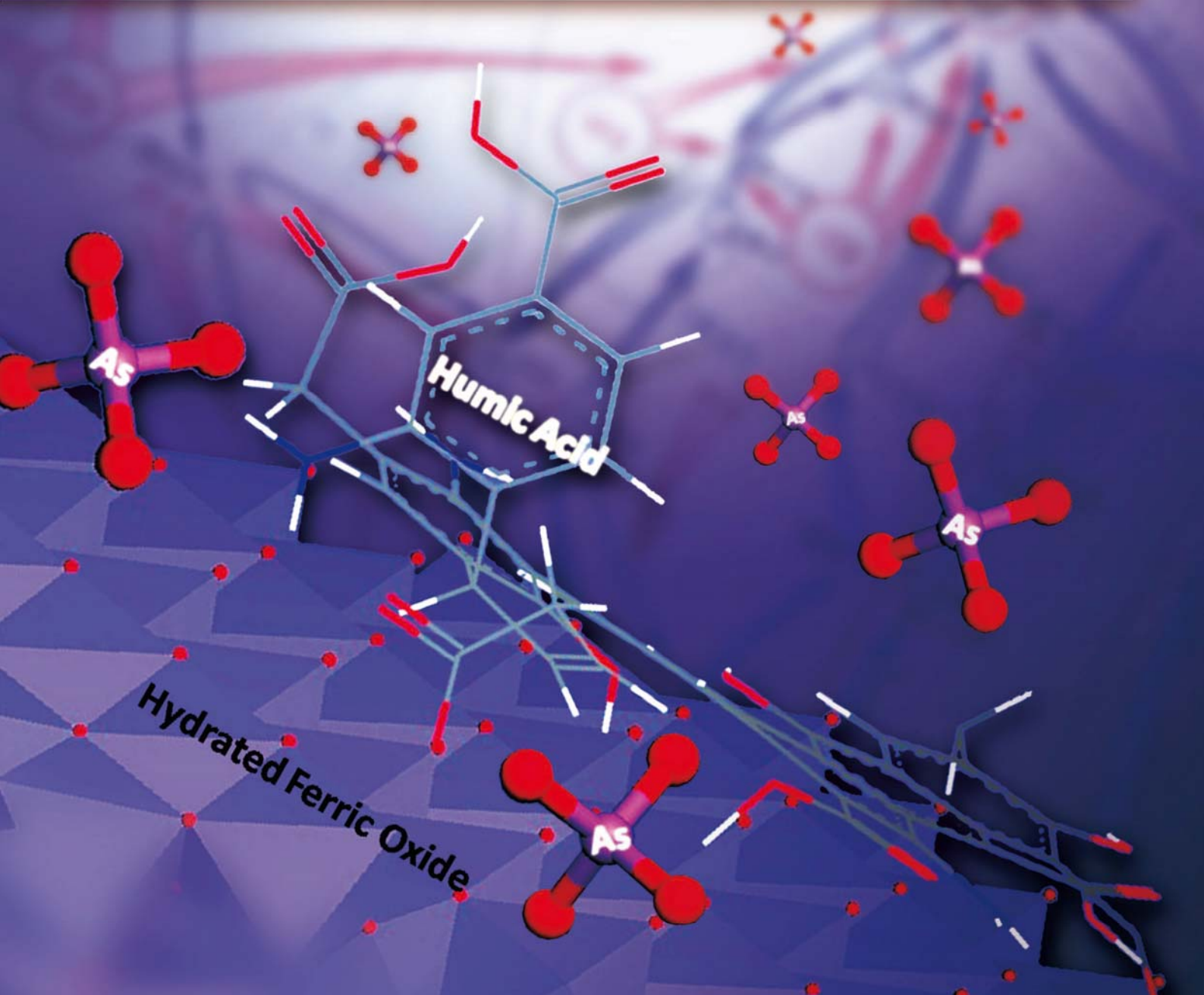
JES
JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
February 1, 2014 Volume 26 Number 2
www.jesc.ac.cn



ELSEVIER
Science Press
Sponsored by
Research Center for Eco-Environmental Sciences
Chinese Academy of Sciences

ISSN 1001–0742 **Journal of Environmental Sciences** Vol. 26 No. 2 2014

CONTENTS

Aquatic environment

Atmospheric environment

Environmental health and toxicology

Environmental catalysis and materials

Serial parameter: CN 11-2629/X*1989*m*261*en*P*30*2014-2

Journal of Environmental Sciences 26 (2014) 423–431

Available online at www.sciencedirect.com

Journal of Environmental Sciences

www.jesc.ac.cn

Digestion performance and microbial community in full-scale methane fermentation of stillage from sweet potato-shochu production

Tsutomu Kobayashi[1,2], Yueqin Tang[3], Toyoshi Urakami[1], Shigeru Morimura[1], Kenji Kida[1,3,*]

1. Graduate School of Science and Technology, Kumamoto University, 2-39-1 Kurokami, Chuo-ku, Kumamoto 860-0862, Japan. E-mail: tutomu-kobayashi@kirishima.co.jp

2. Kirishima Shuzo Co., Ltd 4-28-1 Shimokawahigashi, Miyakonojo 885-8588, Japan

3. College of Architecture and Environment, Sichuan University, Chengdu 610065, China

ARTICLE INFO

Article history:
Received 09 March 2013; revised 17 May 2013; accepted 20 June 2013

Keywords:
thermophilic methane fermentation
stillage treatment
full-scale fixed-bed reactor
microbial community

DOI: 10.1016/S1001-0742(13)60423-4

ABSTRACT

Sweet potato shochu is a traditional Japanese spirit produced mainly in the South Kyushu area in Japan. The amount of stillage reaches approximately 8×10^5 tons per year. Wastewater mainly containing stillage from the production of sweet potato-shochu was treated thermophilically in a full-scale treatment plant using fixed-bed reactors (8 reactors × 283 m^3). Following the addition of Ni^{2+} and Co^{2+}, the reactors have been stably operated for six years at a high chemical oxygen demand (COD) loading rate of 14 kg/(m^3·day). Analysis of coenzyme content and microbial communities indicated that similar microbial communities were present in the liquid phase and on the fiber carriers installed in reactors. Bacteria in the phyla Firmicutes as well as Bacteroidetes were dominant bacteria, and *Methanosarcina thermophila* as well as *Methanothermobacter crinale* were dominant methanogens in the reactors. This study reveals that stillage from sweet potato-shochu production can be treated effectively in a full-scale fixed-bed reactor under thermophilic conditions with the help of Ni^{2+} and Co^{2+}. The high diversity of bacterial community and the coexistence of both aceticlastic and hydrogenotrophic methanogens contributed to the excellent fermentation performance.

Introduction

Sweet potato shochu is a traditional Japanese spirit that is mainly produced in the South Kyushu area in Japan. Two major hurdles in shochu production are how to treat stillage discharged during production and how to reduce the huge consumption of fossil fuel during the distillation process. The amount of stillage, which is generally twice as much as the volume of sweet potato shochu produced, reaches approximately 8×10^5 tons/yr. However, dumping it into the ocean or reutilizing it as manure has already been prohibited. As a result, entrepreneurs have to develop efficient shochu making plants that cannot only solve these problems but also combat the global warming caused by carbon dioxide emitted during shochu making.

