

Brief Report

Biological soil disinfestation compatible with renewable energy production for sustainable agriculture

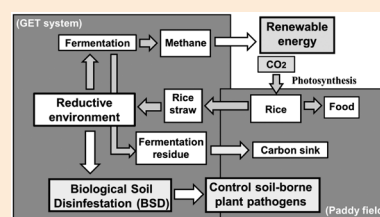
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S Supplementary material

Biological soil disinfestation (BSD) is biotechnology to control soil-borne plant pathogens based on the anaerobic-reducing environment in soil and the functions of indigenous microbes. A new sustainable agricultural technology, the GET system, which produces and recovers methane as renewable energy from paddy fields, has a structure and principles similar to those of BSD technology. To confirm the potential of the GET system as BSD technology, the microbial community structures in the GET system were analyzed using next-generation sequencing. Thirty-four phyla were detected: 31 bacterial and 3 archaeal. *Firmicutes* dominated during the experimental period, which plays an important role in BSD functions such as organic decomposition, nitrate removal, and soil-borne pathogen elimination. The ability of the GET system to control soil-borne pathogens as well as produce renewable energy was demonstrated.



Keywords: biological soil disinfestation (BSD), GET system, sustainable agriculture, soil-borne pathogens, greenhouse gases, renewable energy.

Introduction

According to the FAO (Food and Agriculture Organization of the United Nations), by 2050, the global population will reach 9.2 billion, creating a severe imbalance between food supply and demand, and about 1 billion people worldwide are food insecure.¹⁾ Since diseases caused by soil-borne plant pathogens account for 15% of the global food crop losses, resolving crop loss due to soil-borne plant pathogens has become a global challenge.²⁾

Until now, chemical fungicides have been the primary means of soil pest control, and they contribute significantly to the stability and efficiency of global food production.³⁾ However, since the early 1970s, the ability of pesticides to control soil pests has declined worldwide.⁴⁾ Plant pathogenic fungi began to develop resistance to these fungicides. For example, fungicides such as QoI have excellent efficacy against filamentous fungi. However,

QoI has been less effective in controlling diseases such as seedling blight.^{5–10)} The development of resistance to chemical pesticides has always been a concern.

With the advent of soil fumigation technology, the use of soil fumigants has become the most cost-effective way to control soil-borne diseases. In chemical fumigation, chloropicrin, metam-sodium, dimethyl disulfide, and 1,3-dichloropropene have commonly been used to replace methyl bromide.¹¹⁾ Although methyl bromide was considered most effective in controlling soil-plant pathogens, its use in agriculture was prohibited in 2015 due to its destructive effects on the ozone layer (with exemptions for essential uses).¹²⁾ On the other hand, the strong and growing global demand for “green organic food” reflects general consumer resistance to the use of chemical pesticides in agricultural production, although non-toxic, low-residue pesticides and, particularly, environmental fumigants have been developed and used widely.¹³⁾ Two main types of environmentally friendly fumigation measures have been created. The first is physical fumigation, which uses heat sources such as solar energy, steam, hot water, electrical treatment, microwaves, and far-infrared radiation for soil disinfection.¹⁴⁾ The second is biological fumigation, which uses volatile substances released by the digestion of organic materials to control soil-borne pathogens.¹⁵⁾ However, such techniques are expensive, and the control effects are unstable—no matter which fumigation method adversely af-

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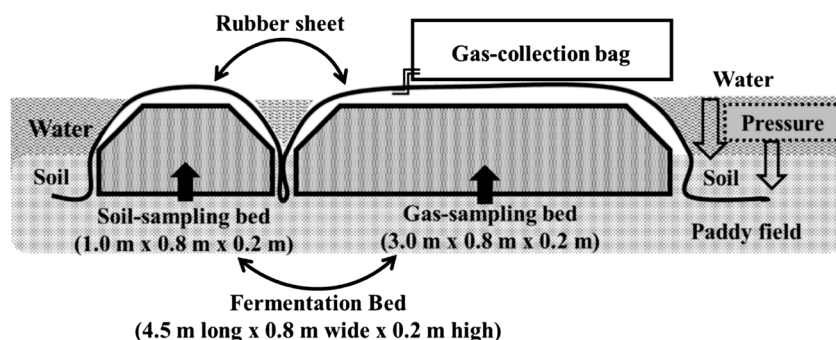


Fig. 1. Overview of the GET system's schematic.²¹⁾

fects non-target microorganisms.

