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Research article

Earthworm cast as a promising filter bed material and its methanotrophic contribution to methane removal

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ABSTRACT

The use of biocovers is a promising strategy toward mitigating CH_4 emission from smaller and/or older landfills. In this study, a filter bed material consisting of a mixture of earthworm cast and rice paddy soil in a biocover was evaluated. Although the CH_4 oxidation rate of the enriched paddy soil was 4.9 μg g-dry soil^{-1} h^{-1}, it was enhanced to 25.1 μg g-dry soil^{-1} h^{-1} by adding an earthworm cast with a 3:7 ratio of earthworm cast:soil (wet weight). CO_2 was found as the final oxidation product of CH_4, and the mole ratio of CO_2 production to CH_4 consumption was 0.27. At a moisture content range of 15–40% and a temperature range of 20–40 °C, the CH_4 oxidation rates of the enriched mixture were more than 57% of the maximum rate obtained at 25% moisture content and 25 °C. By denaturing gradient gel electrophoresis analysis employing primers for the universal bacterial 16S rRNA gene, and terminal-restriction fragment length polymorphism analysis using primers for the *pmoA* gene, the bacterial and methanotrophic communities in the enriched mixture were mainly originate from paddy soil and earthworm cast, respectively. Both type I (mainly *Methylocaldum*) and type II methanotrophs (mainly *Methylocystis*) played important roles in CH_4 oxidation in the enriched mixture.

1. Introduction

CH_4 and CO_2 are the most prominent gases involved in global warming; But the global warming potential of CH_4 is more than 20-fold that of CO_2 per molecule [1]. At least as much focus has been placed on CH_4 removal as toward CO_2 in attempts to ameliorate global warming [1]. Landfills are known to be one of the most important sources of anthropogenic CH_4 [1]. The amount of CH_4 produced from the world's landfills is 10–70 Tg (teragrams) per year, around 11% of the total anthropogenic CH_4 produced per year [2,3]. CH_4 oxidation by methanotrophs in the aerobic phase of landfill cover soil can be an important and natural contribution to the prevention of CH_4 emission from landfill cover soil into the air [3,4]. It has been reported that 10–20% of CH_4 emitted from landfill cover soil is oxidized by methanotrophs, whose maximum CH_4 removal efficiency is 60% [5,6]. Landfill cover soil can play an important role in oxidizing CH_4 [7,8]. Especially in small and/or old landfills, stimulation of biooxidation of CH_4 can be a more effective and economical method of CH_4 removal than physical gas treatment methods using gas extraction wells [9].

Previous studies have employed various kinds of materials as filters in biocover beds to control CH_4 emission in landfills [4]. The CH_4 removal efficiencies of biocovers using mature compost, earthworm cast, powdered activated carbon, or pine bark, have been shown to range from 68% to 100% [4,9–12]. Since the filter bed is a key factor determining the biocover's performance, there is a need to develop new filter bed materials with strong potential for effective CH_4 removal at low cost. At the same time, environmental factors such as temperature, pH, and moisture content need to be monitored, as they also affect the CH_4 removal capacity of the biocovers using each material [4,9,13–15]. Moreover, since there is also a lack of information on microbial communities in biocovers, there is a need for a database of microbial communities that include the methanotrophs responsible for CH_4 oxidation in biocover materials.

In this study, CH_4 removal by a mixture of earthworm cast and rice paddy soil as a filter material for a biocover was evaluated, and the effects of mixing ratio, moisture content, and temperature on CH_4 removal were investigated. Analysis of the bacterial community including the methanotrophs in the enriched mixture containing CH_4 was conducted using denaturing gradient gel electrophoresis (DGGE) and terminal-restriction fragment length polymorphism (T-RFLP). The dominant methanotrophs in the enriched mixture were also identified by cloning and sequencing. The results obtained in this study can be used in creating

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strategies, and/or to develop biotechnology, for the mitigation of CH_4 emissions from landfills.

2. Materials and methods

2.1. Soil and earthworm cast

The soil used in this study was from a rice paddy in Gyeonggi province, Korea, at a depth of 30 cm from the surface. The earthworm cast was supplied from a sewage treatment plant in Seoul, Korea. In the sewage treatment plant, earthworms were cultivated using dewatered sewage sludge, and earthworm casts were dried over more than 6 months under natural conditions. The paddy soil and earthworm cast sampled were sieved using a 2-mm sieve before being used. The pH values of the soil and earthworm cast were 6.5 ± 01 and 5.2 ± 0.1, respectively. Their moisture contents were $2.3 \pm 0.1\%$ and $38.1 \pm 1.4\%$, respectively, and their organic matter contents were $1.7 \pm 0.2\%$ and $37.4 \pm 1.2\%$, respectively.

