J. Microbiol. Biotechnol. (2014), 24(11), 1464–1472
http://dx.doi.org/10.4014/jmb.1404.04043

jmb

Microbial Community Diversity in Anaerobic Reactors Digesting Turkey, Chicken, and Swine Wastes

Elvira E. Ziganshina[1], Dmitry E. Belostotskiy[2], Roman V. Shushlyaev[2], Vasili A. Miluykov[2], Petr Y. Vankov[1], and Ayrat M. Ziganshin[1*]

[1]*Department of Microbiology, Kazan (Volga Region) Federal University, Kazan 420008, Republic of Tatarstan, Russia*

[2]*Department of Technologies, A.E. Arbuzov Institute of Organic and Physical Chemistry, Russian Academy of Sciences, Kazan 420088, Republic of Tatarstan, Russia*

Received: April 24, 2014
Revised: June 11, 2014
Accepted: July 11, 2014

First published online
July 15, 2014

**Corresponding author*
Phone: +7-843-233-7872;
Fax: +7-843-238-7121;
E-mail: a.ziganshin06@fulbright-mail.org

pISSN 1017-7825, eISSN 1738-8872

Copyright© 2014 by
The Korean Society for Microbiology and Biotechnology

The microbial community structures of two continuous stirred tank reactors digesting turkey manure with pine wood shavings as well as chicken and swine manure were investigated. The reactor fed with chicken/swine wastes displayed the highest organic acids concentration (up to 15.2 g/l) and ammonia concentration (up to 3.7 g/l ammonium nitrogen) and generated a higher biogas yield (up to 366 ml/g_{VS}) compared with the reactor supplied with turkey wastes (1.5–1.8 g/l of organic acids and 1.6–1.7 g/l of ammonium levels; biogas yield was up to 195 ml/g_{VS}). The microbial community diversity was assessed using both sequencing and profiling terminal restriction fragment length polymorphisms of 16S rRNA genes. Additionally, methanogens were analyzed using methyl coenzyme M reductase alpha subunit (*mcrA*) genes. The bacterial community was dominated by members of unclassified Clostridiales with the prevalence of specific clostridial phylotypes in each reactor, indicating the effect of the substrate type on the community structure. Of the methanogenic archaea, methanogens of the genus *Methanosarcina* were found in high proportions in both reactors with specific methanosarcinas in each reactor, whereas the strict hydrogenotrophic methanogens of *Methanoculleus* sp. were found at significant levels only in the reactor fed with chicken/swine manure (based on the analyses of 16S rRNA gene). This suggests that among methanogenic archaea, *Methanosarcina* species which have different metabolic capabilities, including aceticlastic and hydrogenotrophic methanogenesis, were mainly involved in anaerobic digestion of turkey wastes.

Keywords: Anaerobic digestion, agricultural wastes, 16S rRNA genes, *mcrA* genes

Introduction

The livestock industry is the active sector with generation of large amounts of wastes. The existing ecological problems dictate the need for the development of safe and at the same time effective methods for the residual biomass disposal. Undoubtedly, one of such ways avoiding the accumulation of wastes in the environment is the anaerobic digestion of biomass with concomitant production of methane, and after the digestion process the effluent can be used as a fertilizer [2, 8, 15]. Anaerobic digestion of multifarious organic waste materials is also a promising way of reducing greenhouse gas emissions. In accordance with European objectives regarding the partial substitution of fossil fuels with alternative energy sources and limitation of global warming, the bioenergy production from renewable sources, including biogas, is among the main priorities [4]. However, in order to reduce gas emissions, it is also recommended to focus on digestates storage and exhaust of the co-generation [27].

Various agricultural and industrial waste materials can be anaerobically converted to energy-rich biogas by complex microbial consortia [8, 15, 29, 36]. The first three stages of anaerobic digestion – hydrolysis, acidogenesis and acetogenesis – are mediated by various functional groups of bacteria, whereas the last step – methanogenesis

– is performed by methanogenic archaea. Many research works in the field of biogas generation process are devoted to the investigation of the diversity and abundance of bacterial and archaeal communities, which play the key role in the anaerobic treatment of a variety of organic materials. Many different molecular techniques allow the reliable evaluation of biodiversity in anaerobic digesters as well as the monitoring of community shifts. Thus, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Synergistetes*, *Proteobacteria*, *Tenericutes*, *Spirochaetes*, and *Chloroflexi* as well as some other taxa are found as the abundant bacterial phyla in various anaerobic digesters. Of the *Euryarchaeota*, methanogens of the orders *Methanosarcinales*, *Methanomicrobiales*, *Methanobacteriales*, and *Methanococcales* are known to be present in different biogas reactors [23, 30−33, 38, 40, 43]. In addition, metaproteome analysis is also used to characterize microbial community changes in biogas reactors [19, 21]. Monitoring of the microbial community structure and dynamics during anaerobic treatment of biowastes can lead to the development of stable and adapted communities, what can finally increase the maximum methane yield.

Bioconversion of recalcitrant substrates (*e.g.*, lignocellulosic biomass, wood chips, and wood shavings used as the bedding materials) under anaerobic conditions in most cases is limited; what is required is the development and implementation of new technologies for the production of biogas from such materials [35, 42]. Enhancement of the anaerobic digestion process can be achieved through co-digestion of several substrates [15, 22, 36, 45]. Swine manure is often used as the feedstock for many large-scale biogas plants to produce methane for energy purposes. However, owing to the low carbon/nitrogen ratio (deficiency of carbon), the anaerobic treatment of swine manure individually is not effective for the full-scale production of methane [13, 37]. Poultry manure is a good source for biogas production; however, excess of ammonia in the biogas reactors treating poultry dung may represent a digestion inhibition risk as well [1, 31]. Co-digestion of poultry dung with some other livestock manures can improve the biogas production [18, 29].

Therefore, for the efficacious production of biogas, we have to deepen our knowledge in the area of anaerobic digestion process optimization. This involves the study of the main biotechnological parameters of biogas generation as well as the investigation of microbes responsible for the effective anaerobic transformation of distinct organic materials into methane. In this work, bacteria and methanogenic archaea of two continuous stirred tank reactors treating farm residues, namely turkey manure with pine wood shavings as well as chicken manure and swine manure, were investigated by sequencing and profiling terminal restriction fragment length polymorphisms of 16S rRNA genes. Additionally, methanogens were analyzed based on the *mcrA* (methyl coenzyme M reductase) gene profiles.