Methane fermentation of industrial wastewater is now commonly used all over the world as an environmentally friendly process, because not only is the waste and wastewater treated but the process is energy producing. During this process, organic matter in wastewater is converted to methane though four steps, hydrolysis, acidogenesis, acetogenesis and methanogenesis, which are conducted by different groups of microorganisms (McCarty, 1982). Among the four steps, acetogenesis and methanogenesis are key to controlling the whole methane fermentation process. The rate of acetogenesis is generally low because the degradation of volatile fatty acids (VFAs) is thermodynamically unfavorable. In addition, acetogenesis can only happen when combined with methanogenesis. Unfortunately, the growth rates of microorganisms respon-

* Corresponding author. E-mail: kida@scu.edu.cn; kida@gpo.kumamoto-u.ac.jp

sible for acetogenesis and methanogenesis are relatively low, which limits the methane fermentation rate especially when completely stirred tank reactors (CSTRs) are employed. Therefore, in order to achieve a high loading rate of methane fermentation, the following is required: (1) retention of a high concentration of microorganisms in the reactor; (2) optimal conditions for syntrophic growth of VFA-degrading bacteria and methanogens; and (3) stimulation of methane conversion of methanogens. A fixed-bed reactor fulfills both (1) and (2) since carriers installed in the reactor can not only retain microorganisms in the reactor but also provide the space for adjacent growth of microorganisms. Stillage from barley shochu- and awamori-making processes has been successfully treated at high chemical oxygen demand (COD) loading rates with fixed-bed reactors under thermophilic conditions (Kida and Sonoda, 1993; Tang et al., 2007a). There are also several reports of using fixed-bed reactors in full-scale plants treating wastewater with high organic content (Andreottola et al., 2005; Colleran et al., 1998; Mokhtarani et al., 2012). In spite of the type of reactor, the addition of cofactors may be necessary for biochemical reactions as they may significantly stimulate reaction rates. Nickel and cobalt ions, which are cofactors of methyl-S-CoM reductase and coenzyme M (CoM) methylase, respectively, are necessary for methane production in both aceticlastic methanogens and hydrogenotrophic methanogens (Speece et al., 1986; Takashima et al., 1990). Addition of Ni^{2+} and Co^{2+} to the reactors drastically improved methanogenic activity, increased biogas evolution rates and led to much higher loading rates (Schink, 1997; Kida et al., 2001).

A pilot-scale plant was initially constructed to treat sweet-potato shochu stillage using novel fixed-bed reactors. By the addition of Ni^{2+} and Co^{2+}, a high COD loading rate of 15 kg/(m^3·day) was achieved (Tatara et al., 2004; Togo et al., 2000). Based on the results of this pilot-scale plant, a full-scale plant was constructed to treat stillage as well as all other wastes and wastewater from sweet-potato shochu production. Here, we report on the performance of the full-scale plant, which has now been operating stably for more than six years. We examined the activity of methanogens in the wastewater from the full-scale plant by measuring coenzymes F_{430}, corrinoids, and F_{420}. Methyltransferase and methylreductase are key enzymes in methane production pathways. Methyltranderase has corrinoid as coenzyme with Co^{2+} ligand. Methylreductase has F_{430} as coenzyme with Ni^{2+} ligand. The addition of Ni^{2+} and Co^{2+} improves the activity of these enzymes and hence increases the methane production rate. In addition, methane is produced from H_2 and CO_2 via C1 cycle pathway. F_{420} was a key coenzyme with intrinsic fluorescence in C1 cycle pathway. The microbial communities attached in the fiber carriers and suspended in liquid phase within the reactors were also analyzed by using denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene clone library techniques. Finally, the stability and the structure of microbial community are discussed.

1 Materials and methods

1.1 Slurry and raw wastewater

Stillage, wastewater and sweet potato waste with a volumetric ratio of 88:8:6 (hereafter called raw wastewater) were discharged from the shochu making process at the Kirishima Shuzo Co., Ltd. (Miyakonojo City, Japan). Sweet potato waste was suspended with water and crushed with a hammer mill before being added into the storage tank. The mixture of stillage, wastewater and sweet potato waste was fed into fixed-bed reactors through a cutting pump, which cut fiber into a mixture. **Table 1** shows the representative composition of stillage and raw wastewater. The concentrations of COD, total nitrogen and total phosphate were very high.

1.2 Thermophilic methane fermentation

Figure 1 shows the schematic diagram of the full-scale plant for thermophilic methane fermentation using fixed-bed reactors. The plant was constructed by Kajima Corporation (Tokyo, Japan). Each fixed-bed reactor with a total volume of 406 m^3 was made of steel. Two hundred tubes of nonwoven carbon fiber (100 mm in diameter, 20 m long) arranged vertically were fixed into a cage with a volume of 283 m^3 and installed in each reactor for microorganism retention. Eight reactors were arranged in parallel as shown in **Fig. 1**. Specified volume of raw wastewater was fed from Service tank2 to each reactor by using automatically controlled valve and pump. Anaerobic garbage digesting sludge was added into each reactor as seed sludge and acclimated at 55°C for 33 days. Trace element solution was prepared to maintain the methanogenic activity as follows: 150 kg of 40% $FeCl_3·4H_2O$ solution, 10 kg of 35% $NiCl_2·6H_2O$ solution and 10 kg of 35% $CoCl_2·6H_2O$ solution were filled up to 400 L with water.

Table 1 Representative characteristics of stillage and raw wastewater

Parameter	Stillage	Raw wastewater
Total solid (mg/L)	37000	38000
Volatile total solid (mg/L)	NM	35000
Suspended solid (mg/L)	5000	28000
BOD_5 (mg/L)	32000	38000
COD_{Cr} (mg/L)	70000	80000
Total nitrogen (mg/L)	2400	2800
Total phosphate (mg/L)	280	300
Water content (%)	92	93
pH	4.2	4.0

NM: not measured.