2.2. CH_4 removal evaluation

The earthworm cast was mixed with paddy soil (5:5, w/w, wet weight), and then distilled water was added to the mixture to fit a final moisture content of 25%. 50 g of the mixture was placed in a 600-mL serum bottle, and the serum bottle was sealed with a butyl-rubber stopper. From a gas cylinder containing a gas mixture of 40% CH_4 and 60% CO_2 (Daedeok Gas Co., Inchon, Korea), the gas was sampled using a syringe and injected into the serum bottle to a final concentration of 5% (v/v) CH_4 (50,000 ppm). The serum bottle was incubated at 25 °C without shaking, and the gas in the headspace of the serum bottle was periodically sampled using a gas-tight syringe to measure CH_4 and CO_2 concentrations. When the CH_4 concentration dropped below 200 ppm, the serum bottle was opened in the laboratory for 0.5–1 h to replace the gases inside the bottle with air. The bottle was then re-sealed with a butyl rubber stopper. After CH_4 was re-injected to a final concentration of 50,000 ppm, the bottle was re-incubated at 25 °C, and the CH_4 concentration measured periodically. CH_4 re-injection was repeated in this way 9 more times. The resulting enriched mixture of the earthworm cast and paddy soil (ECPS) was used as the sample for the analysis of the bacterial community including methanotrophs. Also, to verify CO_2 formation as a final product of CH_4 degradation by the ECPS, the concentrations of CH_4 and CO_2 were monitored in the bottles simultaneously during the batch test for CH_4 removal. Since CO_2 could be produced from other organics contained in the ECPS, CO_2 concentration was also monitored in the bottles supplemented with only CO_2. From a CO_2 cylinder (99.9%, Daedeok Gas Co., Inchon, Korea), the gas was sampled using a syringe and injected into the serum bottle to a final concentration of 8% (v/v) CH_4 (80,000 ppm).

In addition, to confirm the physico-chemical removal of CH_4 by the mixture of earthworm cast and paddy soil, the mixture was autoclaved two times at 121 °C for 30 min. The test for CH_4 removal by the autoclaved sample was conducted using the same methods as described above. All tests were carried out in triplicate.

2.3. Effects of mixing ratio, moisture content and temperature on CH_4 removal

To investigate the effect of mixing ratio on CH_4 removal rate in the soil mixtures, six different mixing ratios of earthworm cast to paddy soil were tested: 0:10, 1:9, 2:8, 3:7, 4:6 and 5:5 (w/w, wet weight). After the moisture content of each mixture was adjusted to 25% by adding distilled water, CH_4 removal from the mixtures was measured at 25 °C using the same methods described in Section 2.2.

The effects of moisture content and temperature on CH_4 removal were investigated as follows. Thirteen 1200-mL serum bottles containing 100 g of the mixture of earthworm cast and paddy soil (5:5, w/w, moisture content of 25%) were prepared. After CH_4 was injected to a final concentration of 50,000 ppm, the bottles were incubated at 25 °C without shaking. When the CH_4 concentration in each bottle decreased below 200 ppm, CH_4 was re-injected after replacing the inner gases of the bottle with air. After the re-injection of CH_4 was repeated five times, the mixture of earthworm cast and paddy soil from each bottle was taken out, and all of the samples were manually mixed well. The homogeneously mixed sample (in total ca. 1300 g) was air-dried at room temperature for 3 days until its moisture content dropped below 15%.

50 g of each air-dried sample was placed into a 600-mL serum bottle, and its moisture content adjusted to 15%, 20%, 25%, 30%, 35% and 40% with distilled water. Each serum bottle was sealed with butyl-rubber stoppers. CH_4 removal from the bottles with differing moisture content was evaluated at 25 °C by the same methods as described in Section 2.2. To test the effect of temperature, the moisture content of each air-dried sample was adjusted to 25% with distilled water, and then each 50 g of each sample was placed into 600-mL serum bottles. CH_4 removal in the bottles at differing temperatures (15, 20, 25, 30, 35, and 40 °C) was also evaluated by the same methods as described in Section 2.2. These tests were duplicated.

2.4. Total DNA extraction

To analyze the bacterial community including methanotrophs in the enriched mixture of earthworm cast and paddy soil with CH_4 (ECPS), genomic DNA was extracted using Fast DNA® SPIN for Soil Kit (MP Biomedicals LLC, Solon, USA) as specified by the manufacturer. For control samples, genomic DNA from raw earthworm cast (REC) and raw paddy soil (RPS) were also extracted. All DNA samples were stored at −20 °C before use.

2.5. Analysis of universal bacterial community by DGGE

The genomic DNA extracted from ECPS, REC, and RPS were used as PCR templates to amplify the 16S rRNA gene to analyze the universal bacterial community. The fragments of the 16S rRNA gene were amplified by PCR with the primer set 341f (CCT ACG GGA GGC AGC AG) [16] attached with a 40-bp GC clamp (CGC CCG CCG CGC GCG GGC GGG GCG GGG GCA CGG GGG G) and 518r (ATT ACC GCG GCT GCT GG) [17]. PCR was carried out in a 50-μl (total reaction volume) mixture containing ca. 100 ng of the template DNA, 5 μg of bovine serum albumin, 0.4 μM of each primer, 1× Ex Taq buffer, 0.75 U Takara Ex Taq DNA polymerase (TaKaRa Bio Inc., Shiga, Japan), and 200 μM dNTP. The reaction was performed in a GeneAmp® PCR system Model 2700 (Applied Biosystems Inc., Foster City, USA). Initial denaturation was done at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s and elongation at 72 °C for 30 s, and the final elongation step at 72 °C for 10 min. The size of each PCR product was checked on 1% agarose gel, and then each amplicon was purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, USA) as specified by the manufacturer.

The purified 16S rRNA gene amplicons (ca. 200 bp) were run in 8% polyacrylamide gel with a denaturation gradient of 40–60% of urea and deionized formamide. Approximately 500 ng of the 16S rRNA gene amplicons were run in the gel in 1× TAE buffer (10 mM Tris base, 20 mM sodium acetate, 1 mM EDTA) at 60 °C for 15 h at 50 V using a DCode™ System (Bio-Rad, Hercules, USA). The separated amplicon bands in the gel were visualized on a UV-illuminator after staining with SYBR GOLD solution (Invitrogen Co., Carlsbad, USA).