Materials and Methods

Performance of the Biogas Reactors and Analytical Methods

Mesophilic (38ºC) anaerobic experiments were carried out in two laboratory-scale continuous stirred tank reactors with a working volume of 10 L (Table 1). The reactor R.1 was supplied with wastes containing turkey manure and pine wood shavings, whereas the reactor R.2 was fed with chicken manure and swine manure. Turkey wastes were received from a poultry farm located in the Zelenodolsky district, Republic of Tatarstan. Chicken and swine wastes were obtained from poultry and pig farms located in

Table 1. Operating conditions and process parameters of the two laboratory-scale biogas reactors.

Reactor	Sample	Organic loading rate, g_{VS} l^{-1} day^{-1}	Inlet, g/day ($_{VS}$, g/day)				Biogas yield, ml/g_{VS}	Methane content, %	pH	Acid capacity, g/l	NH_4^+-N, g/l	Hydraulic retention time, days
			Turkey wastes	Chicken manure	Swine manure	Total						
R.1	1	2.60	71.5 (26.0)	–	–	360.5[a] (26.0)	193 ± 14	60.6 ± 0.6	7.90 ± 0.08	1.47 ± 0.09	1.71 ± 0.06	27.7
	2						195 ± 21	61.6 ± 0.5	7.91 ± 0.07	1.82 ± 0.11	1.70 ± 0.08	27.7
	3						189 ± 12	61.9 ± 0.6	7.92 ± 0.07	1.68 ± 0.09	1.63 ± 0.09	27.7
R.2	1	3.53	–	79.6 (29.0)	365.9 (6.3)	445.5 (35.3)	342 ± 37	56.3 ± 0.6	8.09 ± 0.07	15.2 ± 0.14	3.71 ± 0.12	22.4
	2						366 ± 41	56.8 ± 0.7	8.04 ± 0.06	14.8 ± 0.17	3.26 ± 0.14	22.4
	3						345 ± 32	58.1 ± 0.6	8.05 ± 0.06	13.9 ± 0.17	3.32 ± 0.17	22.4

Parameters at three distinct sampling times are presented (19 February 2013; 26 February 2013; 5 March 2013).

Biogas yield, methane content, and pH are shown as weekly mean values (3 days before and 3 days after sampling; n = 7). Acid capacity and NH_4^+-N data are presented as the average values of a sample measured three times (n = 3).

[a]Water was added to a final concentration of 360.5 g/day.

the Tukayevsky district, Republic of Tatarstan. Substrates were added to the digesters every day; digestates were removed from the reactors every day as well. Biogas yield, methane content, and pH were analyzed every day, while organic acids and NH_4^+-N levels were measured twice a week. All parameters were analyzed as described by us previously [43−46]. Briefly, biogas production was analyzed with milligascounters MGC-1 (Ritter, Germany), methane content was measured by a gas analyzer GA 94 (Ansyco, Germany), NH_4^+-N levels were analyzed with Nessler's reagent (Sigma-Aldrich, Germany) in a SPECOL 1300 spectrophotometer (Analytik Jena, Germany), and acid capacity was estimated by a titration method with 0.025−0.1 M H_2SO_4 in a pH of 4.5 and 3.5. Samples for microbial community analysis were taken on 19 February 2013 (day 72), 26 February 2013 (day 79), and 5 March 2013 (day 86).

Molecular Techniques

The total DNA was extracted and purified from digestates (about 250 µl) using a PowerSoil DNA Isolation Kit (MO BIO, USA). The eluted total DNA was checked by agarose gel electrophoresis and quantified with a NanoVue Plus UV-Vis spectrophotometer (GE Healthcare, USA).

Bacterial 16S rRNA gene fragments were PCR-amplified using the primers UniBac27F and Univ1492R; archaeal 16S rRNA gene fragments were amplified with the primers UniArc21F and UniArc931R; and *mcrA* genes were amplified using the primers mcrA-mlas and mcrA-rev as described previously [46]. For bacterial 16S rRNA genes, the cycle parameters were as follows: an initial denaturation at 94°C for 4 min, 30 cycles of 45 sec at 94°C, 1 min at 58°C, and 2 min at 72°C, with a final 20 min 72°C elongation step. The cycle parameters during archaeal 16S rRNA genes amplification were as described for the bacterial 16S rRNA genes but using an annealing temperature of 54°C. The *mcrA* genes were amplified with an initial denaturation at 95°C for 3 min; 5 cycles of 30 sec at 95°C, 45 sec at 48°C, 30 sec at 72°C; and 30 cycles of 30 sec at 95°C, 45 sec at 52°C, 30 sec at 72°C; followed by a 20 min final extension step at 72°C.

Amplicons were checked by 1.5% gel electrophoresis, purified with a QIAquick PCR Purification Kit (Qiagen, Germany), and cloned using the InsT/Aclone PCR Cloning Kit (Fermentas, Lithuania). Constructed clone libraries were screened by restriction analysis, and representative clones from large clusters were sequenced as described previously [44, 46]. The BLAST tool was then used for finding the similar sequences in the GenBank sequence database (http://www.ncbi.nlm.nih.gov/BLAST), the RDP Classifier (http://rdp.cme.msu.edu) was used for taxonomic assignment of the identified 16S rRNA fragments, and data were checked for chimeric sequences using Bellerophon (http://comp-bio.anu.edu.au/bellerophon/bellerophon. pl). The received sequences were deposited in the GenBank database under the accession numbers KJ004729–KJ004747.

T-RFLP analysis of 16S rRNA and *mcrA* amplicons was performed in accordance with our recent work [46]. Bacterial 16S rRNA genes were cleaved with the restriction endonucleases *Hae*III and *Rsa*I, archaeal 16S rRNA genes were digested with *Hae*III and *Mse*I, and *mcrA* genes were cut with *Hae*III and *Msp*I. T-RFLP was performed with three replicates for each restriction analysis to ensure reproducibility. Noise removal, peak binning, and normalization of signal intensity were done using an R script (R ver. 2.12.2; http://www.r-project.org).