At the beginning of the 21st century, Japanese and Dutch scientists independently found that soils with flood and drought rotations had stronger disease resistance, and they developed reductive soil disinfestation (RSD), also known as anaerobic soil disinfestation (ASD) or biological soil disinfestation (BSD).¹⁶⁾

The basic principles of BSD technology are: to create a strong soil reduction environment (-100 to -200 mV) in a short time, to use denitrification reaction to eliminate the accumulation of nitrate in the soil, and to generate hydrogen sulfide and other reducing sulfides to remove sulfate,¹⁶⁾ all to achieve the purpose of desalination. OH^- produced in the process of reduction was used to neutralize soil acidity. The poor survival of many plant pathogens under anoxic soil conditions¹⁷⁾ suggests that the development of highly reducing conditions in the soil may cause damage or death in various soil-borne aerobic pathogens.

Anaerobic bacteria that grow in BSD-treated soil may release antagonistic substances or show activity against soil pathogens.^{18–20)} Volatile fatty acids (VFAs), such as acetate or butyrate, released in the soil have been suggested to contribute to the inactivation of pathogens.¹⁶⁾ Additionally, enzymes with antifungal or anti-pathogenic activities may be produced by anaerobic bacteria grown in BSD-treated soil. Research has shown that BSD using organic materials as a carbon source has a good effect on controlling soil-borne pathogens. However, the potential for methane emission is a growing concern from a climate-change perspective.

To make agriculture sustainable by reducing methane emissions and simultaneously producing renewable energy from paddy fields, we have established a novel technology called the GET system.^{21–24)} The GET system enables the efficient production of biomethane gas (G) as renewable energy (E) from a *tanbo* (T), which means “paddy field” in Japanese. With the optimum amount of rice straw application (14 kg/m^2) as a renewable energy resource, the GET system could generate up to 100 L/day m^{-2} of biomethane at a methane concentration of more than 60% in a mesophilic environment (20 – 30°C). Moreover, 25.7% of the carbon in the straw remains in the soil, acting as a carbon sink for atmospheric CO_2 and improving soil fertility during the next rice-growing season.²¹⁾

Since the GET system creates a strongly reducing soil environment similar to the principles and construction procedures

of BSD, the GET system is also expected to have a BSD effect. To confirm the ability of the GET system for BSD, the dynamics of changes in the microbial community at each stage of the GET system have been analyzed by next-generation sequencing (NGS), and the results suggest that the GET system has the potential to control soil-borne plant pathogens.

Materials and methods

1. Test field

The experiments were conducted in unfertilized paddy fields ($\text{NH}_4^+\text{-N}$: $2.66 \text{ mg/kg-dry soil}$; $\text{NO}_3^-\text{-N}$: $0.00 \text{ mg/kg-dry soil}$, light clay; clay: 27.2%, silt: 25.0%, sand: 47.8%)²⁵⁾ at Meijo University, Kasugai-shi, Aichi Prefecture, Japan ($35^\circ 16' 10''\text{N}$, $136^\circ 58' 0''\text{E}$) in June to July 2017, during which time the soil temperature was 20 – 30°C .