The similarities between the DGGE profiles of samples and band intensities of each lane were analyzed by GelCompa II software version 3.0 (Applied Maths Inc., Austin, USA) to calculate cluster similarity from the composite data set of relative abundance values on DGGE bands by UPGMA (unweighted pair group method with arithmetic mean) and Pearson coefficient values. Based on the relative signal intensity of each band, principal component analysis (PCA) was performed using SPSS software, version 12.0K (SPSS Inc., Chicago, USA).

2.6. Analysis of methanotrophic community by T-RFLP

To characterize the methanotrophic community in the ECPS, REC, and RPS samples, the fragments of the *pmoA* gene encoding particulate methane monooxygenase (pMMO) (ca. 510 bp) were amplified with the primer set, A189f (GGN GAC TGG GAC TTC TGG) [18] labeled with 6-carboxyfluorescein (6-FAM) and mb661r (CCG GMG CAA CGT CYT TAC C) [19]. The genomic DNAs of the ECPS, REC, and RPS samples were used as templates, and PCR was conducted under the same conditions as the analysis of universal bacterial community as described in Section 2.5 except for an annealing temperature of 60 °C.

The purified *pmoA*-gene amplicon of approximately 200 ng was digested with 20 U of restriction endonuclease MspI and HhaI (BEAMS Biotechnology, Seongnam, Korea) at 37 °C overnight. Analysis of the terminal-restriction fragment (T-RF) size composition of each sample was done by electrophoresis in denaturing polyacrylamide gel (6 M urea and 5% polyacrylamide), using an ABI 377 DNA auto sequencer (GMI Inc., Ramsey, USA). The T-RFs smaller than 47 bp or larger than 511 bp were excluded in T-RFLP analysis using GENESCAN analytical software (Applied Biosystems Inc., Foster City, USA). The selected T-RFs (47–511 bp) were quantified by their peak area, with the minimum peak height threshold at 50 relative fluorescent units (RFU). The relative abundance (%) of the methanotrophic population was assessed as the percentage of the individual peak area of the total peak area. Based on the relative abundances of T-RFs, PCA was performed using the software SPSS version 12.0K (SPSS Inc., Chicago, USA).

2.7. Identification of dominant methanotrophs

For the identification of dominant methanotrophs in the ECPS samples, two different methods – cloning/sequencing and DGGE/sequencing – were employed as follows. The fragments of *pmoA* gene were amplified with the primer set A189f and mb661r [18,19] and purified by the same methods as described in Section 2.5.

The purified *pmoA*-amplicons were cloned into a pGEM-T-Easy vector system (Promega Co., Medison, USA), following the manufacturer's instructions. The clone-vectors were transformed into *E. coli* DH5α competent cells using cell-pulser electroporator (GIBCO BRL Div., Gaithersburg, USA). Individual white colonies containing the insert-amplicon were picked up by color-based physiological (*LacZ*/X-gal) and antibiotic (ampicillin) selection, suspended in 50 μl of distilled-deionized water, and boiled for 30 min followed by spin-down at 13,000 rpm for 10 min. The supernatant of a 1-μl aliquot was applied to the PCR mixture with the primer set A189f and mb661r to reamplify the insert sequence [18,19]. All the reamplified insert-amplicons were purified and sequenced with the forward primer A189f using BigDye v3.1 and ABI 3730XL DNA analyzer (Applied Biosystems Inc., Foster City, USA).

For DGGE and sequencing of dominant *pmoA* genes, a second PCR was conducted with the primer set A189f added with a 40-bp GC clamp (A189f-GC) and mb661r, using the purified *pmoA*-amplicons as templates. After running, the second *pmoA*-PCR products were extracted from the agarose gel using a QIAquick Gel Extraction Kit (Qiagen, Valencia, USA) as specified by the manufacturer. The purified second extracted *pmoA*-PCR products were subjected to DGGE in 6% polyacrylamide gel with a denaturant gradient of 40–80% of urea and deionized formamide at 60 °C for 15 h under 100 V in a 1× TAE buffer. Three dominant bands were excised and eluted by centrifugation after freezing and thawing three times. Each eluted *pmoA*-amplicon was reamplified with the same primer set, A189f-GC and mb661r. The reamplified dominant *pmoA*-specific amplicons were purified using a QIAquick PCR Purification Kit and sequenced with the reverse primer mb661r using BigDye v3.1 and ABI 3730XL DNA analyzer (Applied Biosystems Inc., Foster City, USA).

All *pmoA* gene sequences obtained in this study were compared to nucleic acid and amino acid sequences of *pmoA* genes in the GenBank database by using the Blastn and Blastx search tools of the BLAST program (www.ncbi.nlm.nih.gov/BLAST). The sequences were aligned using the software BioEdit version 7.0.9.0. (BioEdit Sequence Alignment Editor) and the aligned parts of the sequences (>450 bp) were applied to conduct phylogenetic and molecular evolutionary analysis using the software *MEGA* version 4 [20]. The phylogenetic relations were calculated using the Neighbor-Joining method and the Substitution model; the Poisson amino acid correction was done with 1000 bootstrap replications.

All *pmoA* sequences obtained in this study have been deposited in the GenBank database under the following accession numbers: FJ775131 to FJ775133 for the ECPS *pmoA*-DGGE band sequences and FJ775134 to FJ77513164 for the ECPS *pmoA*-clone sequences.

