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Bioremediation of PCDD/Fs-contaminated municipal solid waste incinerator fly ash by a potent microbial biocatalyst

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Abstract

Removal of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) from fly ash poses a serious problem. In the study presented here, we used a microbial biocatalyst which is a mixture of 4 bacterial and 5 fungal dioxin-degrading strains. The ability of this biocatalyst to bioremediate PCDD/Fs from contaminated municipal solid waste incinerator (MSWI) fly ash was examined by solid-state fermentation under laboratory conditions. Treatment of MSWI fly ash with the microbial biocatalyst for 21 days resulted in a 68.7% reduction in total toxic PCDD/Fs. Further analyses revealed that the microbial biocatalyst also removed 66.8% of the 2,3,7,8-substituted congeners from the fly ash. During the treatment period, the presence of the individual strains composing the microbial biocatalyst was monitored by the amplification of strain-specific DNA sequences followed by denaturing gradient gel electrophoresis (DGGE). This analysis showed that all of the bacterial and fungal strains composing this dioxin-degrading microbial mixture maintained under the dioxin treatment conditions. These results demonstrate that this microbial biocatalyst could potentially be used in the bioremediation of PCDD/Fs from contaminated fly ash.

Keywords: Bioremediation; PCDD/Fs (polychlorinated dibenzo-*p*-dioxins and dibenzofurans); Incinerator fly ash; Microbial biocatalysts; DGGE (denaturing gradient gel electrophoresis) analysis

1. Introduction

Polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) are released into the environment from various sources, such as municipal and industrial waste incineration. They are also unwanted by-products in various chlorinated chemical formulations (e.g., chlorophenols, pesticides), and extremely resistant to both environmental and biological degradation [1–3]. In fact, various polychlorinated compounds, such as dioxins, may be generated during incineration, leading to the contamination of the environment surrounding incineration facilities. Since dioxins are known to be recalcitrant contaminants, their toxic effects can linger for long periods of time after contamination [1,4,5]. Thousands tonnes of fly ash are produced every day worldwide from incinerators [14]. Since PCDD/Fs are the major toxic chlorinated organic contaminant of fly ash, an effective method for the removal of PCDD/Fs from fly ash is needed for proper environmental management.

In Korea, incinerator fly ash is typically buried in landfill sites. This leads to serious dioxin contamination of soil leachates from those landfill sites. There have been strident objections against this type of disposal for fly ash [6]. These objections were further strengthened by the identification of significant amounts of dioxins in the bottom layer of buried fly ash, a finding that indicates that these compounds should no longer be buried in landfill sites. Other approaches for the disposal of fly ash include solidification by cementing, stabilization by chemical treatment, stabilization by solvent treatment for the extraction of acids and the neutralization of gas exhaust, and stabilization by thermal treatment [7]. Chemical treatment of fly ash generates significant amounts of

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by-products which become secondary contaminants that must, in turn, be treated. None of the processes described above actually remove dioxin from fly ash, suggesting it could still lead to site contamination. Compared to the other approaches, thermal treatment uses more energy, costs more, and requires an additional facility for the treatment of gas exhaust. In contrast to the physical and chemical treatments of fly ash outlined above, biological treatment is an eco-friendly and cheaper approach and does not cause secondary contamination. Currently, several countries are attempting to develop a viable biological treatment method for dioxin; however, this technology is still in its infancy.

Bioremediation of toxic compounds, such as PCDD/Fs, from the environment is one of the most important areas in environmental microbiology and biotechnology [8]. A number of studies have suggested a potential for certain microorganisms in bioremediation processes. For example, several microbial species are able to catabolize dibenzo-*p*-dioxin (DD) and dibenzofuran (DF), and in some cases, these microorganisms are able to attack chlorinated analogues as well. Recently, a number of studies on the microbial degradation of dioxin and dioxin-like compounds have been reported [9–13]. In addition, bioaugmentation is a promising, albeit controversial, technology used for the remediation of contaminated areas. Since the 1990s, researchers have tested bioaugmentation for the treatment of soils containing recalcitrant organic compounds.