Results and Discussion

Performance of the Reactors

Table 1 illustrates running conditions of the reactors, process parameters measured during anaerobic digestion of organic substrates, and the sampling times, when digestates were analyzed for prokaryotic community composition.

As can be seen in Table 1, the organic loading rate (OLR) and the hydraulic retention time (HRT) for the reactor R.1 fed with turkey wastes and water were constant between three sampling times and achieved 2.6 g_{VS} l^{-1} day^{-1} and 27.7 days, respectively. The biogas yield was in the range of 189–195 ml/g_{VS} with a methane content of ~61–62% under such conditions. The low biogas yield was probably because of the presence of pine wood shavings in turkey wastes, which were used as the bedding materials. They were not effectively hydrolyzed during the anaerobic digestion process in R.1. The pH of 7.9 was observed in this reactor, organic acids concentrations ranged between 1.5 and 1.8 g/l, and ammonium amounts achieved ~1.6–1.7 g/l values in digestates.

Co-digestion of chicken and swine manures (R.2) resulted in a higher biogas yield (342–366 ml/g_{VS}), but delivered biogas with a lower methane content of ~56–58% compared with the treatment operating with turkey wastes as the feedstock (R.1). The higher OLR value (3.53 g_{VS} l^{-1} day^{-1}) and the lower HRT (22.4 days) resulted in the accumulation of more organic acids (13.9–15.2 g/l) as well as in a higher ammonium concentration (3.3–3.7 g/l) in the reactor R.2 (Table 1). High amounts of organic acids detected in R.2 indicate that they were not efficiently funneled into methanogenesis in this reactor.

Bacterial Community Composition

The research presented herein describes the bacteria and archaea involved in the anaerobic digestion of complex organic substrates (turkey, chicken, and swine wastes) in the two laboratory-scale biogas reactors. The bacterial community composition and diversity were assessed using 16S rRNA gene-based clones libraries (192 clones were collected in total) and T-RFLP analysis at three distinct sampling times. The sequencing results of representative bacterial 16S rRNA gene clones, their taxonomic affiliation

Table 2. Sequencing results of representative gene clones and experimentally determined terminal restriction fragments (T-RF)

[a]OTU	Clone (bp)	Acc. No.	Closest affiliation* (Acc. No.) / % similarity	Taxonomic affiliation according to RDP 10	*Hae*III T-RF (bp)	*Rsa*I T-RF (bp)
OTU 1	bac_07 (468)	KJ004740	*Desulfotomaculum thermocisternum* strain DS2 (HM228398) / 86%	*Firmicutes*	158	312
OTU 2	bac_01 (505)	KJ004736	*Moorella thermoacetica* strain JCM 9320 (AB540984) / 84%	*Firmicutes*	258	501
OTU 3	bac_03 (471)	KJ004738	*Clostridium phytofermentans* ISDg (CP000885) / 89%	*Clostridiales*	85	423
OTU 4	bac_06 (215)	KJ004747	*Alkalibacter saccharofermentans* str. Z-79820 (NR_042834) / 94%	*Clostridiales*	239	470
OTU 5	bac_04 (466)	KJ004739	*Clostridiales* bacterium JN18_A89_K (DQ168653) / 86%	*Clostridiales*	267	-
OTU 6	bac_09 (479)	KJ004742	*Howardella ureilytica* strain GPC 589 (NR_044022) / 83%	*Clostridiales*	297	311
OTU 7	bac_08 (498)	KJ004741	*Moorella thermoacetica* strain JCM 9320 (AB540984) / 85%	*Clostridiales*	316	496
	bac_02 (503)	KJ004737	*Moorella thermoacetica* strain JCM 9320 (AB540984) / 85%	*Sporanaerobacter* sp.	316	496

[b]OTU	Clone (bp)	Acc. No.	Closest affiliation* (Acc. No.) / % similarity	Taxonomic affiliation according to RDP 10	*Hae*III T-RF (bp)	*Mse*I T-RF (bp)
OTU 1	ar_15 (691)	KJ004733	*Methanoculleus bourgensis* (AB065298) / 99%	*Methanoculleus* sp.	67	37
OTU 2	ar_11 (735)	KJ004729	*Methanosarcina barkeri* strain HWS2.1 (JN243319) / 98%	*Methanosarcina* sp.	220	556
	ar_14 (677)	KJ004732	*Methanosarcina barkeri* strain HWS2.1 (JN243319) / 98%	*Methanosarcina* sp.	220	556
	ar_16 (671)	KJ004734	*Methnosarcina siciliae* (FR733698) / 97%	*Methanosarcina* sp.	220	556
OTU 3	ar_12 (730)	KJ004730	*Methanosarcina barkeri* strain HWS2.1 (JN243319) / 100%	*Methanosarcina* sp.	220	857
	ar_13 (715)	KJ004731	*Methanosarcina barkeri* strain HWS2.1 (JN243319) / 100%	*Methanosarcina* sp.	220	857
	ar_17 (730)	KJ004735	*Methanosarcina barkeri* strain HWS2.1 (JN243319) / 99%	*Methanosarcina* sp.	220	857

[c]OTU	Clone (bp)	Acc. No.	Closest affiliation* (Acc. No.) / % similarity	Taxonomic affiliation	*Hae*III T-RF (bp)	*Msp*I T-RF (bp)
OTU 1	m_19 (490)	KJ004744	*Methanosarcina mazei* (ABO42325) / 98%	*Methanosarcina* sp.	125	214
	m_21 (490)	KJ004745	*Methanosarcina mazei* (ABO42325) / 97%	*Methanosarcina* sp.	125	214
	m_23 (490)	KJ004746	*Methanosarcina mazei* (ABO42325) / 98%	*Methanosarcina* sp.	125	214
OTU 2	m_18 (490)	KJ004743	*Methanosarcina mazei* (ABO42325) / 95%	*Methanosarcina* sp.	490	215

[a]Bacterial 16S rRNA gene clones.

[b]Archaeal 16S rRNA gene clones.

[c]*mcrA* gene clones.