2.8. Analytical procedures

CH_4 concentrations were measured using a gas chromatograph (GC, Agilent Technologies Inc., Santa Clara, USA) equipped with a flame ionization detector and a HP-1 column (30 m × 0.32 mm × 0.25 μm). The temperatures for injector, detector and oven were 250, 250, and 100 °C, respectively. For the simultaneous analysis of CO_2 and CH_4 concentrations, a GC (7890A, Agilent Technologies Inc., Santa Clara, USA) equipped with a thermal conductivity detector and a Molesive 5A column (30 m × 0.53 mm × 25 μm) was used. The temperatures for the injector/detector and oven were 200 and 100 °C, respectively. The oven temperature was gradually elevated from 50 °C (3 min hold) to 250 °C (2 min hold) by increasing by 30 °C/min. Calibration curves for CO_2 and CH_4 were established using standard gases of 99.9% CO_2 (Daedeok Gas Co., Inchon, Korea) and 99.9% CH_4 (Daedeok Gas Co., Inchon, Korea), respectively.

3. Results and discussion

3.1. CH_4 removal by the mixture of earthworm cast and paddy soil

CH_4 removal by the mixture of earthworm cast and paddy soil (ECPS) is shown in Fig. 1a. A lag period of 2 days was observed before CH_4 degradation with the 1st injection of CH_4, but in subsequent injections, CH_4 was removed by the mixture without the lag period. When the mixture was autoclaved, no CH_4 removal was found (data not shown). These results indicated that physico-chemical removal of CH_4 by the mixture was not significant, and CH_4 removal by the mixture was caused by microbial activity.

Using the enriched mixture of earthworm cast and paddy soil obtained from the tests shown in Fig. 1a, CO_2 formation as a final product of CH_4 degradation was verified (Fig. 1b). CH_4 concentration decreased while CO_2 concentration increased in the mixture, indicating that CH_4 was degraded to CO_2. The mole ratio of CO_2 pro-

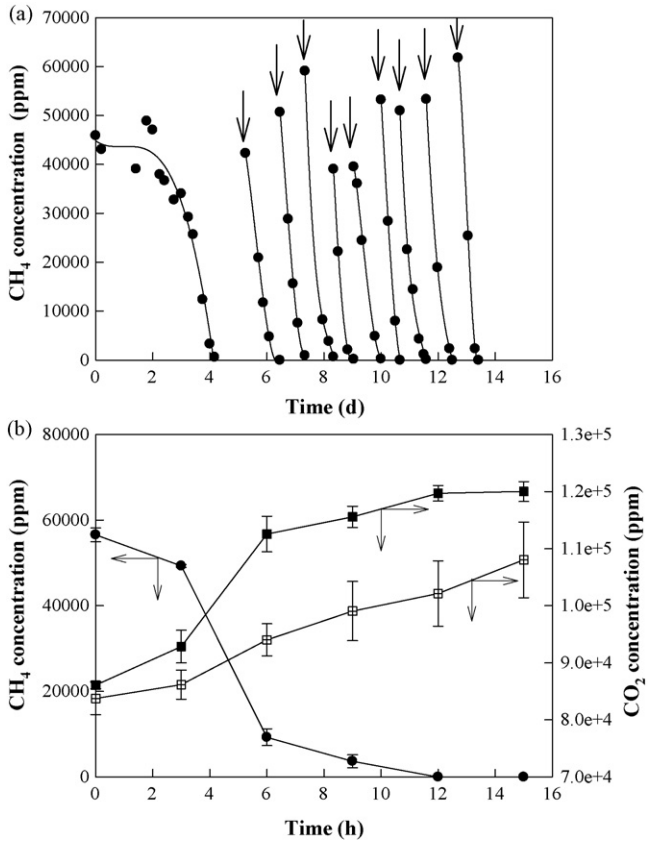

Fig. 1. CH_4 oxidation by mixture of earthworm cast and paddy soil with 1:1 mixing ratio (w/w, wet weight) at 25% moisture content and 25 °C. (a) Change of CH_4 concentrations with incubation times. Arrows indicate the re-injection times of CH_4. (b) Change of CO_2 concentrations during CH_4 oxidation. ●, CH_4 concentration; ■, CO_2 concentration with the addition of CH_4; □, CO_2 concentration without the addition of CH_4.

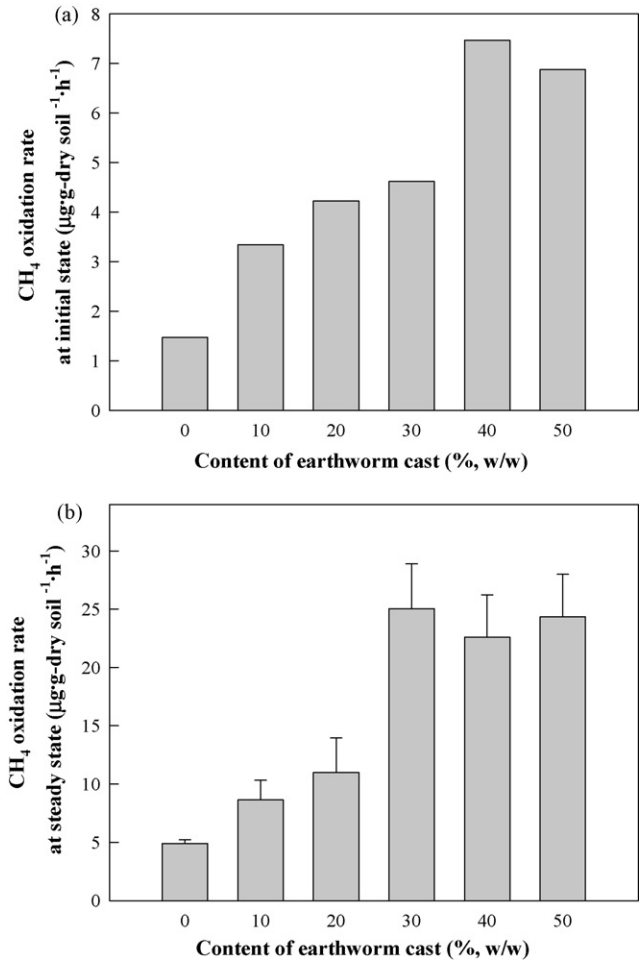

Fig. 2. Comparison of CH_4 oxidation rates by different mixing ratios of earthworm cast and paddy soil. (a) CH_4 oxidation rates at the initial state. (b) CH_4 oxidation rates at a steady state after enrichment culture.

duction to CH_4 consumption was calculated from the data shown in Fig. 1b, to be 0.27.