Recently, we reported the ability of the *Sphingomonas wittichii* strain RW1 to remove polychlorinated dibenzo-*p*-dioxins (PCDDs) from fly ash [14]. To enhance the removal of PCDD/Fs, we used a high population density of degrader organisms. We have also analyzed the removal efficiency of toxic PCDDs and the effects of surfactant addition, repeated inoculation, and pre-adaptation of degrader organism cultures. Our previous results suggested that the *S. wittichii* strain RW1 was a potential candidate for the removal of PCDDs from incinerator fly ash. However, the PCDDs degradation extent was strictly congener specific hence the PCDDs removal was achieved by both biodegradation and adsorption mechanism [14]. In contrast, white rot fungus efficiently degrades several highly chlorinated PCDDs, such as OCDD, and effectively removes PCDD/Fs from fly ash [15,16].

Alternative to axenic culture the use of co-culture would significantly enhance the degradation efficiency against the pollutants which are hard to degrade. Previously, Boonchan et al. [17] reported that the used of co-culture composed of bacterial and fungal strains significantly mineralized the high molecular weight polycyclic aromatic hydrocarbons than in axenic cultures. As far as the authors know, the removal of PCDD/Fs from fly ash using a co-culture, composed of bacteria and fungi has not been reported. In the present paper, such process is reported. The objective was to evaluate the efficacy of mixed microbial biocatalyst for removal of PCDD/Fs, environmentally serious pollutants in the incinerator fly ash. We analyzed the community of the microbial biocatalysts during treatment of fly ash using denaturing gradient gel electrophoresis (DGGE). This analysis clearly showed that the bacterial and fungal strains in the dioxin-degrading microbial mixture were maintained throughout the treatment process and were, therefore, stable under the dioxin treatment conditions.

2. Methods and materials

2.1. Chemicals

An internal standard solution at a concentration of 100 μg/dm^3 was prepared by dissolving 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TCDD) and 1,2,3,7,8,9-hexachlorodibenzo-*p*-dioxin (1,2,3,7,8,9-HxCDD) in nonane. All [^{13}C]-labelled internal and performance standards, as well as the calibration solution, were purchased from Wellington Laboratories. All PCDD/Fs authentic mixtures were purchased from AccuStandard (New Haven, CT) and Ultra Scientific (Kingstown, RI). Dibenzofuran was purchased from Sigma (St. Louis, MO). Toluene, *n*-hexane, methanol, dichloromethane, and acetone (all organic solvents were trace analysis grade) were purchased from Merck (Darmstadt, Germany). Sulfuric acid, phosphoric acid, silica, aluminum oxide, granulated agar, nutrient broth, and potato dextrose broth were purchased from Merck. All chemicals were of the highest commercially available purity.

2.2. Fly ash preparation

Fly ash used in this study was obtained from a MSWI (Municipal Solid Waste Incinerator) in Daejon, South Korea. The fly ash samples were sieved through a 0.2–2.6 μm mesh and pooled together. The pooled samples were then mixed for 24 h using a jar-tester and tubular mixer. These homogeneous mixtures were stored in desiccators and maintained at a temperature of 4 °C until used. Primary characteristics including the total concentration of PCDD/Fs of the fly ash and preparation of PCDD/Fs-free fly ash were as described previously [14].

2.3. Isolation of dioxin-degrading microorganisms

Dioxin-degrading bacteria were isolated from various environmental matrices such as contaminated soil and sediment and even forest soil which contains enormous number of bacteria. They were screened by three or four successive treatments with 1 mM dibenzo-*p*-dioxin (DD) and dibenzofuran (DF) followed by several rounds of enrichment in minimal salts medium (MSM, pH 7.0) in which DD and DF were the sole source of carbon and energy [18]. The enrichment culture was serially diluted with the minimal salts medium, and aliquots were spread on nutrient medium. Approximately 30 colonies were randomly selected and transferred from the solid agar medium, and then tested for their DD- and DF-degrading ability in liquid MSM. A single bacterial isolate was found to be efficient in the degradation of DD and DF. This isolate was characterized by the semi-automated microbial ID/characterization (Biolog, Hayward, CA) test system, fatty acid analysis, and comparative 16S rRNA gene analysis. For this analysis, the 16S rRNA gene was amplified by polymerase chain reaction (PCR; Mycycler, Bio-Rad, Hercules, CA) and its nucleotide sequence was determined [19]. Searches for nucleic acid similarities were

Table 1
List of bacteria and fungi with high dioxin-degrading activities combined into a single microbial agent