*Uncultured/environmental sample sequences were excluded from the analyses.

in accordance with the RDP Classifier (confidence threshold of 50%) as well as experimentally determined terminal restriction fragments (T-RF) are presented in Table 2. According to the obtained sequencing results and T-RF values, the bacterial phylum *Firmicutes* dominated in our samples. The detected phylum comprised seven OTUs (based on the analysis of the clone library), among which representatives of the order *Clostridiales* dominated in the two laboratory-scale reactors. Thus, within bacteria, two OTUs were associated with unknown *Firmicutes* (OTUs 1 and 2) and five OTUs were affiliated with unknown Clostridiales (OTUs 3–7), including representatives of the genus *Sporanaerobacter*. In some other works, the class *Clostridia* was also detected as the dominant taxonomic group in different biogas reactors [17, 24].

In addition, the bacterial community diversity at three distinct sampling times was characterized by T-RFLP analysis using two different restriction enzymes, *Hae*III and *Rsa*I (Fig. 1 demonstrates only the results received with *Hae*III). The numbers of detected bacterial T-RFs in the reactor R.1 fed with turkey manure and pine wood shavings and the reactor R.2 co-digesting chicken and swine wastes were 9 and 7 for *Hae*III, accordingly. The bacterial community in R.1 operating at lower values of organic acids (between 1.5 and 1.8 g/l) and ammonium (in the range of 1.6–1.7 g/l) compared with R.2 was dominated by the fragments *Hae*III-239 (OTU 4) and *Hae*III-267 (OTU 5), whereas the fragment *Hae*III-297 (OTU 6) was found in high proportions in R.2, where accumulation of more organic acids (13.9–15.2 g/l) as well as a higher ammonium

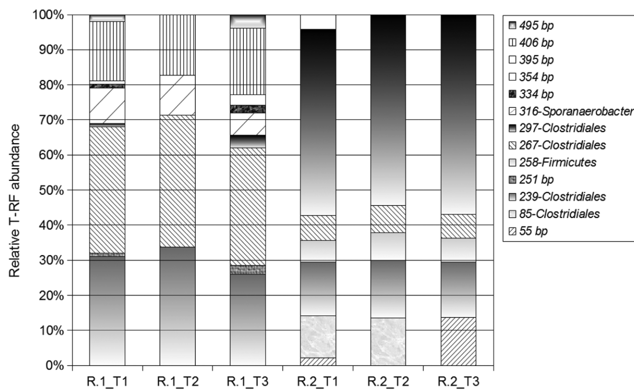

Fig. 1. Bacterial community structure and dynamics in the two anaerobic reactors at three distinct sampling times (T1, T2, and T3) in accordance with T-RFLP profiles of bacterial 16S rRNA amplicons digested with the restriction enzyme *Hae*III.

concentration (3.3–3.7 g/l) were detected (Fig. 1).

Bacterial 16S rRNA gene clone belonging to OTU 4 (clone KJ004747) was identified as a member of *Clostridiales* with a 99% similarity to uncultured clones Bac_SB_84 (JQ739074) from Lonar crater basalts (unpublished study) and SYNH02_C3-13B-042 (JQ245580) retrieved from a terrestrial mud volcano [9]. This gene clone also shared a 94% homology with a cultured obligate anaerobic alkaliphilic saccharolytic bacterium *Alkalibacter saccharofermentans* str. Z-79820 (NR_042834) [16]. OTU 4 was found to be one of the dominant taxonomic groups in the first reactor with the relative T-RF abundances of 26–34%, whereas its proportion in the second reactor did not exceed 15–16% of the total T-RF peak area throughout the experiments (Fig. 1). *A. saccharofermentans* was originally isolated as an obligately anaerobic alkaliphilic saccharolytic bacterium from the cellulolytic community of the alkaline Lake Nizhnee Beloe (Transbaikal region, Russia) [16]. This bacterium is able to ferment some sugars, with the generation of acetate, ethanol, formate, H_2, and CO_2; therefore, the main role of such clostridial phylotypes in our reactors is the supply of both aceticlastic and hydrogenotrophic methanogens with necessary compounds for biogas production. In both reactors, where the pH value was kept under weak alkaline conditions, we determined the presence of such alkaline-tolerant bacteria.

Another major group, representatives of which were also found in high proportions in our treatments, was the order Clostridiales with the T-RF 267 (OTU 5), comprising 33–38% of the total T-RF peak area in the reactor R.1, whereas this phylotype was assigned to non-dominant communities in the reactor R.2 (7–8%; Fig. 1). The fragment *Hae*III-267 (clone KJ004739) shared 95% homology with an uncultured *Clostridiales* bacterium clone A1435 (EU283551) (unpublished studies) and had only very low homology level of 86% to a cultured *Clostridiales* bacterium JN18_A89_K (DQ168653) [5].

The next major taxonomic groups detected only in the samples from R.1 treating turkey wastes were the phylotypes with the T-RFs 316 (clones KJ004737 and KJ004741) and 406. The fragment *Hae*III-316 (comprised 6–12% of the total T-RF peak area; Fig. 1) shared 99% rRNA identity with uncultured bacterial clones B043 (HG007923) and ATB-KS-1485 (EF686971), collected from reactors supplemented with mineral clay residual and maize silage/bovine manure, respectively (unpublished studies), and the fragment *Hae*III-316 was identified as *Sporanaerobacter* sp. using RDP Classifier. A representative of the genus *Sporanaerobacter*, *Sporanaerobacter acetigenes*, belongs to the strictly anaerobic, moderately thermophilic, and sporulating bacteria; it was firstly isolated from an upflow anaerobic sludge blanket reactor in Mexico [20]. It is a heterotrophic microorganism that is able to utilize some sugars, peptides, and distinct single amino acids, and can reduce elemental sulfur to hydrogen sulfide. *S. acetigenes* is able to ferment glucose with the accumulation of acetate, whereas fermentation of peptone occurs with the concomitant formation of acetate together with isobutyrate and isovalerate. A survey of the literature led to the conclusion that members of *Sporanaerobacter* sp. are commonly observed during anaerobic utilization of municipal wastes [39], activated sludge [10], and decomposition of entombed pigs [41] as important members of the bacterial community for destruction of the protein fraction of the complex substrates with the formation of volatile fatty acids (VFA). A high abundance of the T-RF 406 in the reactor R.1 was identified as well, but this phylotype could not be assigned taxonomically.