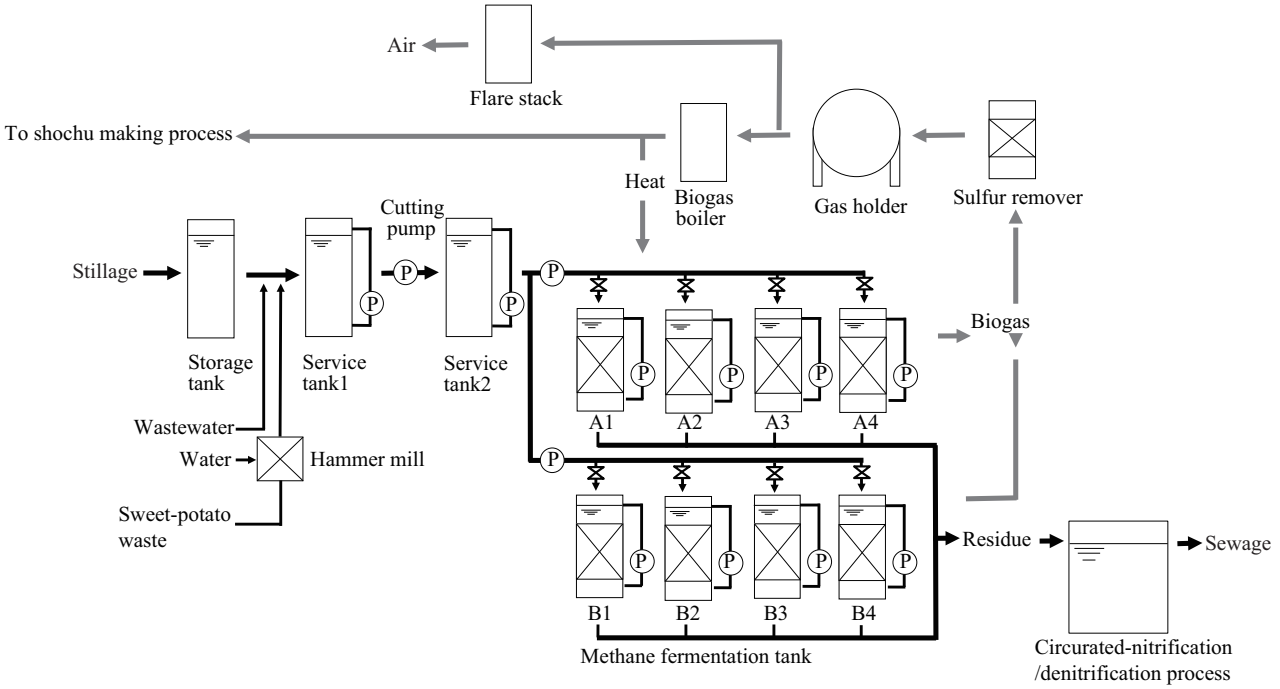


Fig. 1 Schematic diagram of the full-scale plant for treating stillage by thermophilic methane fermentation using fixed-bed reactors. "P" means pump, "A1–A4, B1–B4" were names of eight bioreactors.

Fifty liters of the solution was added to each reactor to give the final concentrations of 8.8 mg/L of Fe^{3+}, 0.9 mg/L of Ni^{2+} and 0.9 mg/L of Co^{2+}. The trace element solution was added every two weeks for the first 24 months-operation after which it was added only when the concentration of volatile fatty acid (VFA) in the reactors increased. Raw wastewater was fed continuously to each reactor and the dilution rate was increased step-wise to achieve increasing loading rate. Biogas was stored temporarily in a gas holder after the removal of sulfate. Steam was generated from the biogas with a boiler and was used to maintain the temperature of the reactors and for the shochu-making process.

1.3 Determination of coenzyme content

Microorganisms were collected separately from the fiber carrier and the suspended liquid phase in the reactor A4 at the 64th, 65th and 66th months, where the reactor was operated at dilution rates of 0.15, 0.16 and 0.12 day^{-1}, respectively. In order to detach the microorganisms attached to the fiber carrier, the carriers were suspended in 10 mmol/L hate buffer solution and vortexed. The determination of coenzymes F_{430} (nickel tetrapyrrole), corrinoids and relative concentrations of F_{420} (8-hydroxy-5-deazaflavin) were carried out according to methods reported previously (Kida et al., 2001). Corrinoids and F_{430} were extracted from microorganisms using acetate buffer containing potassium cyanide and distilled water, respectively. After the removal of insoluble materials, the extracted corrinoids and F_{430} were purified using an Amberlite XAD-2 column (Organo, Tokyo, Japan). Corrinoids and F_{430} in the samples were determined separately as cobalt and nickel concentrations analyzed with an atomic absorption spectrophotometer (AA-6600G, Shimadzu, Kyoto, Japan). F_{420} was extracted from microorganisms with distilled water at 120°C for 20 min. After the removal of insoluble materials, the relative concentrations of F_{420} in the samples were estimated using a spectrofluorophotometer (RF-5300PC, Shimadzu, Japan) based on fluorescent strength determined at a wavelength of 460 nm after excitation at a wavelength of 425 nm. The fluorescent strength of the sludge from the CSTR being fed with acetate as sole carbon source at a dilution rate of 0.025 day^{-1} was defined as a 1 time concentration as described in previous study (Kida et al., 2001).

1.4 Denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene clone analysis

Sludge in the liquid phase at the 64th-, 65th- and 66th-month and sludge in fiber carrier at the 65th-month were sampled from reactors A4 and B4. Community DNAs were extracted using the FastDNA® spin kit for soil (MP Biomedicals, Cleveland, USA). DGGE analysis of these eight samples was carried out using methods described previously (Tang et al., 2005). Clones of methanogens obtained from a dry thermophilic methanogenic digester were used to identify the bands of DGGE for Archaea (Tang et al., 2011). Samples collected from the fiber carriers in the 65th-month at the dilution rate of 0.16 day^{-1} were used to construct 16S rRNA gene clone libraries using methods described previously (Shigematsu et al., 2003,

2006). The primer sets Ar109F and Ar915R (Lueders and Friedrich, 2003) as well as Eu27F and 1490R, were used for amplification of the 16S rRNA genes of Archaea and Bacteria, respectively. One archaeal-16S rRNA gene library (SWSA library) and one bacterial-16S rRNA gene library (SWSB library) were constructed using extracted community DNA. Ninety positive clones in SWSA library and 70 positive clones in SWSB library were randomly selected for sequencing (Takara Bio Inc., Dragon Genomics Center, Mie, Japan). Clones with sequence similarities over 98% were considered as the same operational taxonomic unit (OTU). The OTUs were designated SWSA01 and SWSA02 for clones of the archaeal library, and SWSB01 to SWSB30 for clones of the bacterial library. The phylogenetic analyses of these OTUs were carried out as described previously (Tang et al., 2011).

1.5 Other analytical methods

Total solids (TS), volatile total solids (VTS), suspended solids (SS), biochemical oxygen demand (BOD), total nitrogen (TN), total phosphate (TP), water content and pH were analyzed in accordance with standard methods (Hirakawa, 1998).

Chemical oxygen demand (COD) was analyzed using the HACH method (DR2800, Hach Co., Loveland, Colorado, USA). Concentrations of volatile fatty acids (VFAs) and total alkalinity (T-Alk) were also analyzed using supernatants obtained after centrifugation at 4500 ×*g* for 20 min in accordance with standard methods (Hirakawa, 1998). Biogas evolution rate was analyzed using an ultrasonic gas flow meter (GM868, General Electric Co., USA). The methane content of the biogas was measured by using a gas density meter (GD300S, Yokogawa Electric Corporation, Tokyo, Japan). Hydrogen sulfide was measured using Kitagawa precision gas detector tubes (Komyo Kitagawa, Kanagawa, Japan).

1.6 Nucleotide sequence accession numbers

The nucleotide sequences determined in this study have been deposited in the GenBank database under accession numbers AB772284 to AB772315.