2. Experimental setup

Since methane is emitted from rice paddies, our studies were performed as follows^{21–24)}: briefly, (1) air-dried rice straw (carbon content: 40.7%; nitrogen content: 1.5%) cut to approximately 15 cm by a conventional cutting machine was placed on the paddy field (14 kg/m^2), mixed with the soil by a tiller, (2) and then shaped into ridges as a fermentation bed ($4.5 \text{ m long} \times 0.8 \text{ m wide} \times 0.2 \text{ m high}$), consisting of a gas-sampling bed ($3.0 \text{ m} \times 0.8 \text{ m} \times 0.2 \text{ m}$) and a soil-sampling bed ($1.0 \text{ m} \times 0.8 \text{ m} \times 0.2 \text{ m}$) (Fig. 1). Then, pH/Eh meters (Orion 3-Star plus, Thermo Fisher Scientific, Waltham, MA, USA) were placed in the center of the gas-sampling bed. (3) The shaped ridges were then covered with an impervious sheet ($2.0 \text{ m wide} \times 10 \text{ m long} \times 1 \text{ mm thick}$) (Taiyo Kogyo, Osaka, Japan), and the peripheral edges of the sheet were wholly buried in the soil to create an anaerobic environment and prevent methane leakage. (4) An outlet for the gas collection was attached to the center of the sheet, from which a hose was connected to a biogas collection bag. (5) After the fermentation beds were completed, the paddy field was flooded as in typical rice cultivation. All experiments were conducted in triplicate.

3. Sampling and DNA extraction

To analyze the microbial community structure, soil samples were collected from the soil-sampling beds at six points based

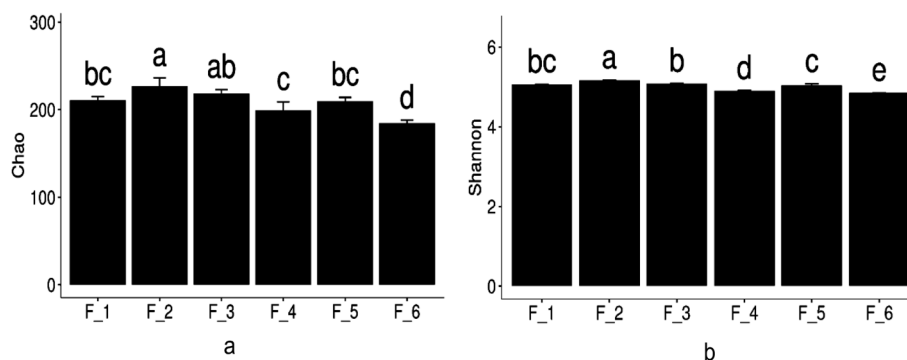


Fig. 2. a. Microbial community richness (Chao) index values with different letters (a, b, c, and d) represent significant differences between samples ($p < 0.05$); b. Microbial community diversity (Shannon) index values with different letters (a, b, c, d, and e) represent significant differences between samples ($p < 0.05$) (The analysis of differences between groups was carried out using the multiple comparison method of LSD).

on the volume and concentration of bio-methane production²¹⁾ (Fig. S1). The soil's redox potential (Eh) and pH at the time of sample collection are shown in Table 1.

The total genomic DNA of all samples was extracted using the FastDNA™ SPIN Kit for Soil (catalog no. 6560200; FastDNA™). The concentration and purity of the extracted DNA were measured by a UV/visible spectrophotometer (DU®/800; BECKMAN).

4. Sequencing and bioinformatics analyses

The Illumina MiSeq system was used for library construction and the sequencing of each extracted genomic DNA. The primer sets 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') were used to amplify the V3–V4 hypervariable region to characterize the microbial community structure and diversity in the GET system. The ASV table is obtained with a data noise reduction processing by DADA2. The species annotation was performed by comparing the ASV representative sequence with the database (Greengenes; confidence threshold set to 0.6) with MOTHUR classify (version 1.39.5). Diversity statistics and charting were produced by R (version 3.6.0).

