Journal of Bioscience and Bioengineering
VOL. 122 No. 3, 334–340, 2016

www.elsevier.com/locate/jbiosc

Variation of the microbial community in thermophilic anaerobic digestion of pig manure mixed with different ratios of rice straw

Sheng Zhou,[1,*] Marcell Nikolausz,[2] Jining Zhang,[1] Shohei Riya,[3] Akihiko Terada,[3] and Masaaki Hosomi[3]

Eco-environmental Protection Research Institute, Shanghai Academy of Agricultural Sciences, No. 1000 Jinqi Road, Shanghai 201403, China,[1] Department of Environmental Microbiology, UFZ – Helmholtz Centre for Environmental Research, Permoserstraße 15, 04318 Leipzig, Germany,[2] and Institute of Engineering, Tokyo University of Agriculture and Technology, 2-24-16 Naka-cho, Koganei, Tokyo 184-8588, Japan[3]

Received 5 November 2015; accepted 23 February 2016
Available online 9 April 2016

The effect of pig manure mixed with rice straw on methane yield and the microbial community involved in a thermophilic (55°C) anaerobic digestion process was investigated. Three substrates composed of mixed pig manure and rice straw at different ratios (95:5; 78:22 and 65:35 w/w, which resulted in C/N ratios of 10:1, 20:1 and 30:1) were used for the experiment. The substrate type had a major influence on the total bacterial community, while the methanogens were less affected. The members of the class *Clostridia* (phylum *Firmicutes*) were predominant regardless of mixture ratio (C/N ratio), but at species level there was a major difference between the low and high C/N ratio samples. The hydrogenotrophic methanogenic genus of *Methanothermobacter* was predominant in all samples but higher C/N ratio sequences affiliated to the genus *Methanosarcina* were also detected. The appearance of *Methanosarcina* sp. is most likely due to the less inhibition of ammonia during the anaerobic digestion.

[**Key words:** Ammonia; Bacterial community; Thermophilic; Hydrogenotrophic methanogen; Terminal restriction fragment-length polymorphisms]

Anaerobic digestion is a promising technology that can be widely applied to the treatment of various types of waste, including animal manure (1). Digestion of some agricultural wastes, such as pig and chicken manure, as sole substrate is usually unsuccessful because of their high ammonium nitrogen concentrations (NH_4^+–N) and low C/N ratio (2,3). Although the N from ammonia is an essential nutrient for bacterial growth, high concentrations of ammonia might inhibit methanogenesis during anaerobic digestion processes and potentially inhibit anaerobic digestion (2,4,5). Inhibition indicates a detrimental effect that substance causes on the activity of a microbial population, which usually denotes an impairment of a particular bacterial function (6). In particular, high concentration of free ammonia is a powerful inhibitor in anaerobic digesters, and can easily cause process instability, which is indicated by a decrease in both biogas and methane yields, and can eventually lead to reactor failure. Therefore, control of ammonia concentrations in substrates and the digester enables safe and stable conditions for the anaerobic digestion of substrates such as manure. Hence, animal manure is preferably co-digested with organic wastes containing high amounts of carbon to improve the C/N ratio and to further increase biogas production (7,8). It has been shown that biogas production could be significantly enhanced using energy crops (maize and grass silage) or crop residues (corn stalks, oat straw, and wheat straw) as co-substrates of pig manure (9,10).

Compared with mesophilic digestion processes, thermophilic digestion achieves higher rates of digestion, greater conversion of organic waste to gas, and minimizes bacterial and viral pathogen accumulation (11). Thermophilic digestion has also been shown to more effectively during the start-up period in a high-solid anaerobic digestion process (12). Anaerobic digestion with high solid content is more beneficial than wet anaerobic digestion for compact reactors because the process has lower water content and higher methane production. In addition, this process produces less leachate, and the residues can be more easily treated by composting processes or used directly as organic fertilizer (13).

Thermophilic and high-solid anaerobic digestion processes differ in their biological and biochemical processes from conventional wet mesophilic anaerobic digestion. One essential problem associated with thermophilic anaerobic digestion is the impact of ammonia inhibition. The effect of ammonia inhibition on the microbial communities involved in anaerobic digestion was investigated by several recent studies using molecular biology techniques. The effect of various substrates on the microbial community structure and dynamics was investigated using amplicon sequencing and profiling by terminal restriction fragment-length polymorphisms (T-RFLP) of 16S rRNA genes of bacteria and archaea (14). Mono-digestion of nitrogen-rich chicken manure under partial inhibition of the whole anaerobic digestion process resulted in distinct and less diverse bacterial and methanogenic communities compared with communities involved in the digestion of conventional substrates (14). Most previous studies focused on analysis of methanogenic activity and mainly determined the sensitivity of acetoclastic methanogens (5). However, the

* Corresponding author. Tel.: +86 21 37195163; fax: +86 21 62202503.
E-mail address: zhous@outlook.com (S. Zhou).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2016.02.012

dominance of *Methanosarcina* and acetoclastic methanogenesis was observed using molecular and isotope tracing approaches in the case of a nonacclimatized thermophilic culture exposed to high ammonia levels (15). As an alternative to PCR-based investigation of the methanogenic communities, stable isotope fingerprinting of biogas was also applied to assess the predominant methanogenic pathways in reactors treating chicken manure together with maize silage (8). The stable isotope composition of the produced biogas indicated a complete shift towards hydrogenotrophic methanogenesis in one reactor before complete process failure, while methane production had stabilized in another reactor with the contribution of both main pathways even at very high ammonia levels.