The fragment *Hae*III-297 (clone KJ004742) shared 99% similarity with various uncultured bacterial clones (*e.g.*, HQ727562, JN998141, and EF586010; unpublished studies) and showed BLAST identity of 83% with the obligately anaerobic non-fermentative *Howardella ureilytica* strain GPC 589 isolated from the rumen fluid of a sheep (NR_044022) [12]. It is essential to emphasize that members of bacterial community with the T-RF 297 (OTU 6) were prevalent only in the reactor R.2. This reactor was fed with chicken manure and swine manure and displayed the highest organic acids concentration (up to 15.2 g/l) and ammonia concentration (up to 3.7 g/l ammonium nitrogen) and generated a higher biogas yield (342–366 ml/g_{VS}) compared with R.1 (Table 1). Within all Firmicutes detected in our research, OTU 6 was the major group detected in all samples removed from R.2, and reached 57% of the total T-RF peak

area in the last sample; but it was assigned to non-dominant communities in R.1 (1–4%; Fig. 1). It can be assumed that this phylotype can tolerate the presence of high VFA and ammonium concentrations. In addition, some other members of the bacterial community with T-RFs 55, 85, and 258 were found to be unique for the reactor R.2 (Fig. 1).

Therefore, members of *Clostridia*, which are intensively involved in the hydrolysis stage, are the major acidogens and acetogens in our treatments providing aceticlastic and hydrogenotrophic methanogenic archaea with necessary compounds for biogas production. Furthermore, members of *Clostridia* could also be involved in syntrophic acetate oxidation (SAO) at high ammonia concentrations in our systems.

Archaeal Community Composition

The compositions of methanogenic archaea in the two laboratory-scale biogas reactors were examined using clone libraries and T-RFLP analyses targeting 16S rRNA and *mcrA* genes (a total of 192 clones were utilized, 96 clones for one gene). The coverage of the reactor biodiversity by the two genes analysis was compared. Table 2 shows the results obtained from the clones and T-RFLP analyses. The T-RFLP technique based on the use of *Mse*I and *Hae*III for archaeal 16S rRNA amplicons as well as based on the application of *Msp*I and *Hae*III for *mcrA* amplicons allowed detecting of the main participants in methanogenesis in the reactors treating turkey wastes and chicken/swine wastes. Figs. 2 and 3 demonstrate the methanogenic community diversity according to T-RFLP profiles of archaeal 16S rRNA amplicons digested with *Mse*I and *mcrA* amplicons digested with *Msp*I, respectively. Similar results were received with *Hae*III (not shown). The numbers of detected T-RFs for both genes in the reactor R.1 treating turkey manure and pine wood shavings and in the reactor R.2 digesting chicken manure and swine manure were comparable with the bacterial data (Figs. 1–3).

Based on the analyses of 16S rRNA gene T-RFLP profiles, members of *Methanosarcina* genus with the T-RF 857 predominated (OTU 3) in the archaeal communities of the reactor R.1 (57–73% of the total T-RF peak area), whereas the methanogens of the same genus with the T-RF 556 (OTU 2) dominated in the reactor R.2 (up to 65%). Furthermore, OTU 2 in the first reactor comprised 23–30% of the total T-RF peak area, whereas OTU 3 with the T-RF 857 in the second reactor was found only in minor proportions (5–7%). The phylotype with the T-RF 857 was also found in high proportions in the bioreactors treating cattle manure and maize straw (T-RFs 859/860 in Ziganshin *et al*. [43]). In addition, the phylotype with the T-RF 556 was specifically abundant in the reactors digesting *Jatropha* wastes and operating in the presence of high levels of ammonia (T-RFs 557/559 in Ziganshin *et al*. [43]). Interestingly, the phylotype of methanogenic community with the T-RF 37 (OTU 1) affiliated with hydrogenotrophic *Methanoculleus* genus was found at high levels (up to 22% of the total peak area) only in R.2; in R.1 its amount never exceeded 4% (Fig. 2).

All 16S rRNA gene clones of OTUs 2 and 3 (clones KJ004729, KJ004732, KJ004734, KJ004730, KJ004731, and KJ004735) were found to be highly similar (97–100%) to several uncultured clones and cultured species of *Methanosarcina*, which are aceticlastic, methylotrophic, and hydrogenotrophic

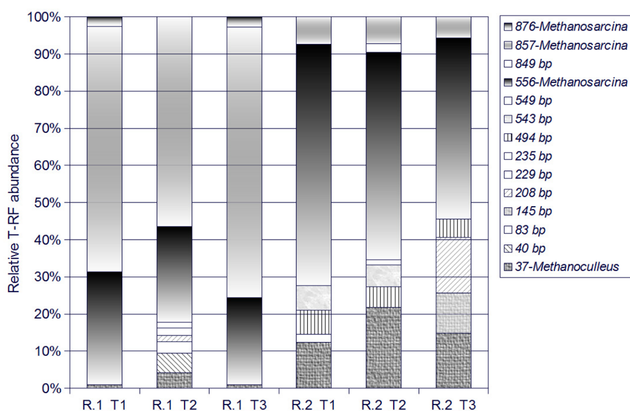

Fig. 2. Archaeal community structure and dynamics in the two anaerobic reactors at three distinct sampling times (T1, T2, and T3) in accordance with T-RFLP profiles of archaeal 16S rRNA amplicons digested with the restriction enzyme *Mse*I.