2 Results and discussion

2.1 Performance of anaerobic fixed-bed reactor treating raw wastewater under thermophilic conditions

Figure 2 shows monthly changes of the COD loading rate, the quality of effluent, the removal efficiency of COD and the digestion efficiency of VTS during the six years of treatment. COD loading rate was increased step-wise to 15 kg/(m^3·day) during the first 20 months operation. After that, COD loading rate was kept at 14 kg/(m^3·day), which corresponding to dilution rates of 0.15–0.17 day^{-1}. Though SS content of raw wastewater was higher than 28 g/L blockages did not occur during the whole operation period. The pH in the reactor was approximately 7.0–7.7, and the VFA concentration was approximately 450–550 mg/L. VFA accumulated to over 2000 mg/L only once, as a result of mis-operation at the 25th-month of operation, however, it decreased sharply by the addition of trace element solution. Following this episode, the trace element solution was added to the reactor when the VFA concentration

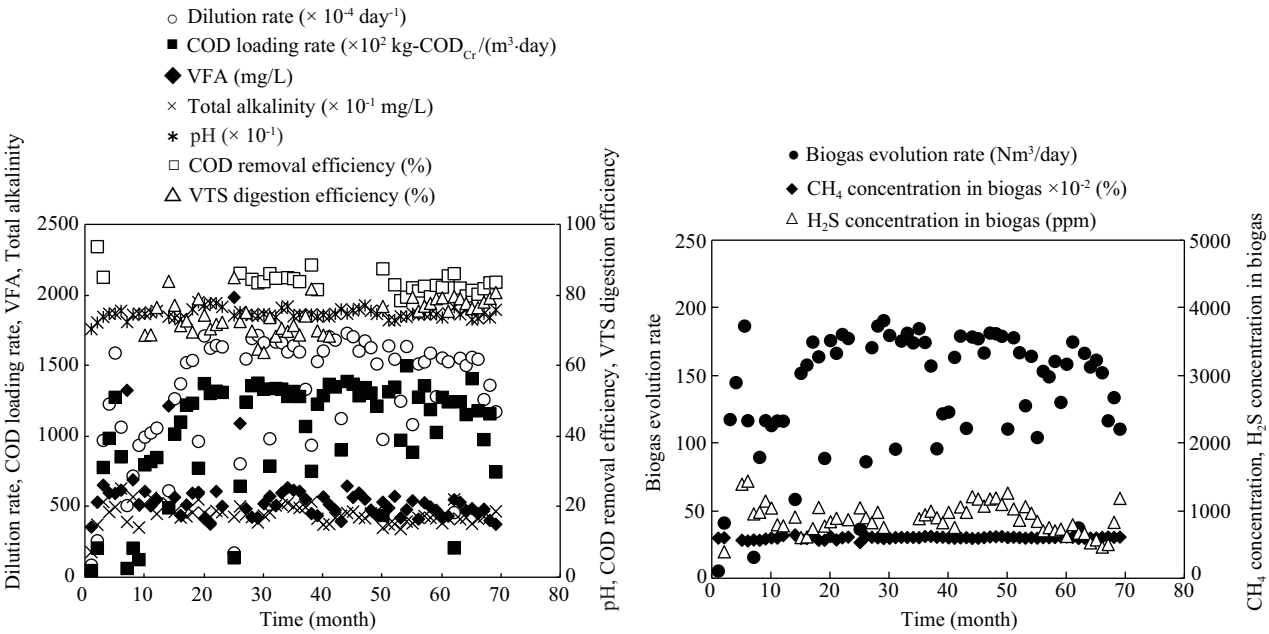


Fig. 2 Monthly changes in the quality of effluent, the removal efficiency of COD and the VTS digestion efficiency during the six-year treatment of raw wastewater with fixed-bed reactor.

tended to increase (**Fig. 3**). As a result, the raw wastewater was treated stably for six years. At the COD loading rate of 14 kg/(m^3·day), the COD removal efficiency and the VTS digestion efficiency were kept at more than 80% and 75%, respectively. Biogas evolution rate was 15000–20000 Nm^3/day raw wastewater, and the methane content and H_2S concentration in the biogas was approximately 60% and 250–820 ppm, respectively.

The maximum COD loading rate of 14 kg/(m^3·day) in the present study was almost the same as that when SS-removed stillage was treated using a UASB reactor and stillage with SS was treated using a membrane reactor (Samejima, 2003; Ikeda and Matsushita, 2010). The biogas evolution rate achieved in this study was almost the same as that generated from the stillage with SS treated by using the membrane reactor, while it was more than twice as that generated from the SS-removed stillage treated with a UASB reactor (Samejima, 2003; Ikeda and Matsushita, 2010). This suggests that a fixed-bed reactor with the addition of trace elements can achieve good performance with respect to the loading rate as well as the biogas evolution rate even when treating wastewater containing high SS for long periods. The carbon fiber carrier used in the present study was highly porous and was arranged tangentially along the flow of liquid in the reactor. The capacity of the carbon fiber of retaining syntrophic bacteria with low growth rate might contribute to the excellent treatment performance (Tang et al., 2007a; Tatara et al., 2008).

The evolution rate of biogas with a methane content of 60% was 45 Nm^3/m^3 raw wastewater, corresponding to the energy production of 968,200 kJ/m^3 raw wastewater. As the amount of raw wastewater produced per day was 188 m^3 (which is equivalent to the stillage of 165 m^3), the energy generated from methane would reach 182,000,000 kJ/day. Since the amount of saturated steam (pressure, 0.3 MPa; specific enthalpy, 2738 kJ/kg) needed for the distillation was 65,000 kg/day in the Kirishima Shuzo plant, the amount of heat supplied per day should be 197,800,000 kJ/day (=178,000,000/0.9), assuming that boiler efficiency is 90%. Therefore, 92% of the energy needed for the distillation could be supplied by methane fermentation of raw wastewater.

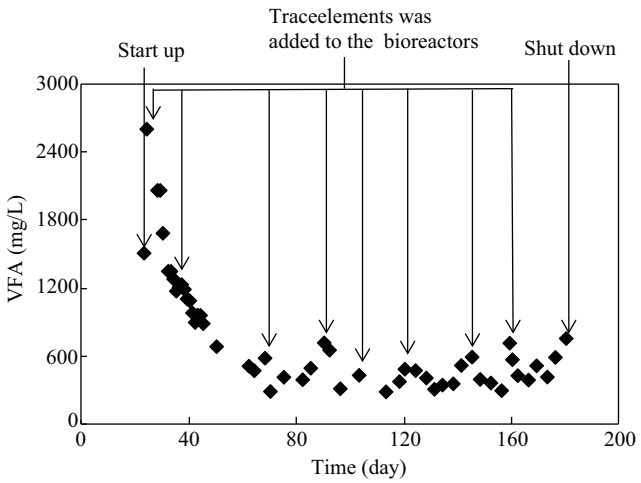


Fig. 3 Changes in VFA concentration in the fixed-bed reactor from the 25th through to the 30th-month operation.