There is little published data on anaerobic digestion of pig manure with rice straw under thermophilic and high solid conditions (9). In particular, there is little evidence of the effect of the ratio of pig manure to rice straw on the microbial community in a thermophilic anaerobic digestion process. In this study, the anaerobic co-digestion of pig manure and rice straw at different ratios was evaluated under thermophilic and high solid conditions. Characterization of community structures of bacteria and methanogenic archaea at different mixture ratios was performed using T-RFLP coupled with cloning and sequencing of 16S rRNA and *mcrA*/*mrtA* genes. Correlations between process parameters and community structures were studied by multivariate statistical analysis.

MATERIALS AND METHODS

Characterization of the biomass Inoculum, pig manure and rice straw were characterized by their total solid (TS), volatile solid (VS), total nitrogen (TN) and total carbon (TC) contents, as shown in Table 1. The inoculum used in this study was taken from a dry thermophilic anaerobic digestion pilot plant that treats solid garbage including kitchen garbage and office paper, which is run by Tokyo Gas and the Tokyo Environmental Public Service Corporation. To remove the degradable organic matter, the inoculum was incubated before the experiment without any added organic matter. The rice straw (*Oryza sativa* L. Takanari produced for forage) was chopped into 20-mm pieces, and then ground into small particles (Wonder Blender WB-1, Osaka Chemical Ltd. Co., Osaka, Japan), which were further sieved using 10-mesh.

Methane production assays Methane production assays were conducted as semi-continuous culture in triplicate with 500-mL Duran laboratory bottles under thermophilic (55°C) conditions. Three substrates composed of mixed pig manure and rice straw at different ratios (95:5, 78:22 and 65:35 w/w resulting in C/N ratios of 10:1, 20:1 and 30:1) were used for the experiment. For comparison, pure pig manure and rice straw treatments were also included. The treatments were named PM (pig manure alone), PF10, PF20, PF30, and FRS (rice straw alone). The water content of the substrate was adjusted to 73% with distilled water, except in the PF10 (86%) and PM (90%) treatments.

Eighty grams of the inoculum and 20 g of each type of substrate were added to the bottles on 26 January 2011. After mixing the inoculum and the substrate, the bottles were sealed with rubber stoppers and the headspace of the bottle was flushed with N_2 (flow rate: 1.0 L min^{-1}, 2 min). The degassed Tetra Pak bag was then connected to each bottle through a stopper to collect biogas to measure the volume of biogas produced by a syringe. Biogas collection was done approximately every week while digested residue was taken and then the same amount of substrate was added to the bottle. After sampling the residue and adding the substrate, the headspace of the bottle was flushed with N_2 again. The solid retention time (SRT) was 80 days. The total weight of the reactor remained constant over the course of the experiment. After approximate 80 days running for startup, the loading rate of substrate remained relatively stable from the beginning of April to the end of September 2011. The average rate was 2.5 g day^{-1} per bottle.

TABLE 1. Characterization of inoculum, pig manure and rice straw.

	Inoculum	Pig manure	Rice straw
Water content (%)	82.8	90.3	6.6
TS (%, w/w)	17.2	9.7	96.9
VS/TS (%, d/w)	54.7	82.9	84.9
TC (%, w/w)	5.28	4.35	35.1
TN (%, w/w)	0.35	0.59	0.28
C/N ratio	15.1	7.4	125
NH_4^+–N (mg kg^{-1})	1833	2567	–

Methane, NH_4^+, and VFA measurement The percentage of CH_4 and CO_2 in the collected biogas was measured using a GC-8A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a thermal conductivity detector and a 2-m stainless column packed with activated carbon (60/80-mesh). Methane production efficiency was calculated as the volume of methane produced per unit weight of VS substrate loaded.

Three samples of digested residue (wet weight 0.15 g per sample) were taken from each reactor every week and added to 2.0-mL centrifuge tubes. The pH of the samples was determined using a pH meter (Horiba, Kyoto, Japan). Then, 1.5 mL of Milli-Q water was added to the tube and mixed. After that, the sample was separated by centrifugation at 4°C and 15,000 rpm for 10 min. The concentration of NH_4^+–N was measured by ICS-1000 ion chromatography (Dionex, Sunnyvale, CA, USA) after the samples were centrifuged and filtered through a 0.45-μm glass fiber filter. The concentration was calculated per wet weight of each sample (mg-N kg^{-1}).