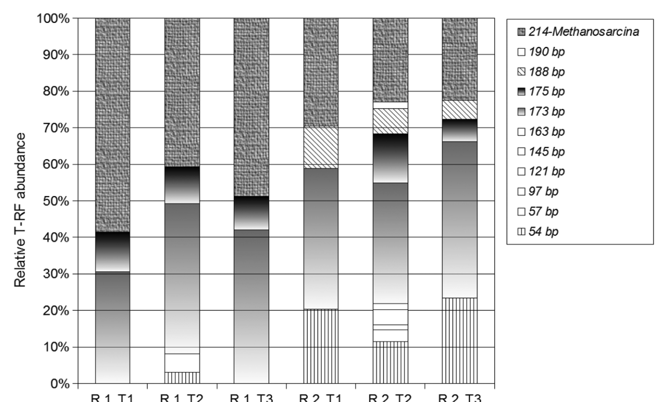

Fig. 3. Methanogenic community structure and dynamics in the two anaerobic reactors at three distinct sampling times (T1, T2, and T3) in accordance with T-RFLP profiles of *mcrA* amplicons digested with the restriction enzyme *Msp*I.

methanogens. Thus, the fragment *Mse*I-857 had a high BLAST identity (99–100%) to uncultured archaea clones (*e.g.*, KC412562, KC412621, and KC412628) [43] retrieved from biogas reactors fed with distinct agricultural waste materials, and shared 99–100% homology with cultured *Methanosarcina barkeri* [28], *Methanosarcina thermophila* [47], and *Methanosarcina siciliae* [14]. OTU 2 was highly similar (99%) to uncultured archaea clone A35_D28_L_A_A05 (EF552189) found in anaerobic solid waste digesters [26], clone 5.5ft C38A (EU369610) from a municipal landfill in Chandigarh, India [25], and clones DA158 (KC846049) and DA126 (KC846046) retrieved from anaerobic sewage sludge (unpublished study). Moreover, OTU 2 shared 97–98% similarity with the same cultured *M. barkeri*, *M. thermophila*, and *M. siciliae* as well as with *Methanosarcina acetivorans* [34]. *M. barkeri* and *M. thermophila* can produce methane from acetate, H_2/CO_2, methanol, and methylated amines, whereas *M. siciliae* and *M. acetivorans* can produce methane from acetate, methanol, and methylamines.

OTU 1 (clone KJ004733) showed a high BLAST identity (99%) to uncultured archaea clone DA71 (KC846039) found in anaerobic sewage sludge (unpublished study), clone AL4_6 (JX101973) from an anaerobic reactor for the treatment of municipal solid waste [7], and shared 99% similarity with a cultured *Methanoculleus bourgensis* (AB065298) [3]. *M. bourgensis* uses H_2/CO_2, formate, and some secondary alcohols as methanogenic substrates. *Methanoculleus* sp. is commonly observed in different anaerobic reactors [17, 24, 43].

As can be seen in Fig. 3, the methanogenic community based on the *mcrA* gene T-RFLP profiles was also represented by phylotypes of the genus *Methanosarcina* (T-RFs 214 and 215). The fragments *Msp*I-214 (OTU 1; clones KJ004744, KJ004745, and KJ004746) and *Msp*I-215 (OTU 2; clone KJ004743) had 97–98% similarity to an uncultured methanogenic archaeon (CAN99777) [11] and shared 95–98% homology with a cultured *Methanosarcina mazei* [6]. In addition, the methanogens with the T-RFs 173–175 were also defined between T-RFLP profiles at high abundances in both reactors. Despite the fact that they were not assigned taxonomically based on the clone library constructed in this research, probably they belonged to *Methanosarcina* and/or *Methanoculleus* genera, as was shown in our previous work during analysis of a microbial community in a pilot-scale anaerobic reactor fed with cattle manure and plant biomass [46].

As has been mentioned earlier, the reactor R.1 was dominated almost completely by the representatives of *Methanosarcina* sp., whereas the strict hydrogenotrophic methanogenic archaea did not exceed 4% from the total T-RF peak area in this reactor (based on the analyses of 16S rRNA gene). Recently, we showed that a reactor R 3.1 utilizing chicken and cattle manure operated in the presence of high concentrations of VFA and ammonium, and the methanogenic community consisted almost completely of two phylotypes associated with the hydrogenotrophic genus *Methanoculleus*, indicating that anaerobic digestion of these wastes relied on syntrophic acetate oxidation as the dominant acetate-consuming process because of the inhibition of the aceticlastic methanogens [43]. In the research described herein, we show that the methanogenic community in R.2 fed with chicken and swine manure and operated almost under the same conditions, with the exception of differences in ammonium concentrations, was represented by dominant methanosarcinas and by minor strict hydrogenotrophic methanogens indicating the possibility of SAO in this reactor also but with *Methanoculleus* and *Methanosarcina* spp. as hydrogen-consuming partners. The absence of strict aceticlastic *Methanosaeta* species in our samples might be explained by the presence of a high concentration of ammonia and VFA [23].

The interaction of bacteria and methanogenic archaea, which are involved in the anaerobic microbial process of complex molecules biodegradation with a mixture of CH_4 and CO_2 formation, is coordinated by environmental and internal factors. A clear understanding of the structure and behavior of such complex communities, including the relationship between consortium representatives and the ability to adapt and respond to a variety of factors, is one of the major goals of current microbiology and biotechnology. Modern molecular biology techniques allow not only the evaluation of the diversity of microbial communities, but also the determination of the relationships between various members of the diverse communities as well as the investigation of environmental factors affecting the biotransformation processes. In practice, the results of such studies will help to optimize the anaerobic digestion process with an increased methane yield.

In conclusion, the research described herein shows the ecological structure of bacterial and methanogenic communities responsible for anaerobic digestion of the agricultural industry wastes, namely turkey (in R.1) and chicken/swine wastes (in R.2). Bacterial and archaeal 16S rRNA genes as well as methanogen *mcrA* genes served as markers for the estimation of microbes. Phylogenetic and functional genes diversity was investigated based on the clone libraries and T-RFLP analysis. The conclusion drawn from the received results is that representatives of the order *Clostridiales* intensively involved in hydrolysis and acidogenesis stages

were found with a remarkably high prevalence in the two reactors; namely, members with the T-RFs 239 and 267 in the reactor R.1 and T-RF 297 in the reactor R.2. Each reactor was dominated by the special group of the class *Clostridia*, indicating the effect of the substrate type on the community structure. In addition, members of *Clostridia* could also be involved in SAO at high ammonia levels in our systems. As for methanogenesis, it was efficiently performed by the methanogens of the *Methanosarcina* sp. in both reactors. Based on the analyses of archaeal 16S rRNA gene T-RFLP profiles, differences between the methanogenic compositions of the two laboratory-scale reactors were observed in the proportions of dominant methanogenic species within the *Methanosarcina* genus. In addition, *Methanoculleus* methanogens that have strict hydrogenotrophic metabolism were presented at significant levels only in the reactor R.2.