Results

1. Soil physicochemical state dynamics in the GET system

The redox potential (Eh) was decreased to -157 mV after two

Table 1. Sampling information

| Sample name | After treatment (days) | Eh (mV) | pH |
|-------------|------------------------|-----------------------|----------------------|
| F_1 | 2 | -157.16 ± 73.28^a | 6.37 ± 0.17^b |
| F_2 | 9 | -192.60 ± 38.38^a | 6.43 ± 0.08^{ab} |
| F_3 | 19 | -196.76 ± 32.92^a | 6.51 ± 0.05^{ab} |
| F_4 | 27 | -217.80 ± 23.05^a | 6.68 ± 0.10^a |
| F_5 | 40 | -226.53 ± 27.18^a | 6.51 ± 0.11^{ab} |
| F_6 | 54 | -227.85 ± 34.69^a | 6.48 ± 0.06^{ab} |

The analysis of differences between groups was carried out using the multiple comparison method of LSD. Values with different letters (a and b) in a row indicate that they are significantly different from each other ($p < 0.05$).

days of treatment, and then it remained at about -200 mV throughout the experiment in the GET system (Table 1). This was consistent with the soil Eh in BSD, which was usually maintained at -200 mV.²⁶⁾

pH was also an important indicator of BSD. The value of pH in the GET system fluctuated over time from 6.4 to 6.7 (Table 1). This was because in a reducing environment, with interactions between microbial populations, the production of volatile fatty acids through the degradation of organic matter could lower the pH of the soil, while the reduction of NO_3^- and the production of NH_4^+ could increase the pH.¹⁵⁾

2. Dynamics of microbial community compositions when using the GET system

2.1. α -diversity

After quality filtering, a total of 57,186 good-quality sequences with an average length of 414 bp from all samples were obtained, and the number of reads per sample ranged from 8,653 to 10,616. At the 97% identity level, all good-quality sequences were distributed into 1,589 OTUs.

The diversity index is an important indicator describing the change in microbial communities in the samples. The Chao index proves whether the sequencing depth has covered all species in a sample,²⁷⁾ and the Shannon index reflects community diversity.²⁸⁾

The Shannon index is influenced by the species richness and uniformity of the sample community, thus reflecting the diversity of the community. For example, for the same abundance, greater uniformity results in greater species diversity and a higher Shannon index. As shown in Fig. 2, the microbial richness (Fig. 2a) and diversity (Fig. 2b) indices of microbial communities in the GET system increased significantly from F_1 to F_2, and then notably decreased from F_2 to F_6 ($p < 0.05$).

The fact that the Chao index did not increase as days passed after the start of the experiments meant that no new species had appeared, indicating that the depth coverage of the sequence had reached saturation.

In the Shannon index, microbial enrichment began after the start of the experiment, and microbial diversity reached its