For VFA measurement, the stored supernatant was mixed with H_2SO_4 (10 mmol L^{-1}) at a ratio of 1:1 to maintain the concentration of the mobile phase. The mixture was then filtered through a 0.45-μm membrane Dismic filter (Advantec Toyo Kaisha, Ltd., Tokyo, Japan). Following that, the concentration of each organic acid was analyzed by HPLC (LC-10AD, Shimadzu) with a Differential Refractive Index Detector (RID) (Aminex HPX-87H column, 300 mm × 7.8 mm). The column temperature was 55°C and the injection volume was 20 μL H_2SO_4 (5 mmol L^{-1}) was used as the mobile phase and the flow rate was 0.7 mL min^{-1}.

DNA extraction and purification After stabilization of the anaerobic digestion process, samples from all digesters were collected into sterile 15-mL Falcon tubes immediately before the addition of the substrate and used immediately for DNA extraction on 17 May and 6 June 2011. Biomass was harvested by centrifugation of the samples at 20,000 × *g* for 10 min, and the total DNA was extracted and purified using a FastDNA SPIN Kit for soil (MP Biomedicals, Germany). DNA quantity and purity were determined photometrically using a NanoDrop ND-1000 UV/Vis spectral photometer (PeqLab, Germany) and by agarose gel electrophoresis.

PCR was carried out using the HotStarTaq Plus DNA Polymerase (Qiagen, Hilden, Germany) in 12.5 μL final volume with 0.5 μL isolated DNA. The forward primer *mlas* (5′-GGTGGTGTMGGDTTCACMCARTA-3′) and reverse primer *mcrA*-rev (5′-CGTTCATBGCGTAGTTVGGRTAGT-3′) were used for *mcrA*/*mrtA*-specific PCR amplification as described by Steinberg and Regan (16). Bacterial 16S rRNA gene fragments were amplified using the primers 27F and 1492R (17). The thermal cycling parameters were as follows: an initial denaturation at 94°C for 4 min, 30 cycles of 45 s at 94°C, 30 s at 51°C, 2 min at 72°C, followed by a final elongation at 72°C for 20 min.

PCR for subsequent T-RFLP analysis was conducted with FAM-labeled (phosphoramidite fluorochrome 6-carboxyfluorescein) reverse or forward primer in the case of *mcrA*/*mrtA* or 16S rRNA gene-specific PCR, respectively. PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide and visualized with UV excitation. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) and quantified photometrically using a NanoDrop ND-1000 UV/Vis spectral photometer (PeqLab).

T-RFLP analysis FAM-labeled bacterial 16S rRNA and *mcrA*/*mrtA* gene PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and quantified photometrically using a NanoDrop ND-1000 UV/Vis spectral photometer (PeqLab). Five ng of *mcrA*/*mrtA* and 20 ng of bacterial 16S rRNA gene PCR products were subsequently digested with the restriction endonucleases *Hae*III or *Msp*I (New England Biolabs, Schwalbach, Germany) in separate digestion reactions using ten units of the respective enzymes. Samples were incubated at 37°C overnight and then precipitated with 0.1 volumes of 3 M sodium acetate (pH 5.5) and 2.5 volumes of absolute ethanol. Dried DNA samples were re-suspended in 20 μL HiDi formamide containing 1.5% (v/v) GeneScan-500 ROX standard (Applied Biosystems) and were denatured at 95°C for 5 min and chilled on ice. Terminal restriction fragments (T-RFs) were separated on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems) with POP-7 polymer as described previously (18). Further analysis was performed using the GeneMapper V3.7 software (Applied Biosystems). Relative T-RF abundances were calculated by dividing the individual T-RF areas by the total peak areas. The lengths of the terminal fragments were determined using GeneMapper V3.7 software (Applied Biosystems) and fluorescent data for the range of 50–500 bp was exported to R script and peak areas were normalized and noise filtering was applied ($\sigma = 5$) according to Abdo and co-workers (19).

Multivariate statistical analysis was performed using the vegan package of R (http://cc.oulu.fi/~jarioksa/opetus/metodi/vegantutor.pdf) applying the Bray–Curtis dissimilarity index. Non-metric multidimensional scaling (NMDS) was used for visualization of the level of dissimilarities between bacterial community structures of investigated samples by optimizing the object locations for a two-dimensional scatterplot. The major abiotic process parameters correlating with community composition were fitted using the envfit algorithm. The significance of single process parameters on the NMDS results was tested using a Monte–Carlo test with 1000 permutations and only significant parameters are shown ($p < 0.05$).

Cloning and sequence analysis Cloning of purified non-labeled PCR products was carried out with Qiagen PCR Cloning Kit (Qiagen) according to the manufacturer's protocol. Vector-specific M13 primers were used to re-amplify the inserted 16S rRNA amplicons. Before sequencing, the PCR products were screened