2.2 Coenzyme content in methanogens attached to the carbon fiber carrier and those suspended in the liquid phase

The treatment had been carried out at the high COD loading rate for six years. Then, the contents of coenzymes in microorganisms in the reactor were measured at the 64th–66th month. **Table 2** shows the contents of coenzymes in microorganisms attached on the carbon fiber as well as those suspended in the liquid phase in the fixed-bed reactor during the 64th, 65th, and 66th month of operation. F_{430} and corrinoids are coenzymes of key two enzymes, methyl-S-CoM reductase and coenzyme M (CoM) methylase, both of which participate in methane production pathways of both aceticlastic and hydrogenotrophic methanogens. F_{420} is much more involved in methanogenesis from H_2-CO_2 than in methanogenesis from acetate (Kida et al., 2001). No obvious changes in the concentrations of the three coenzymes were detected during the operation time, indicating a stable methanogen structure during the operation time. This was consistent with the stable performance of the methane fermentation. No significant differences in concentrations of the three coenzymes were observed between microorganisms attached on the carbon fiber carrier and those suspended in the liquid phase (**Table 2**), suggesting that the relative ratio of aceticlastic and hydrogenotrophic methanogens was similar between methanogens existing in the carrier and liquid phases.

As shown in **Table 2**, concentrations of coenzyme F_{430}, corrinoids and F_{420} (relative value) were 0.510–0.580 μmol Ni/g VSS, 0.130–0.152 μmol Co/g VSS and 0.176–0.210, respectively. They were even comparable to those in a methane fermentor fed with transparent synthetic wastewater with acetate as the sole carbon and energy source (Kida et al., 2001), suggesting that there was relatively high methanogenesis activity in the fixed-bed reactor that led to good performance of methane fermentation.

2.3 Microbial community revealed by DGGE and clone library analyses

The structures of archaeal and bacterial communities attached on the fiber carrier and those suspended in the liquid phase were compared by DGGE analysis (**Fig. 4**). The bacterial community was more complex than the archaeal community. However, for both archaeal and bacterial communities, in spite of minor differences in the strength of the bands, no obvious differences in band number and band site were observed along the operation time, sug-

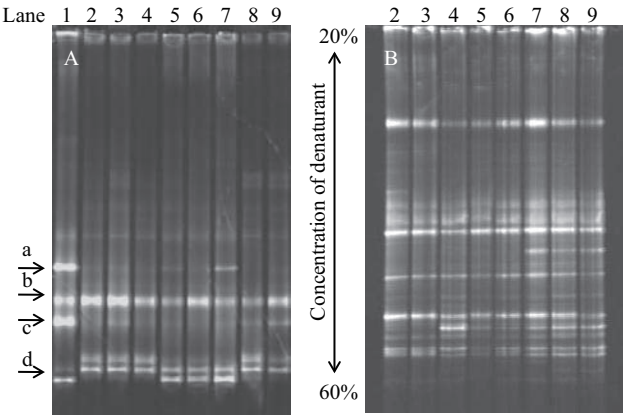


Fig. 4 Comparison using DGGE of archaeal (A) and bacterial (B) communities attached to the fiber carrier and those suspended in the liquid phase. Lane 1: archaeal clone marker (a, *Methanobacterium*; b, *Methanothermobacter*; c, *Methanosarcina*; d, *Methanoculleus*); lane 2: iquid phase in Reactor A4 at the 64th month (0.15 day^{-1}); lane 3: liquid phase in Reactor B4 at the 64th month (0.15 day^{-1}); lane 4: liquid phase in Reactor A4 at the 65th month (0.16 day^{-1}); lane 5: liquid phase in Reactor B4 at the 65th month (0.16 day^{-1}); lane 6: liquid phase in Reactor A4 at the 66th month (0.12 day^{-1}); lane 7: liquid phase in Reactor B4 at the 66th month (0.12 day^{-1}); lane 8: fiber carrier in Reactor A4 at the 65th month (0.16 day^{-1}); lane 9: fiber carrier in Reactor B4 at the 65th month (0.16 day^{-1}).

gesting a relatively stable microbial community which was responsible for the efficient treatment of raw wastewater. In addition, the archaeal and bacterial communities on the fiber carrier and in the liquid phase found in both the same reactor and in different reactors were similar. The stable archaeal community was consistent with the results from the coenzyme content analysis (**Table 2**).

The bands for *Methanothermobacter* and *Methanoculleus* were detected as dominant bands in all samples, while bands for *Methanosarcina* and *Methanobacterium* were weak and only appeared in some of the samples. The DGGE result on the archaeal community in the thermophilic fixed-bed reactor indicated that hydrogenotrophic methanogens might dominate methanogens and that methane was produced mainly from H_2 and CO_2 pathways. However, since biases might occur due to the primers used, it may be necessary to use several other analysis techniques simultaneously.

Since the microbial communities on fiber carriers are thought to play a major role in digestion, microbial communities on the fiber carrier at the 65th month were investigated further by 16S rRNA gene clone library analysis. **Table 3** shows the classification of clones of SWSA and SWSB libraries. Only two OTUs were obtained in the SWSA library. One OTU with 58 clones was closely related to *Methanosarcina thermophila* with a 99% sequence similarity, while another OTU with 29 clones was closely related to *Methanothermobacter crinale* with a 98% sequence similarity. Clones related to *Methanoculleus* were not obtained, though *Methanoculleus*-related bands were detected as dominant bands in DGGE analysis (**Fig. 4**). The biases between DGGE and clone library analysis might be attributed to the different primers used in these two techniques. Altogether, hydrogenotrophic methanogens of *Methanothermobacter* and *Methanoculleus* as well as aceticlastic methanogen *Methanosarcina* were dominant methanogens. In other words, both aceticlastic and hydrogenotrophic pathways for methane production were requisite in the thermophilic fixed-bed reactor treatment of raw wastewater. These methanogens have also been detected as dominant methanogens in other thermophilic methanogenic reactors (Frank et al., 2007; Liu et al., 2009, 2011; Tang et al., 2011), indicating that their presence is ubiquitous in thermophilic reactors.

As shown in **Table 3**, 29 OTUs were obtained from 68 clones in the SWSB library. Twenty four OTUs from 53 clones were classified in the phylum Firmicutes, while one OTU from 10 clones was classified in the phylum Bacteroidetes. In addition, four OTUs from 5 clones were classified in the phyla Thermotogae, candidate division OP9, Tenericutes and Planctomycetes. Species in phyla Firmicutes and Bacteroidetes could be considered as dominant bacteria in the fixed-bed reactor treatment of raw wastewater, which was similar with the bacterial community in a thermophilic fixed-bed reactor treating awamori distillation wastewater (Tang et al., 2007a).