Microorganism	Strain	Source
Bacterium	*Sphingomonas* sp. HH69	DSMZ (Germany)
	Sphingomonas wittichii RW1	DSMZ (Germany)
	Pseudomonas veronii PH-03	POSTECH (Korea)
	Paenibacillus sp. VSE5L	Isolated in this study
Fungus	*Phanerochaete chrysosporium* DSM 6909	DSMZ (Germany)
	Phanerochaete chrysosporium DSM 1556	DSMZ (Germany)
	Irpex sp. KW3	KRIBB (Korea)
	Trametes sp. CH2	Isolated in this study
	Fusarium sp. VSO7	Isolated in this study

performed using the BLAST program in the EMBL and GenBank databases. The bacterial strain was identified as *Paenibacillus* sp. Soil samples with decayed wood biomass were employed from the forest for the isolation of fungi. The soil fungi were isolated using serial dilution method on Potato dextrose agar (PDA) medium. Several fungal strains were isolated from PDA plates and they were subjected to preliminary screening for DD and DF degradation on low nitrogen mineral medium. Finally, we found two efficient fungal strains for DD and DF degradation and we selected those two strains for further study. Based on the molecular identification method we confirmed the isolated strains as *Trametes* sp. and *Fusarium* sp.

2.4. Preparation of microbial biocatalyst

In order to prepare the mixed microbial biocatalyst for the bioremediation of PCDD/F-contaminated fly ash, we mixed the 4 bacterial and 5 fungal dioxin-degrading strains listed in Table 1. For seed cultures of the bacteria, each strain was inoculated independently in 1 dm^3 of minimal salt media containing 1 mM DF as the sole carbon source. The cells were grown for 48 h in the shaking incubator, harvested using a continuous high speed centrifuge (8000 × *g* for 20 min at 4 °C) in exponential growth phase, and then washed 3 times in 20 mM phosphate buffer. The four different pellets of bacterial strains were suspended together in 0.7 dm^3 of the media without DF, mixed with 100 g of vermiculite (Table 2), and then incubated for 24 h at 28 °C. The vermiculite was added to increase the efficiency of mixing of the microorganisms and to maintain aeration. To prepare the fungal inocula, the 5 fungal strains were grown independently in 0.1 dm^3 of potato dextrose broth in 0.5 dm^3 Erlenmeyer flasks for 5–7 days. The strains were harvested and washed with minimal salt medium. An equal weight (50 g wet mycelium) of each of the five fungal strains were combined and homogenized in minimal medium containing 10% molasses and 0.03 kg/dm^3 sawdust. To make the working microbial biocatalyst the bacterial mixture and the fungal mixture were combined with a ratio of 1:4 (w/w) developed experimentally, and it was used as the microbial biocatalyst in the fly ash treatment experiments.

Table 2
Components of vermiculite

Components	Content (%)
SiO_2	32.6
Al_2O_3	8.13
CaO	10.1
Fe_2O_3	7.65
MgO	26.8
Na_2O	0.28
K_2O	4.41
Others	10.03

2.5. Removal of DD and DF from fly ash by mixed microbial biocatalysts

To evaluate the ability of the microbial biocatalyst to remove non-chlorinated dioxins, such as DD and DF, in fly ash, we conducted the experiments in PCDD/F-free fly ash in a solid-state fermentation. Five grams of fly ash were moistened with MSM to a moisture content of 50% and transferred to a 0.1 dm^3 culture bottle. Each bottle was inoculated with 1 g of microbial biocatalyst and mixed well. Then, 1 mM of DD or DF was added. The DD and DF were prepared from crystal form to a final concentration of 1 mM. Two types of control experiments were performed. One control experiment used inactivated microbial biocatalyst generated by autoclaving twice at 121 °C for 15 min. The other control experiment included the use of vermiculite. The samples and corresponding controls were incubated at 28 °C for 96 h, and the moisture content was maintained at 50%. Every 6 or 12 h, samples were collected and stored at −20 °C. At the end of the time course, the samples were thawed, and the contents were extracted and analyzed as described in the "sample extraction and clean-up" and "analytical methods" sections.

2.6. Removal of PCDD/Fs from fly ash

To assess the efficiency of the removal of PCDD/Fs from MSWI fly ash by the manufactured microbial biocatalyst, experiments were carried out using 5 g of fly ash with 50% moisture content and 2 g of microbial biocatalyst (40% portion). The mixed samples were incubated for 21 days at 28 °C in solid-state conditions in a fermentation bottle. The control cultures were maintained as described in the previous section. Throughout the incubation, the moisture content was maintained at 50%. After the 21 days incubation, PCDD/Fs were extracted from the cultures with toluene, and the concentration of the PCDD/Fs was determined using the methods described in the next sections. All data are reported as a mean value of triplicate determinations.