Methanotrophs can convert CH_4 to CO_2 through the oxidation pathway for energy production or the assimilation pathway for cell component production: $CH_4 + 2O_2 \rightarrow$ Biomass $+ CO_2 + 2H_2O$ [21]. In the assimilation pathway, since HCHO (formaldehyde), an oxidized form of CH_4, is used for the synthesis of cell components, less than one molecule of CO_2 is generally produced from one molecule of CH_4. Börjesson et al. [22] has reported that 0.17–0.36 mol of CO_2 per 1 mol CH_4 can be produced in landfill cover soil (moisture content of 29.7%). Megraw and Knowles [23] reported that the mole ratio of CO_2 production to CH_4 consumption was 0.27 in soil humus. The conversion value from CH_4 to CO_2 obtained in this study, being 0.27, is similar to the previous results. This result also consistently supports the hypothesis that biological CH_4 oxidation produces less CO_2 than the amount of oxidized CH_4 itself, suggesting that biological CH_4 oxidation (removal) can contribute to reducing the total production of greenhouse gases.

3.2. *Effects of mixing ratios of earthworm cast, moisture content and temperature on CH_4 removal*

The effect of various mixing ratios of earthworm cast to paddy soil on CH_4 removal was investigated, and the CH_4 oxidation (removal) rates in the mixtures with different mixing ratios were calculated at the initial state (at 1st injection of CH_4) and at the steady state (at 4th–10th injection of CH_4). The paddy soil without the addition of earthworm cast showed a CH_4 oxidation rate of 1.5 μg g-dry soil^{-1} h^{-1} at the initial state (Fig. 2a) and 4.9 μg g-dry soil^{-1} h^{-1} at the steady state (Fig. 2b). When earthworm cast was added to the paddy soil with different mixing ratios ranging from 10% to 50% (w/w), the CH_4 oxidation rates of the mixtures were 3.3–7.5 μg g-dry soil^{-1} h^{-1} at initial states and 8.7–25.1 μg g-dry soil^{-1} h^{-1} (= 6.1–17.9 g m^{-3} h^{-1}) at steady states (Fig. 2). The initial CH_4 oxidation rates of the mixtures increased with increasing ratios of earthworm cast, up to 40% (Fig. 2a). Their CH_4 oxidation rates increased with increasing ratios of earthworm cast under 30% at steady states. They were not significantly different at the mixing ratios of 30–50% earthworm cast (Fig. 2b). This result suggests that earthworm cast plays an important role as an excellent source of microorganisms responsible for CH_4 oxidation [11].

CH_4 oxidation rates in agricultural soil have been reported as 14 g m^{-3} h^{-1} [3] and 6.5 g m^{-3} h^{-1} [24]. In landfill cover soil, CH_4 oxidation rates have been reported as 5.9–20.0 g m^{-3} h^{-1} [3,5,24]. CH_4 oxidation rates in compost biocovers were 9.4–28 g m^{-3} h^{-1} [25,26]. Park et al. [11] demonstrated that CH_4 oxidation rate in a mixture of earthworm cast or powdered activated carbon with soil was 9.7 g m^{-3} h^{-1}. Compared with previous results, our maximum CH_4 oxidation rate of 17.9 g m^{-3} h^{-1} is relatively high.

A variety of useful microorganisms are found in earthworm cast [27,28]. In addition, earthworm cast is an inexpensive material, and it has good potential to lower C/N ratio and to improve porosity in soils [28]. Higher porosity is especially important to CH_4 removal in soils because CH_4 is mainly oxidized by methanotrophs under aer-

obic conditions. Considering these properties of earthworm cast as well as its effectiveness for CH_4 oxidation, the mixing of earthworm cast into landfill cover soil is one available strategy for mitigation of CH_4 emission from landfills.

The moisture content and temperature of filter beds in biocovers significantly influence the efficiency of CH_4 removal [4,13–15]. The effects of moisture content and temperature on the CH_4 removal rate by the mixture of earthworm cast paddy soil are shown in Fig. 3. The CH_4 removal rate for each condition of moisture content or temperature is expressed as a value relative to the maximum obtaining at 25% moisture content and 25 °C.

The CH_4 oxidation rates at moisture contents at 15%, 20%, 35% and 40% were 57–82% of the maximum rate at the moisture content of 25% (Fig. 3a). This result means that the mixture of earthworm cast and paddy soil can display its CH_4 oxidation ability in a relatively broad range of moisture content (15–40%). The optimum moisture content of filter bed depends on the type of filter bed materials such as soil, compost, and others, as well as gas flow rate [4]. The optimum moisture content of filter beds employing composts or biological residues has been reported to be 25–50%, whereas it lies between 10% and 30% for soil materials [4]. If the filter bed moisture content is too high, the flow and transfer of CH_4 and CO_2 is prevented by excess water, resulting in deteriorating efficiency of CH_4 removal [4].