OTUs classified in Thermotogae, Tenericutes, Planctomycetes and Candidate division OP9 showed relatively

Table 2 Contents of coenzymes in microbial communities from thermophilic fixed-bed reactor treating raw wastewater

Month	Dilution rate (day^{-1})	Sample	Concentration		
			F_{430} (μmol Ni/g VSS)	Corrinoids (μmol Co/g VSS)	F_{420}
64th	0.15	Suspended in liquid phase	0.580	0.144	0.182
65th	0.16	Attached on fiber carrier	0.521	0.130	0.176
		Suspended in liquid phase	0.512	0.151	0.201
66th	0.12	Attached on fiber carrier	0.514	0.152	0.194
		Suspended in liquid phase	0.510	0.144	0.210

F_{430} is coenzyme of Methylreductase with Ni^{2+} ligand in methane production pathways; F_{420} is a key coenzyme with intrinsic fluorescence in C1 cycle pathway.

Table 3 Distribution of 16S rRNA gene clones in SWSA and SWSB libraries constructed using community DNA extracted from carbon fiber carrier in the thermophilic fixed-bed reactor treating raw wastewater (65th month, dilution rate 0.16 day^{-1})

Taxon (phylum)	Number of OTUs	Number of clones
Archaea		
Methanosarcina (genus)	1	58
Methanothermobacter (genus)	1	29
Bacteria		
Firmicutes	24	53
Bacteroidetes	1	10
Thermotogae	1	1
Candidate division OP9	1	2
Tenericutes	1	1
Planctomycetes	1	1

low similarities to pure-cultured species but had high similarities to uncultured clones obtained from various anaerobic reactors such as a solid waste degrading packed-bed reactor, a sludge digester and a swine manure digester (Akuzawa et al., 2011; Sasaki et al., 2007; Riviere et al., 2009).

Most of the OTUs in phylum Firmicutes, i.e., those located in Cluster 1 (three OTUs of five clones), Cluster 2 (four OTUs of 12 clones) and Cluster 3 (five OTUs of 14 clones) shown in **Fig. 5**, were not closely related to any pure-cultured species. However, these OTUs showed high sequence similarities of 99%–100% to uncultured clones obtained from various anaerobic reactors such as the full-scale anaerobic sludge digester (for SWSB03–05), and the thermophilic anaerobic cellulose-, glucose-, silage-, and turfgrass-, solid waste-degrading reactors (for SWSB07–11, 14–16) (Tang et al., 2008, 2011; Sasaki et al., 2011). Though the role of microorganisms represented by these OTUs in the reactors are still unclear due to their consistent existence in anaerobic reactors as described above, they might contribute significantly to the acidogenesis and acetogenesis steps in the methane fermentation process.

As shown in **Fig. 5**, among other OTUs in phylum Firmicutes, SWSB01 (six clones) and SWSB02 (one clone) showed 91% and 94% sequence similarities, respectively, to cellulolytic species *Clostridium stercorarium* subsp. *leptospartum*, however, showed 99% sequence similarity to uncultured clone AWA-B7 from a thermophilic upflow anaerobic filter reactor treating awamori distillery wastewater (Tang et al., 2007a) and OTU-B9 from a thermophilic anaerobic digester treating organic solid waste (Sasaki et al., 2011). SWSB30 (three clones) had a 91% similarity to non-cellulolytic *Clostridium* sp. TG60-81 (AB551425) and a 97% similarity to uncultured clone G55_D%_H_B_A06 (DQ887919) from a thermophilic anaerobic solid waste digester. SWSB06 with only one clone was related to *Clostridium* sp. XB90 isolated from anoxic bulk soil of rice paddy microcosms

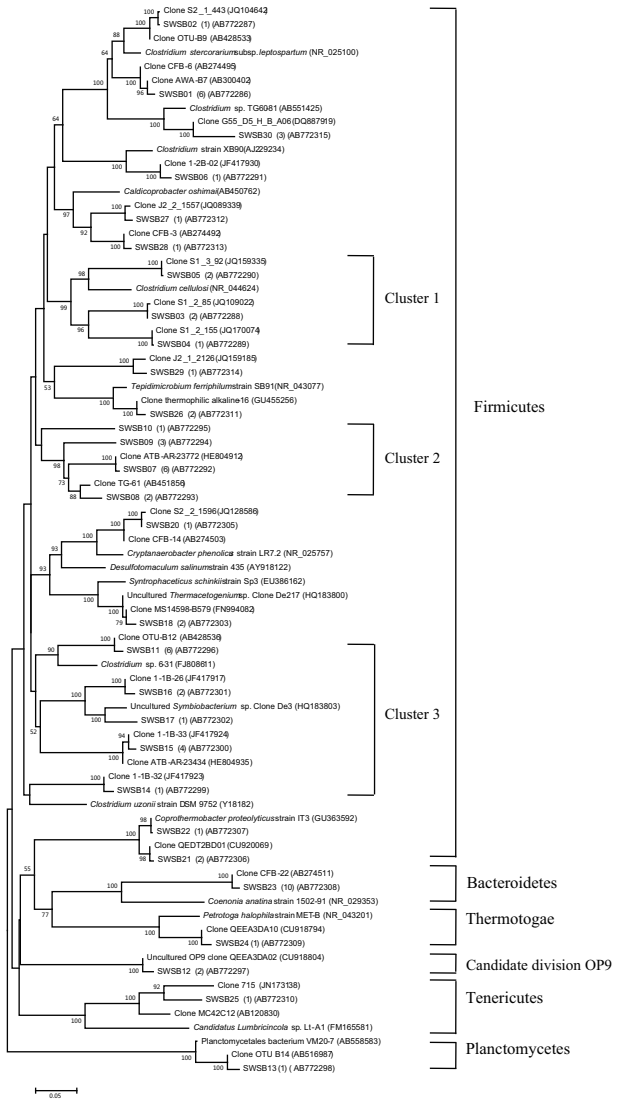


Fig. 5 Phylogenetic tree of bacterial clones of the SWSB library constructed using community DNA from the fixed-bed reactor treating raw wastewater. The tree was constructed by using the Neighbor-Joining method and partial sequences of the 16S rRNA gene. The bar represents two substitutions per 100 nucleotide positions. Bootstrap probabilities <50% are indicated at the branch nodes. Numbers of clones with identical sequences are shown in parentheses. The DDBJ/EMBL/GenBank accession numbers for reference strains and clones obtained in this study are shown in parentheses.

(Chin et al., 1999) with a 90% similarity and to uncultured clone 1-2B-02 from a dry thermophilic methanogenic digester (Tang et al., 2011) with 100% similarity. SWSB27 and 28 (two clones) had a sequence similarity of 90% to xylanolytic thermophilic *Caldicoprobacter oshimai* and similarities of 98% and 99%, respectively, to uncultured clones J2_2_1557 (JQ089339) from a full scale anaerobic reactor and CFB-3 from a pack-bed reactor degrading solid waste (Sasaki et al., 2007). Dedicated from the related species of OTUs, the microorganisms represented by the OTUs described above might contribute to the degradation

of holocellulose component in raw wastewater.

SWSB26 (two clones) had a 96% similarity to *Tepidimicrobium ferriphilum* strain SB91 which is a thermophilic, Fe(III)-reducing bacterium capable of fermenting protein and amino acids (Slobodkin et al., 2006). SWSB21 and 22 (three clones) showed 97% and 100% similarities to protein fermenting *Coprothermobacter proteolyticus*, respectively. The microorganisms represented by OTUs SWSB21, 22 and 26 might therefore play a significant role in protein degradation in the fixed-bed reactor.