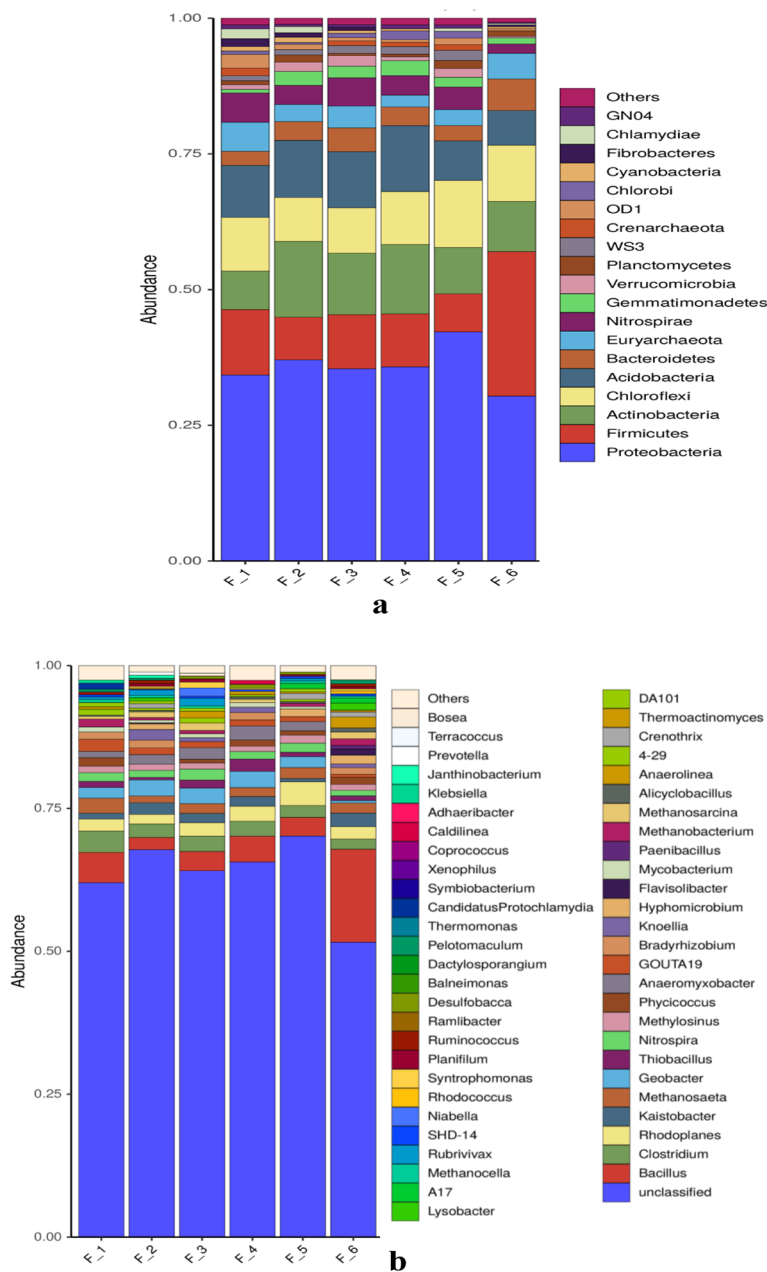


Fig. 3. a. Phylum-level identification of all sequences on each sampling day; b. Genus-level identification of all sequences. Phyla and genera with less than 0.5% relative abundances were included in “others.”

maximum at F_2. Meanwhile, as soil pH increased and Eh decreased, microbial diversity began to decline and reached a minimum at F_6. This is likely due to the enrichment of anaerobic microorganisms, their increased diversity, and the gradual disappearance of some aerobic microbial communities as the anaerobic environment was enhanced.

2.2. The taxonomic classifications of sequences at the phylum level

Overall, a total of 34 phyla, including 31 bacterial and 3 archaeal, were detected; *Proteobacteria* (35.84%), *Firmicutes* (12.22%), *Actinobacteria* (10.49%), *Chloroflexi* (9.78%), and

Acidobacteria (9.38%) were the dominant phyla (representing >9% of total optimized-quality sequences). Other less abundant phyla, including *Bacteroidetes* (3.75%), *Euryarchaeota* (3.72%), and *Nitrospirae* (3.94%), were also found in most GET system samples. Fourteen of 31 phyla were present in all GET system samples, with the shared phyla accounting for 95.71 to 99% of the abundance in each sample (Fig. 3a). Moreover, 14 phyla, represented by less than 0.05% of the total good-quality sequences, were defined here as rare phyla, such as *Elusimicrobia* and *Armatimonadetes*. *Proteobacteria* dominated throughout the experimental process, but the analysis revealed that *Proteobacteria*

were mainly composed of unclassified genera, which meant that the sequences could not be assigned to the genus level.

Firmicutes dominated F_1 and F_6, especially in F_6, and the abundance increased significantly from 7% (F_5) to 26.6% (F_6). Genera analysis (Fig. 3b) indicated that *Firmicutes* were mainly composed of the genera *Bacillus* and *Clostridium*. The change in abundance mainly occurred in the genus *Bacillus*, with the initial abundance increasing from 5 to 16%, while the abundance of the genus *Clostridium* decreased from an initial 3.7 to 1.7%. *Actinobacteria* dominated on the 7th day after the experiment started (F_2), and its abundance increased from 7 to 13%.