2.7. Molecular biological analyses

PCR amplification of the 16S rRNA gene followed by the denaturing gradient gel electrophoresis (DGGE) analysis of the products was used to examine the community of the microbial biocatalyst mixture. DNA was extracted from 1 g of sample using a MoBio kit (Carlsbad, CA). The UltraClean soil DNA kit, used according to the manufacturer's instructions, was also

Table 3
Specific PCR primers for bacteria and fungi

Microorganism	Specific gene	Primer for PCR	Sequences
Bacteria	16S rDNA gene	F1	5′ AGA GTT TGA TCC TGG CTC AG 3′
		R12	5′ GGT TAC CTT GTT ACG ACT T 3′
		GC-338F	5′ CGC CCG CCG CGC GGC GGG CGG GGC GGG GGC ACG GGG GGA CTC CTA CGG GAG GCA GCA G 3′
		518R	5′ ATT ACC GCG GCT GCT GG 3′
Fungi	ITS region of rDNA gene	GC-ITS1-F	5′ CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCT TGG TCA TTT AGA GGA AGT AA 3′
		ITS2	5′ GCT GCG TTC TTC ATC GAT GC 3′

employed. The DNA extraction protocol included a 10 min incubation of the extraction mixture at 70 °C prior to the physical disruption of the cells, a phenol extraction step to remove protein, and a precipitation step. The DNA recovered from each aliquot was combined into a single sample before the quality and quantity of the extracted DNA was estimated by gel electrophoresis. DGGE analysis was performed every 7 days throughout the incubation period using previously described methods [20]. The PCR program consisted of 20 cycles of 60 s at 95 °C, 60 s at 50 °C, and 110 s at 72 °C. The bacteria- and fungi-specific primers used for the PCR are listed in Table 3. For amplification of fungi-specific genes, parts of the ITS region between the 18S rDNA and the 23S rDNA were used. The primers used in this PCR amplification were ITS1-F and ITS2R. The PCR program used for this amplification consisted of 35 cycles of 30 s at 94 °C, 40 s at 55 °C, and 40 s at 72 °C using fungi-specific primers. The recovered DNA was estimated by agarose gel electrophoresis. DGGE analysis was performed using the Bio-Rad DCode system (Richmond, CA). PCR products were loaded onto a 7% (w/v) polyacrylamide gel (37.5:1 acrylamide:bisacrylamide ratio) in TAE buffer (pH 7.4). The gels were made with a denaturing gradient from 30 to 70% (100% denaturant was 7 M urea and 40% formamide). The gel was run for 15 h at 60 V and 60 °C, then stained with ethidium bromide (0.5 mg/dm^3) for 30 min and destained in water for 15 min.

2.8. Sample extraction and clean-up

After the 21-day incubation, the experimental and control samples were transferred to an accelerated solvent extractor (ASE200, DIONEX, Sunnyvale, CA) and extracted for 3 h with toluene. Before extraction, a mixture of ^{13}C-labelled PCDD/Fs (1 ng each) was added as an internal standard. The ASE conditions used for the toluene extraction were as follows: (1) temperature: 200 °C, (2) pressure: 2000 psi, (3) static time: 15 min, (4) flush volume: 60%, (5) purge time: 100 s, and (6) number of static cycle: 3. The extracted samples were washed with H_2SO_4 until colorless and then with hexane rinsed water to neutralize. These samples were also cleaned using multi silica gel and acid alumina columns [21]. The anhydrous Na_2SO_4 (Kanto Chemical, Japan) was maintained at 450 °C overnight before use. The impregnated silica gel used was 100–200 mesh (Merck Korea Ltd., Korea) prepared by heating at 130 °C for 13 h. Activated silica gel was packed into the column according to the multi-layer silica method. Impregnated alumina was prepared by heating for 18 h at 180 °C. Activated alumina (10 g) was packed into the column. Sample cleanup was done in two stages: (1) a multi-silica gel column (with layers of basic, neutral, acidic, and neutral silica) and (2) an activated acidic alumina column capped with anhydrous Na_2SO_4 and concentrated with N_2 gas. Finally, the [^{13}C]-labelled PCDD/F spiking solution was prepared by dissolving 17-toxic equivalents factor (TEF) PCDD/Fs congeners in nonane such that the concentration of each congener was 100 μg/dm^3. This solution was added to each sample in the experiment.