SWSB18 with two clones showed a 92% similarity to syntrophic acetate-oxidizing *Syntrophaceticus schinkii* strain Sp3 (Westerholm et al., 2010) and a 99% similarity to uncultured *Thermacetogenium* sp. clone De217 (Liu et al., 2011). In the fixed-bed reactor, the existence of acetate-oxidizing bacteria indicated the existence of a methane production pathway by acetate oxidization combined with hydrogenotrophic methanogenesis (Shigematsu et al., 2004). Since acetate-oxidizing bacteria grow slowly, they are generally washed out from CSTR-type reactors which were operated at high dilution rates (Shigematsu et al., 2004). The dilution rates of the fixed-bed reactors in the present study were 0.12–0.18 day^{-1}. The acetate-oxidizing bacteria may have been retained in the reactors due to the carriers installed in the reactors. This should therefore be considered one of the requirements of a fixed-bed reactor as it enables those microorganisms with low growth rates to stay in the reactor, despite the high retention times when treating wastewaters. It is generally reported that under thermophilic conditions, methanogenesis through the hydrogenotrophic pathway becomes more dominant than at mesophilic conditions (Tang et al., 2005, 2007a, 2007b, 2008, 2011), which means that the role of acetate-oxidizing bacteria is quite critical. Since fixed-bed reactors were used for the treatment, sludge retention time was extended intensively. Though the doubling time of acetate-oxidizing bacteria were reported to be around 30 days, those slow growing acetate-oxidizing bacteria could be retained on the carrier. As a result, syntrophic methane production from H_2 and CO_2 by acetate-oxidizing bacteria and hydrogenotrophic methanogens became easier in the fixed-bed reactor. Hence, to achieve high loading rates, reactors like fixed-bed type reactors should be employed when treating wastewater under thermophilic conditions.

3 Conclusions

The stillage discharged from sweet potato-shochu production has been treated stably for about six years in a full-scale methane fermentation plant using a novel fixed-bed reactor with the addition of trace elements. The COD loading rate reached 14 kg/(m^3·day) and a biogas of 45 Nm^3/m^3 raw wastewater was generated effectively. Analysis of the coenzyme content and the microbial community suggested similar microbial communities existed in the liquid phase and on the fiber carrier in the reactors. The coexistence of both aceticlastic and hydrogenotrophic methanogens and a bacterial community with high diversity contributed to the excellent performance of the treatment.

REFERENCES

Akuzawa, M., Hori, T., Haruta, S., Ueno, Y., Ishii, M., Igarashi, Y., 2011. Distinctive responses of metabolically active microbiota to acidification in a thermophilic anaerobic digester. Microb. Ecol. 61, 595–605.

Andreottola, G., Foladori, P., Nardelli, P., Denicolo, A., 2005. Treatment of winery wastewater in a full-scale fixed bed biofilm reactor. Water Sci. Technol. 51, 71–79.

Chin, K. J., Hahn, D., Hengstmann, U., Liesack, W., Janssen, P. H., 1999. Characterization and identification of numerically abundant culturable bacteria from the anoxic bulk soil of rice paddy microcosms. Appl. Environ. Microbiol. 65, 5042–5049.

Colleran, E., Pender, S., Philpott, U., O'Flaherty, V., Leahy, B., 1998. Full-scale and laboratory-scale anaerobic treatment of citric acid production wastewater. Biodegradation 9, 233–245.

Frank, D. N., St, Amand, A. L., Feldman, R. A., Boedeker, E. C., Harpaz, N., Pace, N. R., 2007. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc. Natl. Acad. Sci. USA 104, 13780–13785.

Hirakawa, K., 1998. Testing methods for industrial wastewater, JIS K0102-1998. Japanese Standards Association, Tokyo, Japan.

Ikeda, K., Matsushita, N., 2010. Thermal recycling for manufacturing process by producing biogas from shochu post distillation slurry. Cleanenergy 19, 61–68.

Kida, K., Shigematsu, T., Kijima, J., Numaguchi, M., Mochinaga, Y., Abe, N. et al., 2001. Influence of Ni^{2+} and Co^{2+} on methanogenic activity and the amounts of coenzymes involved in methanogenesis. J. Biosci. Bioeng. 91, 590–595.

Kida, K., Sonoda, Y., 1993. Influence of mineral nutrients on high performance during anaerobic treatment of distillery wastewater from barley-shochu making. J. Ferment. Bioeng. 75, 235–237.

Liu, J. J., Wu, W. X., Chen, C. J., Sun, F. Q., Chen, Y. X., 2011. Prokaryotic diversity, composition structure, and phylogenetic analysis of microbial communities in leachate sediment ecosystems. Appl. Microbiol. Biotechnol. 91, 1659–1675.

Liu, K., Tang, Y. Q., Matsui, T., Morimura, S., Wu, X. L., Kida, K., 2009. Thermophilic anaerobic co-digestion of garbage, screened swine and dairy cattle manure. J. Biosci. Bioengin. 107, 54–60.

Lueders, T., Friedrich, M. W., 2003. Evaluation of PCR amplification bias by terminal restriction fragment length polymorphism analysis of small-subunit rRNA and *mcrA* genes by using defined template mixtures of methanogenic pure cultures and soil DNA extracts. Appl. Environ. Microbiol. 69, 320–326.

McCarty, P. L., 1982. Anaerobic Digestion 1981, One Hundred Years of Anaerobic Treatment. Elsevier Biomedical Press B.V., Amsterdam. pp. 3.

Mokhtarani, N., Bayatfard, A., Mokhtarani, B., 2012. Full scale performance of compost's leachate treatment by biological anaerobic reactors. Waste Manage. Res. 30, 524–529.

Riviere, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J. et al., 2009. Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. ISME J. 3, 700–714.

Samejima, Y., 2003. Construction of the recycling system for shochu by-product. J. Brew. Soc. Japan, 98, 481–490.

Sasaki, D., Hori, T., Haruta, S., Ueno, Y., Ishii, M., Igarashi, Y., 2011. Methanogenic pathway and community structure in a thermophilic anaerobic digestion process of organic solid waste. J. Biosci. Bioeng. 111, 41–46.

Sasaki, K., Haruta, S., Ueno, Y., Ishii, M., Igarashi, Y., 2007. Microbial population in the biomass adhering to supporting material in a packed-bed reactor degrading organic solid waste. Appl. Microbiol. Biotechnol. 75, 941–952.

Schink, B., 1997. Energetics of syntrophic cooperation in methanogenic degradation. Microbiol. Mol. Biol. Rev. 61, 262–280.

Shigematsu, T., Era, S., Mizuno, Y., Ninomiya, K., Kamegawa, Y., Morimura, S. et al., 2006. Microbial community of a mesophilic propionate-degrading methanogenic consortium in chemostat cultivation analyzed based on 16S rRNA and acetate kinase genes. Appl. Microbiol. Biotechnol. 72, 401–415.