Chloroflexi dominated in F_5 (12.3%), and its abundance gradually decreased from 9.5% (F_1) to 6.4% (F_6). The abundance of *Bacteroidetes* increased from 2.5 to 5.8%, indicating that the GET system's phylum was enriched. The abundance of *Nitrospirae* was significantly reduced in F_6, while the abundance of *Firmicutes* was increased substantially during this stage, implying that there was a possible competitive effect between *Nitrospirae* and *Firmicutes*.

2.3. The taxonomic classifications of the sequences at the genus level

Genus-level analysis (Fig. 3b) determined that some genera are relevant to the function of BSD. The genus *Janthinobacterium*, which has fungicidal activity against pathogenic fungi such as white scab fungus, was increased in F_2. The production of VFAs plays an important role in BSD.²⁹⁾ In the GET system, the following bacteria related to VFA production were detected. The butyric acid-producing genus *Coprococcus* was present in all processes and especially increased at F_6. The genus *Clostridium*, acetic acid, and butyric acid-producing bacteria were detected in all processes. The genus *Pelotomaculum*, a propionic acid-oxidizing bacteria, was present in F_5 and F_6. The genus *Ruminococcus*, a butyric acid-producing bacteria, was present in F_3, F_5, and F_6. The acetic acid-producing bacteria *Syntrophomonas* was also present in F_3 and F_6. *Lysobacter* increased from F_1 to F_6, and the genus *Thiobacillus* was present in all processes in which *Bacillus* was present. The methanogens in this experiment were mainly genus *Methanosaeta* (1.8%), genus *Methanobacterium* (0.6%), genus *Methanosarcina* (0.7%), and genus *Methanocella* (0.3%). The genus *Hyphomicrobium*, a methane-oxidizing bacteria, increased in F_6.

Discussion

BSD is a sustainable biological control method that inhibits plant pathogens by adding organic matter and stimulating the activity of endemic microorganisms in the soil. The steps of BSD treatment are as follows: (1) after drying and crushing the carbon source, evenly till material into the soil (or apply liquid organic material such as ethanol to the soil); (2) cover it with a thin film, flood it, and maintain the anaerobic condition. A large amount of irritating gas will be produced during treatment, meaning that the BSD treatment is complete when the treated soil no longer has a foul odor.¹⁷⁾ Therefore, in terms of organic

matter treatment methods, the GET system is almost identical to the BSD treatment. As to functionality, the GET system can be considered an upgraded version of the BSD treatment because the BSD system does not recover the generated biogas, while the GET system can recover and use it as renewable energy.

The development of reducing conditions or a decrease in the oxidation-reduction potential (ORP) in the soil is the essential feature of BSD treatment.¹⁷⁾ In the first stage of BSD treatment, after the organic matter is incorporated into the soil, a reduction process proceeds with the consumption of O₂ by aerobic microorganisms, resulting in an anoxic soil with Eh of -100 to -200 mV due to the higher moisture.²⁶⁾ Similarly, in the GET system, soil Eh decreased quickly right after the start of the experiment, reaching -200 mV. The pH gradually increased, with a continuous decrease in Eh, and reached neutral at F_4 (6.68). These values are consistent with those of the physicochemical state of the BSD treatment.

As the decomposition of treated organic matter by anaerobic (including facultatively anaerobic) hydrolytic and fermentative bacteria proceeds, the diverse and heterogeneous anaerobic bacterial community proliferates to a high population density. Consequently, fermentation products such as volatile fatty acids, alcohols, and gases are generated. A lot of fermentative anaerobic bacteria species are known to yield these products from saccharides, proteins, or amino acids. Some anaerobic bacteria such as *Clostridium* spp. produce skatole, indole, cresol, and other phenolic compounds by the decomposition of amino acids such as tryptophan and tyrosine.³⁰⁾ The production of VFAs may cause a decrease in the pH of the soil, whereas NO₃⁻ reduction and NH₄⁺ production may increase it. Thus, the anaerobic bacterial community should play a key role in changes in the soil condition, thereby inactivating soil-borne plant pathogens.