2.9. Analytical methods

The extracted samples were treated and analyzed as previously described [14]. PCDD/Fs were analyzed using high-resolution gas chromatography/mass spectrometry (Hewlett–Packard Model 6890 series II/JMS 700T) with a DB-5 MS column (60 m, 0.25 mm i.d., 0.25 μm film thickness). The temperature program of the capillary column was as follows: (1) 140 °C initial temperature for 4 min, (2) increased 15 °C/min to an isothermal hold at 220 °C for 3 min, (3) increased 1.5 °C/min to an isothermal hold at 240 °C for 2 min, and (4) increased 4 °C/min to an isothermal hold at 310 °C for 6 min. The sample was introduced by splitless injection. The MS was operated over 10,000 resolution under positive EI conditions (38 eV electron energy), and data were obtained in the single ion monitoring (SIM) mode. During the GC/MS data acquisition, perfluorokerosene was bled into the mass spectrometer to obtain high accuracy assignments over 10,000 mass resolutions. Two ions were monitored, including M^+, M^{2+}, and M^{4+}. The toxic 2,3,7,8-substituted congeners were quantified as well as the totals for the tetra to octachlorinated homologues. The peaks were quantified when the criteria were the following: (1) the isotope ratios were within ±15% of theoretical values and (2) the signal noise ratio ≥2.5. Recoveries of the ^{13}C-labelled PCDD/Fs internal standards in the environmental samples ranged from 50 to 120%, a satisfactory range according to the EPA method 1613 [6,14,22,23]. Leaching tests for heavy metals were performed using the methods of the Korea Standard Leaching Test which is based on the US EPA method 1311 [14,24]. Fifty grams of sample was mixed with 500 ml of distilled water (pH 7.0). The mixture was continuously shaken at 200 rpm for 6 h. The

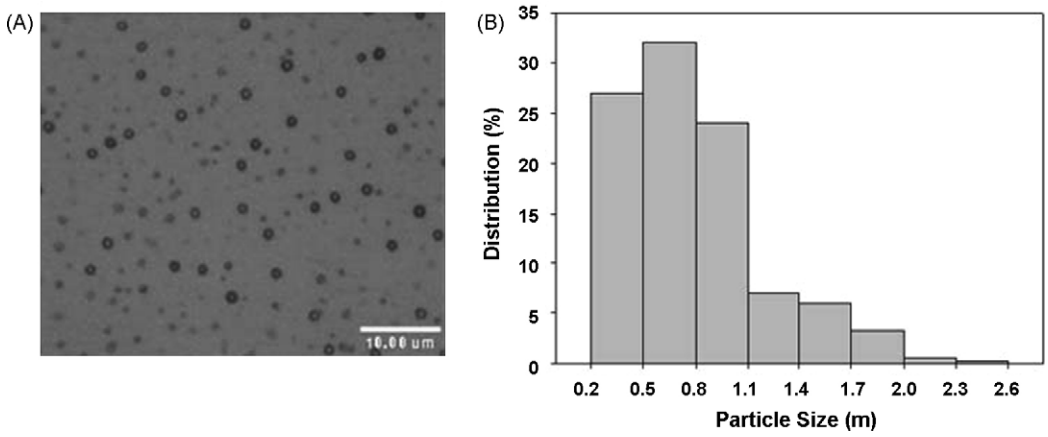

Fig. 1. (A) Size and morphology of MSWI fly ash observed by light microscopy. (B) Particle size distribution of municipal solid waste incinerator fly ash used in this study.

leachate was filtered using 0.1 μm glass–wool fiber filter and analyzed using inductively coupled plasma-atomic emission spectroscopy (Thermo Elemental, Franklin, MA). The total organic carbon was measured using a total organic carbon analyzer (TOC-VCPH, Shimadzu Co., Kyoto, Japan) equipped with a solid sample module (SSM-5000A, Shimadzu Co., Kyoto, Japan). Glucose (40% for total carbon) and Na_2CO_3 (11.2% for inorganic standard) were used as the authentic standards.