Acknowledgments

This work was performed according to the Russian Government Program of Competitive Growth of Kazan Federal University. Partial financial support was provided by the Russian Foundation for Basic Research, Grant No. 14-08-31768. Lastly, the insightful comments received during the review process are gratefully acknowledged.

References

1. Abouelenien F, Fujiwara W, Namba Y, Kosseva M, Nishio N, Nakashimada Y. 2010. Improved methane fermentation of chicken manure *via* ammonia removal by biogas recycle. *Bioresour. Technol.* **101:** 6368-6373.
2. Antoni D, Zverlov VV, Schwarz WH. 2007. Biofuels from microbes. *Appl. Microbiol. Biotechnol.* **77:** 23-35.
3. Asakawa S, Nagaoka K. 2003. *Methanoculleus bourgensis*, *Methanoculleus olentangyi* and *Methanoculleus oldenburgensisare* subjective synonyms. *Int. J. Syst. Evol. Microbiol.* **53:** 1551-1552.
4. Bacenetti J, Negri M, Fiala M, González-García S. 2013. Anaerobic digestion of different feedstocks: impact on energetic and environmental balances of biogas process. *Sci. Total Environ.* **463-464:** 541-551.
5. Bedard DL, Bailey JJ, Reiss BL, Jerzak GV. 2006. Development and characterization of stable sediment-free anaerobic bacterial enrichment cultures that dechlorinate aroclor 1260. *Appl. Environ. Microbiol.* **72:** 2460-2470.
6. Blotevogel KH, Fischer U. 1989. Transfer of *Methanococcus frisius* to the genus *Methanosarcina* as *Methanosarcina frisia* comb. nov. *Int. J. Syst. Bacteriol.* **39:** 91-92.
7. Cardinali-Rezende J, Colturato LF, Colturato TD, Chartone-Souza E, Nascimento AM, Sanz JL. 2012. Prokaryotic diversity and dynamics in a full-scale municipal solid waste anaerobic reactor from start-up to steady-state conditions. *Bioresour. Technol.* **119:** 373-383.
8. Chamy R, Vivanco E, Ramos C. 2011. Anaerobic mono-digestion of turkey manure: efficient revaluation to obtain methane and soil conditioner. *J. Water Resource Protect.* **3:** 584-589.
9. Cheng TW, Chang YH, Tang SL, Tseng CH, Chiang PW, Chang KT, *et al*. 2012. Metabolic stratification driven by surface and subsurface interactions in a terrestrial mud volcano. *ISME J.* **6:** 2280-2290.
10. Cirne DG, Bond P, Pratt S, Lant P, Batstone DJ. 2012. Microbial community analysis during continuous fermentation of thermally hydrolysed waste activated sludge. *Water Sci. Technol.* **65:** 7-14.
11. Conrad R, Klose M, Noll M, Kemnitz D, Bodelier PLE. 2008. Soil type links microbial colonization of rice roots to methane emission. *Glob. Chang. Biol.* **14:** 657-669.
12. Cook AR, Riley PW, Murdoch H, Evans PN, McDonald IR. 2007. *Howardella ureilytica* gen. nov., sp. nov., a gram-positive, coccoid-shaped bacterium from a sheep rumen. *Int. J. Syst. Evol. Microbiol.* **57:** 2940-2945.
13. Cuetos MJ, Fernández C, Gómez X, Morán A. 2012. Anaerobic co-digestion of swine manure with energy crop residues. *Biotechnol. Bioprocess Eng.* **16:** 1044-1052.
14. Elberson MA, Sowers KR. 1997. Isolation of an aceticlastic strain of *Methanosarcina siciliae* from marine canyon sediments and emendation of the species description for *Methanosarcina siciliae*. *Int. J. Syst. Bacteriol.* **47:** 1258-1261.
15. El-Mashad HM, Zhang R. 2010. Biogas production from co-digestion of dairy manure and food waste. *Bioresour. Technol.* **101:** 4021-4028.
16. Garnova ES, Zhilina TN, Tourova TP, Kostrikina NA, Zavarzin GA. 2004. Anaerobic, alkaliphilic, saccharolytic bacterium *Alkalibacter saccharofermentans* gen. nov., sp. nov. from a soda lake in the Transbaikal region of Russia. *Extremophiles* **8:** 309-316.
17. Goberna M, Insam H, Franke-Whittle IH. 2009. Effect of biowaste sludge maturation on the diversity of thermophilic bacteria and archaea in an anaerobic reactor. *Appl. Environ. Microbiol.* **75:** 2566-2572.
18. Güngör-Demirci G, Demirer GN. 2004. Effect of initial COD concentration, nutrient addition, temperature and microbial acclimation on anaerobic treatability of broiler and cattle manure. *Bioresour. Technol.* **93:** 109-117.
19. Hanreich A, Heyer R, Benndorf D, Rapp E, Pioch M, Reichl U, Klocke M. 2012. Metaproteome analysis to determine the metabolically active part of a thermophilic microbial community producing biogas from agricultural biomass. *Can. J. Microbiol.* **58:** 917-922.
20. Hernandez-Eugenio G, Fardeau ML, Cayol JL, Patel BK, Thomas P, Macarie H, *et al*. 2002. *Sporanaerobacter acetigenes* gen. nov., sp. nov., a novel acetogenic, facultatively sulfur-reducing bacterium. *Int. J. Syst. Evol. Microbiol.* **52:** 1217-1223.
21. Heyer R, Kohrs F, Benndorf D, Rapp E, Kausmann R,

Heiermann M, *et al*. 2013. Metaproteome analysis of the microbial communities in agricultural biogas plants. *N. Biotechnol.* **30:** 614-622.

