OPEO_6$ degradation, strain BSN22 had a two-step degradation process: (1) degradation of $OPEO_n$ to $OPEO_3$ by exo-oxidative biodegradation, (2) degradation of $OPEO_3$ to OP by oxidative biodegradation without the accumulation of $OPEO_2$ (**Figure 2**). Moreover, these results suggested that the degradation of $OPEO_2$ to $OPEO_1$ by strain BSN22 was faster than that of $OPEO_3$ to $OPEO_2$. $NPEO_n$ was also degraded via the same degradation mechanism (data not shown).

Importantly, most endocrine active OP was produced after accumulation of the intermediate product from $OPEO_1$, so this key intermediate product was identified to verify their biodegradation mechanism quantitatively. The fact that the key intermediate product was $OPEC_1$ revealed that biodegradation from $OPEO_1$ to OP was caused by an oxidative reaction in a certain alcohol dehydrogenase. There is need for further study to identify the enzymes induced by the degradation process because each oxidation step may involve different enzymes.

In this study, our previously reported microscale biodegradation method (*17*) was not adequate for long-term quantitative biodegradation experiments because of water loss; therefore, we developed a new long-term and microscale biodegradation method using polyethylene film, which prevents water loss from the medium. This study also demonstrated that a biodegradation study using a stable isotope like ^{18}O-labeled water is a very effective approach not only to determine the degradation mechanism, but also to identify key products with very similar spectra.

Since this is the first report on the quantitative analysis of aerobic degradation of long EO-chained $APEO_n$ to AP and their biodegradation profile significantly depended on the medium condition (**Figures 1** and **2**), our interest next focused on the interaction between $APEO_n$ degradation and environmental elements. Although there is evidence that the coexistence of a carbon and energy source, such as yeast extract, inhibited ether cleavage of the EO chain (*27*), this study revealed that the coexistence of yeast extract enhanced bacterial growth 2-fold, and stimulated the $APEO_n$ biodegradation activity of strain BSN22 (**Table 1**, **Figure 4**).

Recently, we reported that *P. putida* S5 supplemented with Ca^{2+} promoted the biodegradation of $APEO_n$ to $APEC_n$ rather than that of Mg^{2+} (*13*). In strain BSN22, the coexistence of Mg^{2+} and Ca^{2+} stimulated cell growth and $APEO_n$-degrading activity. Moreover, the addition of Fe^{3+} promoted the increase of cell growth, accelerated $APEO_n$ biodegradation activity, and produced more potent endocrine active metabolites (**Table 1**, **Figure 4**). Furthermore, the fact that cell growth in TX-B medium with Mg^{2+} and Ca^{2+} was higher than in TX-A medium with Mg^{2+}, Ca^{2+}, and Fe^{3+}, but the biodegradation rate of $APEO_n$ was lower, suggested that $APEO_n$ biodegradation was promoted by the presence of Fe^{3+}, which induced and/or stimulated enzymes. The arithmetic mean of the amount of iron in Japanese soil is 53 g kg^{-1} (range 0.53−252 g kg^{-1}) (*28*); therefore, the concentration of Fe^{3+} in the environment is sufficient to accelerate the biodegradation of $OPEO_n$. Taken together, the activity of $APEO_n$ degradation and especially the production of endocrine active chemicals like AP, $APEO_1$, and $APEC_1$ depended on the concentration of Mg^{2+}, Ca^{2+}, and Fe^{3+} in the environment. Therefore, this means that the ecotoxicity caused by endocrine active metabolites was significantly influenced by the type of minerals which affect the concentration of accumulated biodegradation products in the environment, suggesting that trace amounts of minerals as well as carbon and nitrogen sources play an important role in the biodegradation of $APEO_n$ in the environment. This means that it is important not only to isolate $APEO_n$-degrading bacteria but also to clarify the $APEO_n$ biodegradation mechanism based on the identification of degraded compounds under different medium conditions.

In conclusion, the microbial degradation of man-made chemicals in the environment has been classified into three main types as follows: (1) cell growth stimulated by chemicals such as carbon and energy sources enhances the biodegradation rate, (2) cell growth stimulated by minerals enhances the biodegradation rate, (3) enzymes induced and/or stimulated by minerals accelerate the biodegradation rate. As $APEO_n$ biodegradation was significantly influenced by minerals, this may enable a significant advance in technologies for the control and prevention of ecotoxicity by $APEO_n$ pollution. Furthermore, our findings advance the idea that external inputs, such as minerals in the environment, modulate a specific response of the microbe community by regulating the population size of a specific microbe involved in controlling the biodegradation of man-made chemicals. Therefore, microbe plasticity in the environment can cause the weakening or strengthening of communication between microbes and external inputs under the stress of man-made chemicals.

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