3. Results and discussion

3.1. Determination of the physico-chemical properties of incinerator fly ash and its PCDD/Fs concentration

We investigated the particle size distribution of fly ash collected from municipal solid waste incinerators (Fig. 1). The results indicate that fly ash contains mostly small sizes particles which are the major source of PCDD/Fs. Therefore, the smaller particles contribute to the significant TEQ levels of PCDD/Fs in fly ash. The density and particle size of the municipal waste incinerator fly ash used in this study were 2.97 kg/dm^3 and 0.3–1.0 μm (85%), respectively. The total concentration of PCDD/Fs was 27.3 μg/kg-ash, and the toxicity equivalency (TEQ) was 1.02 WHO-TEQ ng/kg-ash. The fly ash contained $(32.5 \pm 8) \times 10^{-3}$% total carbon (TC) and $(1.43 \pm 4) \times 10^{-3}$% total organic carbon (TOC). Previously, Halden et al. [25] reported that high TOC content in soil correlated with a lower dioxin removal rate from soil when the bacterium *Sphingomonas* sp. strain RW1 is used. Recently, we observed that the strain RW1 could remove a significant level of PCDDs from fly ash which contained a low TOC content [14]. In this study, the concentration of PCDD/Fs in the fly ash was considered to be at a sufficient level for microbial biocatalyst test and trace amount analysis, and the TOC content was also low. Therefore, we predicted that the PCDD/Fs concentration and the TOC content of fly ash would not inhibit the activity of the microbial biocatalyst used in the treatment.

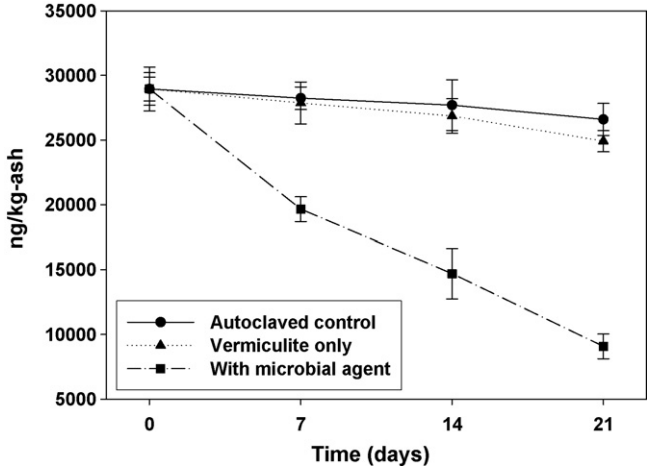

Fig. 2. Time course of the removal of PCDD/Fs from incinerator fly ash during the 21 days incubation process. The total PCDD/F concentrations (w/w) were determined every 7 days by triplicate determinations. Autoclave was performed by heating for 15 min at 121 °C.

3.2. Removal of DD/DF by mixed microbial biocatalyst from fly ash

Prior to examining our microbial biocatalyst's efficacy to remove PCDD/Fs from fly ash, we tested its metabolic activity under the fly ash conditions using PCDD/Fs free fly ash supplemented with non-chlorinated dioxins, such as DD and DF. The manufactured microbial biocatalyst completely degraded DD and DF during a 96 h incubation period. We did not detect any remaining DD or DF residues in the GC chromatograms demonstrating that the remaining analytes were below the detection limit. However, in control samples in which the microbial biocatalyst was autoclaved twice, all of the supplemented DD and DF (1 mM) was remained (data not shown). Because the microbial biocatalyst contains efficient DD and DF degraders, the complete degradation of DD and DF is not surprising. However, these results demonstrate that the microbial strains used in this study are able to maintain their degradation activities even in the solid-state fly ash environment. Previously, several studies have shown that bacterial RW1 and PH-03 can effectively degrade DD and DF and its metabolic intermediates (such as sal-

Table 4
Removal of PCDD/Fs from incinerator fly ash by the newly manufactured microbial agent (consisting of five fungi and four bacteria)[a]

Homolog	Congener	WHO-TEQ conc. (ng/kg-ash)[b]		Decrease (%)
		Control[c]	Sample	
TCDD	2378	70.4	31.4	55.40
PeCDD	12378	96.3	40.9	57.53
HxCDD	123478	12.2	5.7	52.79
HxCDD	123678	104.4	45.9	56.03
HxCDD	123789	69.6	31.1	55.32
HpCDD	1234678	83.6	33.8	59.57
OCDD	12346789	10.8	3.6	66.67
TCDF	2378	18.5	8.7	52.97
PeCDF	12378	9.4	4.0	57.45
PeCDF	23478	353.6	66.7	81.14
HxCDF	123478	38.0	12.9	66.05
HxCDF	123678	39.2	13.6	65.31
HxCDF	123789	73.8	26.2	64.50
HxCDF	234678	19.0	6.8	64.21
HpCDF	1234678	15.1	5.2	65.56
HpCDF	1234789	2.3	0.9	60.87
OCDF	12346789	1.4	0.4	71.43
SUM		1018.3	338.5	66.76

[a] PCDD/Fs were determined after 21 days incubation.
[b] All values were determined in triplicate ($p = 0.001$).
[c] Autoclaved control.

icylate and catechol) in axenic cultures in MSM liquid medium, model soils, and fly ash slurry [14,18,26]. This report implied that the type of culture medium (i.e. liquid, solid, or slurry) had little effect on the biological activity of the inoculated strain.