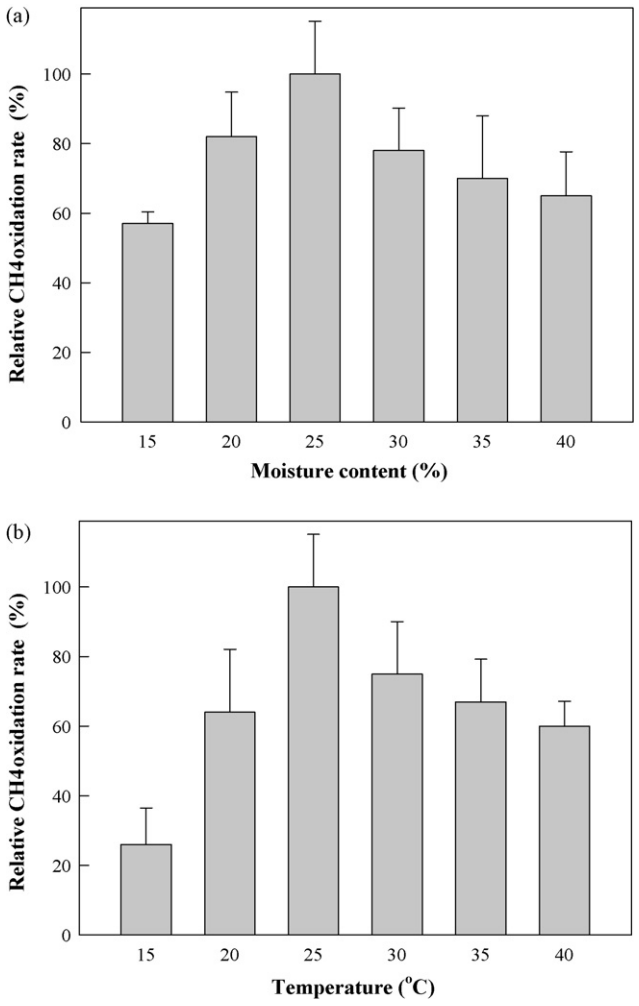

Fig. 3. The effects of moisture content (a) and temperature (b) on CH_4 oxidation rate by the mixture of earthworm cast and paddy soil with 1:1 mixing ratio (w/w, wet weight).

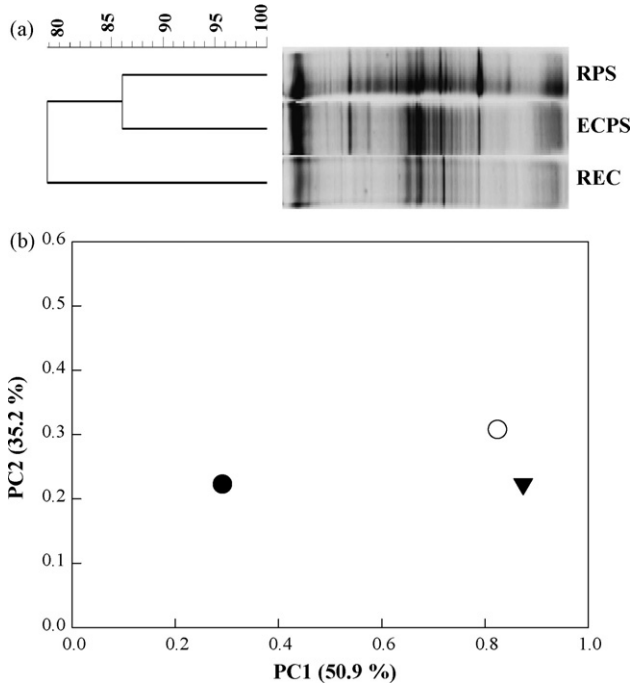

Fig. 4. Comparison between universal bacterial communities in ECPS, REC, and RPS samples using a 16S-rDNA PCR and DEEG method. (a) DGGE fingerprints and their similarity. (b) PCA pattern based on the DGGE fingerprints.

The CH_4 oxidation rates at 20, 30, 35, and 40 °C were higher than 60% of the theoretical maximum rate obtained at 25 °C (Fig. 3b). There was evidence of CH_4 oxidation activity at temperatures even as low as 15 °C, although its oxidation rate was only 26% of the maximum value. This result suggests that CH_4 oxidation in the mixture was carried out by mesophilic methanotrophs [21], whose methanotrophic activity was effective at a relatively broad range of temperatures, from 15 to 40 °C.

Because the landfill cover surface is in direct contact with air, there is significant seasonal temperature change [29]. The average temperature of the surface layer of the landfill cover in winter drops down to around 10 °C [30,31]. Considering *in situ* temperature change in the landfill, the mixture of earthworm cast and paddy soil can be applicable as an effective biocover material, even though there is some deterioration in performance of CH_4 removal in winter.

The optimum temperature for the mixture of earthworm cast and paddy soil was 25 °C, whereas it has been usually found to be 29–30 °C for composts and 20–36 °C for soils [4]. When the temperature declined from 30 to 20 °C or from 29 to 24 °C, the efficiency of CH_4 removal fell to around 50% [4]. Compared with previous results, the CH_4 oxidation activity of the mixture employed in this study seems to be less inhibited by temperature change.