According to microbial analysis results (Fig. 3b), the genera *Clostridium* and *Bacillus* dominated after the GET system was launched (F_1). After 9 days of treatment (F_2), the abundance of these two genera decreased significantly, but the total diversity within the GET system was the highest (Fig. 2b). In F_3 and F_4, the genera *Clostridium* and *Bacillus*, respectively, began to pick up, and the abundance of the genus *Bacillus* increased significantly in F_6.

It is known that species in the genus *Clostridium* (*C. saccharobutylicum*, *C. xylanovorans*, and *C. pasteurianum*) contribute to the production of antibacterial substances such as acetic acid and butyric acid.^{30–33)} The genus *Bacillus* contains denitrification species, particularly *Bacillus subtilis*, which is often used as a highly effective biocide.³⁴⁾

The formation of a strongly reducing soil environment with increased organic matter has allowed anaerobic microorganisms to enrich in large numbers.

Since aerobic microorganisms still could survive briefly in a strong redox environment, diversity remained maximized. Then, the aerobic microbial communities present in *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* were dead or damaged due to such a strong redox environment, and anaerobic denitrifying bacteria

were enriched in large quantities, eliminating the accumulation of nitrate, neutralizing the soil acidity, and increasing the pH in the soil. At the same time, pathogens in the soil were inhibited due to the extreme redox environment and the production of sulfides, resulting in the lowest diversity of substances at F₆.

Furthermore, most *Proteobacteria* in this study could not be classified; however, the phylum *Proteobacteria* dominated throughout the experiment. At the end of the GET system treatment, the *Proteobacteria* richness of F₆ was reduced as compared to the initial abundance of F₁. The phylum is known to contain some pathogens, such as *Agrobacterium tumefaciens*³⁵⁾ and *Ralstonia solanacearum*.³⁶⁾ Since these pathogens can be inhibited effectively by using BSD,¹⁵⁾ the decline in the abundance of *Proteobacteria* in this study may be related to the reduction of these pathogens in the GET system. In particular, the *Lysobacter* in this phylum was enriched. *Lysobacter enzymogenes* can produce antifungal antibiotics against crop pathogens,³⁷⁾ and the *Lysobacter* sp. SB-K88 strain can suppress damping-off disease.³⁸⁾

In this study, an analysis of the bacterial community revealed that the GET system also plays a vital role in the biological soil disinfestation of paddy fields, constructing a strict anaerobic environment similar to that of BSD. As a result, the dominant phyla and genera were consistent with the known microbial community structure in BSD, implying that the GET system worked as a complete BSD system. As compared to traditional BSD systems, the GET system has the advantage of reducing greenhouse gas emissions, producing renewable energy, and providing a carbon sink for atmospheric CO₂, in addition to biological soil disinfestation (BSD). However, the design intention of the GET system and the indicators in the established process were to maximize the occurrence of methane emissions in paddy fields and collect them as a renewable energy. The current research and application of the GET system are carried out in paddy fields, using rice straw as the only organic material. Especially in upland cultivation, it is necessary to control soil pests. Therefore, to cultivate upland crops in the soil of the GET system, which is anaerobically fermented by applying 14 kg/m² of rice straw, the next issue is how to return the soil of the GET system to an oxidative state on site.

This study clarified the possibility of the BSD effect of the GET system, and experimental studies, including methodologies for upland cultivation, are important in the future.

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Electronic supplementary materials

The online version of this article contains supplementary material (Supplemental Fig. S1), which is available at <https://www.jstage.jst.go.jp/browse/jpestics/>.

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