3.3. Removal rates of PCDD/Fs from fly ash

The ability of the microbial biocatalyst to remove PCDD/Fs from MSWI fly ash was examined using a solid-state fermentation system. The removal rate for total PCDD/F congeners, depicted in Fig. 2, was determined to be 68.7%. The negative controls (i.e. the addition of either inactivated microbial biocatalyst or vermiculite) showed only a slight decrease in PCDD/Fs. This decrease may be due to adsorption to the dead biomass. This result is consistent with previous reports that microbial adsorption plays a major role in the removal of chlorinated recalcitrant pollutants [14,27,28]. PCDD/Fs congeners substituted with 2,3,7,8-chlorination are particularly toxic [1]. We detected seventeen PCDD/Fs congeners with 2,3,7,8-chlorination patterns. For these seventeen toxic congeners, the removal rates were 66.8% (Table 4). We observed that the reduction of TEQ values (average 57.6%) for chlorinated dioxin congeners was smaller than that for chlorinated dibenzofuran congeners (average 64.9%). The TEQ reduction for PCDD/Fs was statistically significant ($p < 0.001$, $n = 3$). The reduction in TEQ of the highly chlorinated congeners suggests that most of highly chlorinated congeners were likely degraded by the microbial biocatalyst's fungal strains which are known to be capable of degrading highly chlorinated PCDD/Fs [14]. This is an important finding, given that the potential use of many microbial dioxin-degraders in the biotransformation or biodegradation of dioxins has been restricted to low-halogenated congeners because of the relatively narrow substrate range of the initial dioxygenase [9,29,30].

In the present study, 55.4% of the most toxic 2,3,7,8-TCDD and 57.5% of the second most toxic 1,2,3,7,8-PeCDD were removed by the microbial biocatalyst. This represents a substantial reduction in the total TEQ values (Table 4) and could be due to the degradation of 2,3,7,8-TCDD and other high chlorinated PCDD/Fs by the fungal strains. These fungal strains are known to secrete lignin peroxidase, manganese peroxidase, and H_2O_2-generating enzymes in response to starvation [31,32]. These enzymes mediate dioxin degradation by a radical reaction which involves the cation-mediated cleavage of the C–O–C bond at the angular position adjacent to the ether bridge of molecules [15,33]. Many researchers have suggested that the peroxidases of these fungi are essential for the oxidative degradation of environmentally persistent compounds, such as polycyclic aromatic hydrocarbons [34,35].

Previous reports have demonstrated the removal of PCDD/Fs from fly ash by pure cultures of either bacteria or fungi [14,16]. However, the degradation extent was limited to only few congeners [16]. Since highly chlorinated PCDD/Fs degradation in bacterial systems is difficult, the chemical or biological pretreatment of fly ash to dechlorinate octa chlorinated DD or DF would aid in the complete removal of PCDD/Fs by bacteria. Microbial-mediated reductive dechlorination has been reported for these chemicals in anaerobic sediments [3,36,37]. Microbial dechlorination is a slow process and this method is not possible to apply to the bulk volume of fly ash that is generated at incinerator sites. Furthermore, reductive dechlorination may increase the toxicity of the samples via the formation of 2,3,7,8-substituted congeners from the *peri*-dechlorination of higher chlorinated congeners [36,38]. Alternatively, white rot fungi can be used for the degradation of PCDD/Fs from fly ash. Suhara et al. [16] reported that a white rot fungus, *Ceriporia* sp. Mz-340, was able to remove 46.5% of the total PCDD/Fs and