3.3. Diversity of universal bacterial community

To characterize the bacterial community in the mixture of earthworm cast and paddy soil after enrichment of the culture with CH_4, the bacterial communities in the ECPS (the enriched mixture of earthworm cast and paddy soil), REC (raw earthworm cast without amended with CH_4), and RPS (raw paddy soil without amended with CH_4) samples were analyzed using 16S-rRNA PCR and a DEEG method (Fig. 4). Based on the DGGE bands' positions and their relative abundances (Fig. 4a), the similarity between the ECPS and RPS samples (86.1%) was slightly higher than between the ECPS and REC samples (84.2%). The similarity between REC and RPS was the lowest (73.4%). The results of the PCA pattern corresponded to the

similarity data; the ECPS bacterial community was closely grouped with the RPS bacterial community, and the REC bacterial community was located far away from the RPS community (Fig. 4b). These results show that the universal bacterial community in the ECPS sample may have originated mainly from the RPS sample rather than the REC sample.

3.4. Diversity of methanotrophic bacterial community

Since pMMO, encoded by the *pmoA* gene, is considered to be very metabolically active as compared to the soluble methane monooxigenase (sMMO) for *in situ* CH_4 oxidation, encoded by the *mmoX* gene [32], diversity of the methanotrophs in the ECPS, REC, and RPS samples was characterized through *pmoA*-based T-RFLP analysis (Fig. 5). MspI-digested T-RFs of 43, 77, 244 and 511 bp in lengths (±2) were detected in all samples (Fig. 5a). Based on *in silico* analysis, the T-RFs of 43, 77, 244 and 511 bp can be representative of the nitrifiers *Methylococcus*/*Methylocaldum*, *Methylocystis*/*Methylosinus* and *Methylobacter* groups, respectively [33,34]. Generally, the genera of methanotrophs are grouped into three types, mainly by assimilatory pathway [4,21]: Type I methanotrophs, including *Methylomonas*, *Methylomicrobium*, *Methylobacter*, *Methylocaldum*, and so on, use the ribulose monophosphate pathway for their formaldehyde assimilation; Type II methanotrophs, including *Methylocystis*, *Methylocella*, *Methylocapsa* and *Methylosinus*, assimilate formaldehyde by the serine pathway; and Type X methanotrophs have both the properties of types I and II: the genus *Methylococcus* belongs to this type.

Nitrifiers (43-bp T-RF) were detected in all samples, and their abundances were 10%, 12%, and 9% for the ECPS, REC, and RPS, respectively. Nitrifiers can be detected due to the similarity of their *amoA* gene sequence to *pmoA* gene sequences [18]. The *Methylococcus*/*Methylocaldum* (77-bp T-RF) group existed in the ECPS and REC samples with 10% and 9% abundances, respectively, whereas

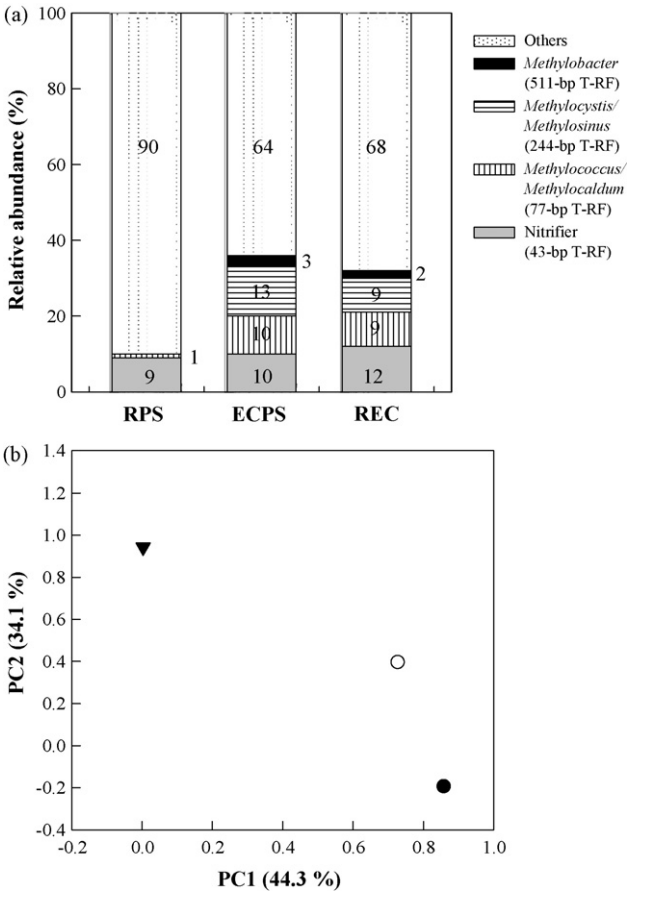

Fig. 5. Comparison of methanotrophic communities in ECPS, REC and RPS samples using a *pmoA*-T-RFLP method. (a) Relative abundance of MspI-digested T-RFs. (b) PCA pattern based on T-RFPL.