Shigematsu, T., Tang, Y. Q., Kawaguchi, H., Ninomiya, K., Kijima, J., Kobayashi, T. et al., 2003. Effect of dilution rate on structure of a mesophilic acetate degrading methanogenic community during continuous cultivation. J. Biosci. Bioeng. 96, 547–558.

Shigematsu, T., Tang, Y. Q., Kobayashi, T., Kawaguchi, H., Morimura, S., Kida, K., 2004. Effect of dilution rate on metabolic pathway shift between aceticlastic and nonaceticlastic methanogenesis in chemostat cultivation. Appl. Environ. Microbiol. 70, 4048–4052.

Slobodkin, A. I., Tourova, T. P., Kostrikina, N. A., Lysenko, A. M., German, K. E., Bonch-Osmolovskaya, E. A. et al., 2006. *Tepidimicrobium ferriphilum* gen. nov., sp. nov., a novel moderately thermophilic, Fe (III)-reducing bacterium of the order Clostridiales. Int. J. Syst. Evol. Microbiol. 56, 369–372.

Speece, R. E., Parkin, G. F., Bhattacharya, S., Takashima, M., 1986. Trace nutrient requirements of anaerobic digestion, in: Proceedings of the European Water Treatment, Industrial Presentations B. V. Schiedam, The Netherlands, pp. 177–188.

Takashima, M., Speece, R. E., Parkin, G. F., 1990. Mineral requirements for methane fermentation. Crit. Rev. Environ. Contr. 19, 465–479.

Tang, Y, Q., Shigematsu, T., Morimura, S., Kida, K., 2007b. Effect of dilution rate on the microbial structure of a mesophilic butyrate-degrading methanogenic community during continuous cultivation. Appl. Microbiol. Biotechnol. 75, 451–460.

Tang, Y. Q., Fujimura, Y., Shigematsu, T., Morimura, S., Kida, K., 2007a. Anaerobic treatment performance and microbial population of thermophilic upflow anaerobic filter reactor treating awamori distillery wastewater. J. Biosci. Bioeng. 104, 281–287.

Tang, Y. Q., Ji, P., Hayashi, J., Koike, Y., Wu, X. L., Kida, K., 2011. Characteristic microbial community of a dry thermophilic methanogenic digester: its long-term stability and change with feeding. Appl. Microbiol. Biotechnol. 91, 1447–1461.

Tang, Y. Q., Matsui, T., Morimura, S., Wu, X. L., Kida, K., 2008. Effect of temperature on microbial community of a glucose-degrading methanogenic consortium under hyperthemophilic chemostat cultivation. J. Biosci. Bioeng. 106, 180–187.

Tang, Y. Q., Shigematsu, T., Morimura, S., Kida, K., 2005. Microbial community analysis of mesophilic anaerobic protein degradation process using bovine serum albumin(BSA)-fed continuous cultivation. J. Bios. Bioeng. 99, 150–164.

Tatara, M., Makiuchi, T., Ueno, Y., Goto, M., Sode, K., 2008. Methanogenesis from acetate and propionate by thermophilic down-flow anaerobic packed-bed reactor. Bioresour. Technol. 99, 4786–4795.

Tatara, M., Yamazawa, A., Ueno, Y., Fukui, H., Goto, M., Sode, K., 2004. High-rate thermophilic methane fermentation on short-chain fatty acids in a down-flow anaerobic packed-bed reactor. Bioprocess Biosyst. Eng. 27, 105–113.

Togo, Y., Kato, H., Tatara, M., Takahashi, K., Moriyama, K., 2000. Japan Patent Kokai 2000-23656. 2000.01.25.

Westerholm, M., Roos, S., Schnrer, A., 2010. *Syntrophaceticus schinkii* gen. nov., sp. nov., an anaerobic, syntrophic acetate-oxidizing bacterium isolated from a mesophilic anaerobic filter. FEMS Microbiol. Lett. 309, 100–104.

Wrighton, K. C., Agbo, P., Warnecke, F., Weber, K. A., Brodie, E. L., DeSantis, T, Z. et al., 2008. A novel ecological role of the Firmicutes identified in thermophilic microbial fuel cells. ISME J. 2, 1146–1156.

Editorial Board of Journal of Environmental Sciences

JOURNAL OF ENVIRONMENTAL SCIENCES

环境科学学报(英文版)

(http://www.jesc.ac.cn)

Aims and scope

Journal of Environmental Sciences is an international academic journal supervised by Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences. The journal publishes original, peer-reviewed innovative research and valuable findings in environmental sciences. The types of articles published are research article, critical review, rapid communications, and special issues.

The scope of the journal embraces the treatment processes for natural groundwater, municipal, agricultural and industrial water and wastewaters; physical and chemical methods for limitation of pollutants emission into the atmospheric environment; chemical and biological and phytoremediation of contaminated soil; fate and transport of pollutants in environments; toxicological effects of terrorist chemical release on the natural environment and human health; development of environmental catalysts and materials.

For subscription to electronic edition

Elsevier is responsible for subscription of the journal. Please subscribe to the journal via http://www.elsevier.com/locate/jes.

For subscription to print edition

China: Please contact the customer service, Science Press, 16 Donghuangchenggen North Street, Beijing 100717, China. Tel: +86-10-64017032; E-mail: journal@mail.sciencep.com, or the local post office throughout China (domestic postcode: 2-580).

Outside China: Please order the journal from the Elsevier Customer Service Department at the Regional Sales Office nearest you.

Submission declaration

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis), that it is not under consideration for publication elsewhere. The submission should be approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out. If the manuscript accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

Submission declaration

Submission of the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis), that it is not under consideration for publication elsewhere. The publication should be approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out. If the manuscript accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

Editorial

Authors should submit manuscript online at http://www.jesc.ac.cn. In case of queries, please contact editorial office, Tel: +86-10-62920553, E-mail: jesc@263.net, jesc@rcees.ac.cn. Instruction to authors is available at http://www.jesc.ac.cn.

Journal of Environmental Sciences (Established in 1989)

Vol. 26 No. 2 2014

Supervised by	Chinese Academy of Sciences	**Published by**	Science Press, Beijing, China
Sponsored by	Research Center for Eco-Environmental		Elsevier Limited, The Netherlands
	Sciences, Chinese Academy of Sciences	**Distributed by**	
Edited by	Editorial Office of Journal of	Domestic	Science Press, 16 Donghuangchenggen
	Environmental Sciences		North Street, Beijing 100717, China
	P. O. Box 2871, Beijing 100085, China		Local Post Offices through China
	Tel: 86-10-62920553; http://www.jesc.ac.cn	Foreign	Elsevier Limited
	E-mail: jesc@263.net, jesc@rcees.ac.cn		http://www.elsevier.com/locate/jes
Editor-in-chief	Hongxiao Tang	**Printed by**	Beijing Beilin Printing House, 100083, China

CN 11-2629/X **Domestic postcode: 2-580** **Domestic price per issue RMB ¥ 110.00**

ISSN 1001-0742