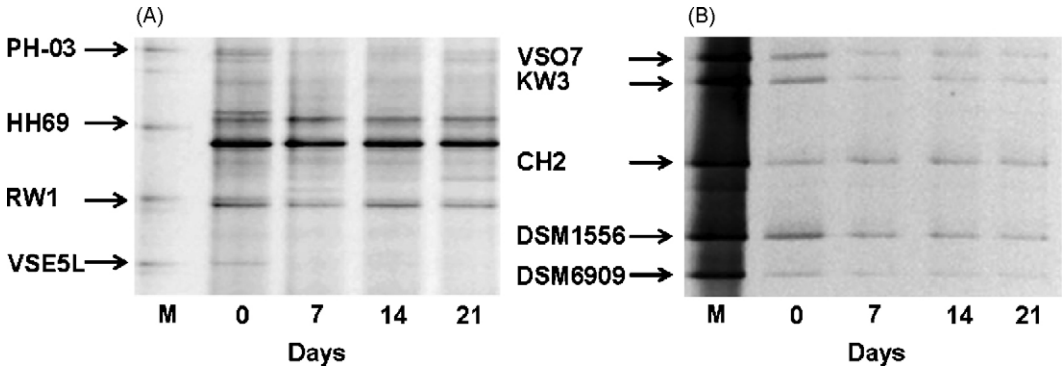

Fig. 3. Denaturing gradient gel electrophoresis (DGGE) analysis of bacteria-specific (A) and fungi-specific (B) genes throughout the fly ash treatment processes. In the left figure (A), PH-03: *Pseudomonas veronii* PH-03, HH69: *Sphingomonas* sp. HH69, RW1: *Sphingomonas wittichii* RW1, VSE5L: *Paenibacillus* sp., and M: Marker. In the right figure (B), VSO7: *Fusarium* sp. VSO7, KW3: *Irpex* sp. KW3, CH2: *Trametes* sp. CH2, DSM1556: *Phanerochaete* sp. DSM1556, DSM6909: *Phanerochaeta* sp. DSM6909, and M: Marker.

46% of total TEQ value from fly ash during a 12 week treatment period.

It has been shown that fungal bacterial mixed culture significantly mineralized the poly aromatic hydrocarbons (PAHs) rather than axenic culture [17]. In the present study, we used mixed microbial biocatalyst composed of 5 different fungal strains and 4 different bacterial strains for fly ash treatment. We demonstrate the removal of the total PCDD/Fs and TEQ and this result suggests that a combined bacterial and fungal microbial biocatalyst is efficient in the removal of PCDD/Fs from fly ash. Additionally, further incubation may yield a more complete removal of PCDD/Fs from the fly ash. It has been previously demonstrated that the use of ashes as a soil amendment resulted in increased growth and yield of agricultural crops, increased pH, and increased potassium and phosphorus availability [39,40]. Our microbial biocatalyst, which is very effective in the removal of toxins from fly ash, could potentially be used to increase the value and use of fly ash in land applications.

3.4. Community analysis

The survival of the microorganisms introduced into the fly ash for the removal of the PCDD/Fs is important for efficient PCDD/F bioremediation [14]. Although, we did not detect the growth of microorganisms we monitored the presence of the members of the microbial biocatalyst using PCR-DGGE analysis at every 7 days during the treatment (Fig. 3). Saw dust, a natural substrate for fungal strains, added to the inoculum could serve as the source for fungal growth and enzyme production which would be required for PCDD/Fs degradation. The products of the fungal-mediated PCDD/Fs degradation might have served as the carbon sources for the bacterial strains. DGGE analysis demonstrated that all of the fungal and bacterial strains in the dioxin-degrading microbial mixture were maintained during the treatment, and therefore, they were stable under these dioxin treatment conditions. However, it should be noted that the decrease in intensity of the DNA bands over time of all of the stains detected by the DGGE analysis is likely due to decreased active cell densities throughout the treatment period. Although all of the fungal and bacterial strains were potential dioxin degraders as determined experimentally in pure culture, the conditions in fly ash are very different from those of liquid culture medium. These differences may account for this observed decrease in cell density. Furthermore, immediate mixing and exposure of cells to highly toxic chlorinated dioxin congeners may alter cell viability. Additionally, fungal degradation products, such as chlorinated catechols, may inhibit the microbial growth by repressing essential enzymes.

4. Conclusion

In the present study, a microbial biocatalyst consisting of various dioxin-degrading bacteria and fungi reduced the total concentration of PCDD/Fs in fly ash from 27.3 to 9.97 μg/kg of fly ash (68.7% of the total PCDD/Fs). Importantly, this study used a short treatment period, and the microbial strains survived in the harsh conditions provided by the fly ash. The results of this study indicate that this microbial biocatalyst could be a potential candidate used in the development of a biological treatment technology for elimination of PCDD/Fs in fly ash and that the combination of several dioxin degraders into a single microbial biocatalyst may be beneficial in the treatment of fly ash contaminated with dioxins.

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