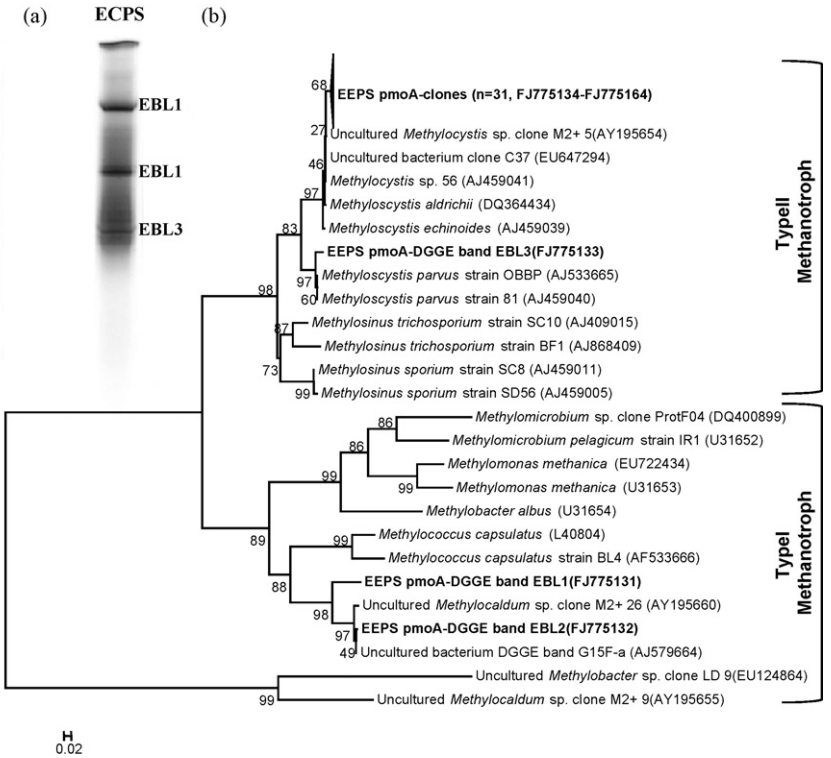

Fig. 6. *pmoA*-DGGE fingerprint (a) and phylogenetic relationship of *pmoA*-clones and *pmoA*-DGGE bands isolated from the ECPS samples (b). Scale bar indicates 2% difference between sequences.

its abundance in the RPS sample was only 1%. The abundances for the *Methylocystis*/*Methylosinus* (244-bp T-RF) group in the ECPS and REC samples was somewhat higher, at 13% and 9%, respectively, even though there is no *Methylocystis*/*Methylosinus* group in the RPS sample. The *Methylobacter* (511-bp T-RF) group was only found in the ECPS and REC samples, with 3% and 2% abundances, respectively. Compared with the REC samples, the abundances for all groups of methanotrophs in the ECPS sample analyzed in this study were slightly higher despite being mixed with paddy soil, which may cause flourishing methanotrophs during enrichment culture with CH_4. The PCA results, based on the abundance of each T-RF, showed that the methanotrophic community in the ECPS was closely related to that in the REC rather than in the RPS (Fig. 5b). This finding supports the hypothesis that the methanotrophic community in the ECPS may mainly originate from the REC. This result is strong evidence for earthworm cast as an excellent source of methanotrophs.

3.5. Identification of dominant methanotrophs

For the identification of dominant methanotrophs in the ECPS sample, 31 clones were randomly selected from the *pmoA*-based clone library, sequenced, and identified (Fig. 6). Interestingly, the sequence identities of 31 clones were most closely related to the genus *Methylocystis* (type II methanotroph). Three *pmoA*-DGGE bands (EBL1–EBL3) were dominantly observed in the DGGE fingerprints of the ECPS-*pmoA* amplicons (Fig. 6a). The ECPS *pmoA*-DGGE bands EBL1 and EBL2 had the highest similarity with the genus *Methylocaldum* (type I methanotroph), and the ECPS *pmoA*-DGGE band EBL3 was most closely related to the genus *Methylocystis* (type II methanotroph) (Fig. 6b). These results correspond well to the T-RFLP results showing that the abundances of *Methylococcus*/*Methylocaldum* (77-bp T-RF) and *Methylocystis*/*Methylosinus* (244-bp T-RF) were relatively higher in the ECPS samples. The results in Figs. 5 and 6 suggest that both type I and type II methanotrophs are responsible for CH_4 oxidation, and especially that *Methylocystis* is the most dominant methanotroph in the ECPS sample. Similarly to our results, *Methylobacter*, *Methylocystis*, *Methylomicrobium*, *Methylosinus*, and *Methylocystis* have been reported as the significant methanotrophs for CH_4 oxidation in landfill soils [35,36]. Moreover, the dominance of *Methylocystis* has been demonstrated in landfill soil and methanotroph biofilters [37–39].

4. Conclusions

Since the filter bed in a biocover is a key factor to determine CH_4 removal efficiency, the characterization of the microbial dynamics and CH_4 oxidation performance of the filter bed material are very important. In this study, CH_4 removal by the mixture of earthworm cast and paddy soil as a filter bed material was evaluated, and the bacterial community including methanotrophs in their enriched mixture with CH_4 was analyzed. The CH_4 oxidation rate of the paddy soil enriched with CH_4 was 4.9 $\mu g\,g\text{-dry soil}^{-1}\,h^{-1}$. When earthworm cast was added to the paddy soil to a 3:7 (wet weight) ratio and enriched with CH_4, the CH_4 oxidation rate was enhanced to 25.1 $\mu g\,g\text{-dry soil}^{-1}\,h^{-1}$. The mole ratio of CO_2 production to CH_4 consumption was 0.27, which is similar to the values reported previously by other researchers. The optimum moisture content and temperature for CH_4 oxidation in the mixture of earthworm cast and soil were 25% and 25 °C, respectively. Under relatively broad ranges of moisture content (15–40%) and temperature (20–40 °C), the enriched mixture of earthworm cast and soil could display CH_4 oxidation rates of more than 57% of the theoretical maximum rate. Both type I (mainly *Methylocaldum*) and type II methanotrophs (mainly *Methylocystis*) were responsible for CH_4 oxidation in the mixture, and the T-RFLP suggested that these methanotrophs originated from the earthworm cast. Based on the identification of dominant methanotrophs by T-RFLP analysis, *Methylocystis* was found to be the most dominant methanotroph. These results suggest that earthworm cast is an effective filter bed material and a promising inoculum source for methanotrophs to improve CH_4 removal performance in landfill biocovers.

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