22. Holm-Nielsen JB, Al Seadi T, Oleskowicz-Popiel P. 2009. The future of anaerobic digestion and biogas utilization. *Bioresour. Technol.* **100:** 5478-5484.
23. Karakashev D, Batstone DJ, Angelidaki I. 2005. Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors. *Appl. Environ. Microbiol.* **71:** 331-338.
24. Krause L, Diaz NN, Edwards RA, Gartemann KH, Krömeke H, Neuweger H, *et al*. 2008. Taxonomic composition and gene content of a methane-producing microbial community isolated from a biogas reactor. *J. Biotechnol.* **136:** 91-101.
25. Krishnamurthi S, Chakrabarti T. 2013. Diversity of bacteria and archaea from a landfill in Chandigarh, India as revealed by culture-dependent and culture-independent molecular approaches. *Syst. Appl. Microbiol.* **36:** 56-68.
26. Li T, Mazéas L, Sghir A, Leblon G, Bouchez T. 2009. Insights into networks of functional microbes catalysing methanization of cellulose under mesophilic conditions. *Environ. Microbiol.* **11:** 889-904.
27. Liebetrau J, Reinelt T, Clemens J, Hafermann C, Friehe J, Weiland P. 2013. Analysis of greenhouse gas emissions from 10 biogas plants within the agricultural sector. *Water Sci. Technol.* **67:** 1370-1379.
28. Maestrojuan GM, Boone DR. 1991. Characterization of *Methanosarcina barkeri* MS^T and 227, *Methanosarcina mazei* S-6^T, and *Methanosarcina vacuolata* Z-761^T. *Int. J. Syst. Bacteriol.* **41:** 267-274.
29. Magbanua BS, Adams TT, Johnston P. 2001. Anaerobic co-digestion of hog and poultry waste. *Bioresour. Technol.* 76: 165-168.
30. Merlino G, Rizzi A, Schievano A, Tenca A, Scaglia B, Oberti R, *et al*. 2013. Microbial community structure and dynamics in two-stage *vs* single-stage thermophilic anaerobic digestion of mixed swine slurry and market bio-waste. *Water Res.* **47:** 1983-1995.
31. Niu Q, Qiao W, Qiang H, Li YY. 2013. Microbial community shifts and biogas conversion computation during steady, inhibited and recovered stages of thermophilic methane fermentation on chicken manure with a wide variation of ammonia. *Bioresour. Technol.* **146:** 223-233.
32. Qiao JT, Qiu YL, Yuan XZ, Shi XS, Xu XH, Guo RB. 2013. Molecular characterization of bacterial and archaeal communities in a full-scale anaerobic reactor treating corn straw. *Bioresour. Technol.* **143:** 512-518.
33. Smith AM, Sharma D, Lappin-Scott H, Burton S, Huber DH. 2013. Microbial community structure of a pilot-scale thermophilic anaerobic digester treating poultry litter. *Appl. Microbiol. Biotechnol.* **98:** 2321-2334.
34. Sowers KR, Baron SF, Ferry JG. 1984. *Methanosarcina acetivorans* sp. nov., an acetotrophic methane-producing bacterium isolated from marine sediments. *Appl. Environ. Microbiol.* **47:** 971-978.
35. Tuesorn S, Wongwilaiwalin S, Champreda V, Leethochawalit M, Nopharatana A, Techkarnjanaruk S, Chaiprasert P. 2013. Enhancement of biogas production from swine manure by a lignocellulolytic microbial consortium. *Bioresour. Technol.* **144:** 579-586.
36. Wan S, Sun L, Douieb Y, Sun J, Luo W. 2013. Anaerobic digestion of municipal solid waste composed of food waste, wastepaper, and plastic in a single-stage system: performance and microbial community structure characterization. *Bioresour. Technol.* **146:** 619-627.
37. Wang G, Gavala HN, Skiadas IV, Ahring BK. 2009. Wet explosion of wheat straw and codigestion with swine manure: effect on the methane productivity. *Waste Manag.* **29:** 2830-2835.
38. Wang W, Xie L, Luo G, Zhou Q, Angelidaki I. 2013. Performance and microbial community analysis of the anaerobic reactor with coke oven gas biomethanation and *in situ* biogas upgrading. *Bioresour. Technol.* **146:** 234-239.
39. Weiss A, Jérôme V, Freitag R, Mayer HK. 2008. Diversity of the resident microbiota in a thermophilic municipal biogas plant. *Appl. Microbiol. Biotechnol.* **81:** 163-173.
40. Wirth R, Kovács E, Maróti G, Bagi Z, Rákhely G, Kovács KL. 2012. Characterization of a biogas-producing microbial community by short-read next generation DNA sequencing. *Biotechnol. Biofuels* **5:** 41-57.
41. Yang SH, Hong SH, Cho SB, Lim JS, Bae SE, Ahn H, Lee EY. 2012. Characterization of microbial community in the leachate associated with the decomposition of entombed pigs. *J. Microbiol. Biotechnol.* **22:** 1330-1335.
42. Yue ZB, Li WW, Yu HQ. 2013. Application of rumen microorganisms for anaerobic bioconversion of lignocellulosic biomass. *Bioresour. Technol.* **128:** 738-744.
43. Ziganshin AM, Liebetrau J, Pröter J, Kleinsteuber S. 2013. Microbial community structure and dynamics during anaerobic digestion of various agricultural waste materials. *Appl. Microbiol. Biotechnol.* **97:** 5161-5174.
44. Ziganshin AM, Schmidt T, Scholwin F, Ilinskaya ON, Harms H, Kleinsteuber S. 2011. Bacteria and archaea involved in anaerobic digestion of distillers grains with solubles. *Appl. Microbiol. Biotechnol.* **89:** 2039-2052.
45. Ziganshin AM, Ziganshina EE, Pröter J, Kleinsteuber S, Ilinskaya ON. 2012. Methanogenic community dynamics during anaerobic utilization of agricultural wastes. *Acta Naturae* **4:** 91-97.
46. Ziganshina EE, Bagmanova AR, Khilyas IV, Ziganshin AM. 2014. Assessment of a biogas-generating microbial community diversity in a pilot-scale anaerobic reactor. *J. Biosci. Bioeng.* **117:** 730-736.
47. Zinder SH, Sowers KR, Ferry JG. 1985. *Methanosarcina thermophila* sp. nov., a thermophilic, acetotrophic, methane-producing bacterium. *Int. J. Syst. Bacteriol.* **35:** 522-523.