ATLANTIS PRESS

Advances in Biological Sciences Research, volume 7

1st International Symposium Innovations in Life Sciences (ISILS 2019)

Microbial Community of Biogas Plant Feeding with Complex Substrate: Archaea/Bacteria Ratio Dynamics by the Stages of Fermentation

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***Abstract*—The technology of anaerobic fermentation of organic substrates and especially of organic wastes with rich microbiota needs effective method to observe the microbial community on the different stages of substrate transformation, including its digestion, fermentation and conversion of its products to methane. The fast and cheap method based on taxon-specific RT PCR is developed to assess density of *Archaea* and *Bacteria* population in complex microbial communities. The data obtained on the samples from the industrial biogas plant on the different technological stages are consistent with processes that should evolve during fermentation.**

Keywords—microbiota, microbial communities, RT PCR, biomethanation, biogas

I. INTRODUCTION

Biogas production is a promising field of alternative energy with substantial economic [1] and ecological [2] potential. Efficiency of biomethanation process totally depends on the structure of the microbiota that performs decomposition of substrates, their fermentation and generation of methane [3, 4]. Complexity and dynamical behavior of microbial communities involved in the anaerobic fermentation technology attracts attention of researchers especially for last decade [5-8].

Due to economic reasons the substrate composition for large biogas plant is really unique and varies throughout the year. It makes impossible to elaborate the universal technological model to govern the parameters of biomethanation process. More, the response of microbial community to some feeding and conditional changes may pass some bifurcation points and be influenced by random fluctuations [9]. So, continuous optimization of the process needs regular analysis of chemical and biological parameters of the fermenting mix and effluent.

Bacteria and *Archaea* play different roles in biomethanation process and their quantities are important parameter to govern it [10, 11]. The aim of our work is to elaborate the fast and cheap method for quantification of *Archaea* and *Bacteria* in complex microbial communities. The common approach implies data standardization using cloned 16S rRNA genes of some representatives of the taxa that are to assess [12]. The same time the data observed remain still method-dependent because of unequal binding capacity of primers chosen to target genes of different representatives of a taxon leading to biases in the resulting data. So, for practical purposes it is of sense to use standardization by such widely used DNA sequence as lambda phage DNA. This way is easier, cheaper and more universal. In the present study we have tested it.

For quantification of the data, the simple equation was formulated based on the equal fluorescence of samples on the chosen threshold line (on the line generated by the softwear of the equipment). It depends on the cycle quantity (Cq) at the chosen matrix dilution, amplification factor and the amplicon length, effecting dye binding capacity.

II. EXPERIMENTAL

To achieve the aim of the work RT PCR technology was used. We employed three primer pairs specific to 16S rRNA genes of *Bacteria* and *Archaea* (from literature [13]) and to lambda phage DNA (based on its genome sequence data) (fig. 1). All three primer pairs showed good amplification factors (close to 2) when use the common annealing temperature 60 ºC (data not shown). Primer pairs specific to the 16S rRNA genes of *Bacteria* and *Archaea* contained one or two degenerate positions necessary to ensure maximum coverage of sequences belonging to these taxa.

Target taxon	Primer name	Primer sequence	Melting temperature used	Amplicon length
Bacteria	E1052f E1193r	TGCATGGYTGTCGTCAGCTCG CGTCRTCCCCRCCTTCC	60	141
Archaea	A967f A1060r	AATTGGCGGGGGAGCAC GGCCATGCACCWCCTCTC	60	101
λ-phage	L13915-F L14061-R	TTTCCGGGACGTATCATGCT ACCGCTCAGGCATTTGCTG	60	147

Fig. 1. Primer pairs used in the research.

311.24 pM lambda phage DNA was added to each 150 µl sample of fermenting mix and effluent prior to microbiota DNA isolation to enable quantification of bacterial and archaeal 16S rRNA genes despite occasional levels of DNA losses. DNA was isolated using diaGene DNA extraction kit (Dia-M, Russia). Reaction mixture for RT PCR contained 2.5x Reaction Mix with SYBR Green I (Syntol, Russia), 5, 0.5 or 0.05 µg/ml of template DNA and 0.3 µM of each primer. The amplification program included initial denaturing step at 95 ºC for 5 min and 40 cycles of three steps at 95, 60 and 72 ºC for 20 s each.

Quantification of bacterial and archaeal 16S rRNA genes was carried out using the formula 1 based on the assumption of equality of lambda phage and bacterial/archaeal amplicon DNA mass amounts in the fluorescence threshold points. It is necessary to take into account the length of amplicons, since it is proportional to the number of fluorescent dye molecules binding to them.

$$C_1 = C_\lambda \frac{A_\lambda^{Cq_\lambda} L_\lambda}{A_1^{Cq_1} L_1} \quad (1)$$

The known concentration of starting lambda DNA added prior to processing of samples was used for quantity determination of the taxon of interest by formula 1. C means molar concentration of recognizable sequences on the template DNA, L – amplicon length, A – amplification factor, Cq – amplification cycle quantity needed to achieve the fluorescence intensity threshold for the chosen template dilution. Index λ corresponds standard DNA (lambda phage), index 1 – DNA to be analyzed.

Amplification factors for three primer pairs were calculated on the base of three 10-fold template dilutions used in triplicate. Despite the presence of DNA recognizable by the primers for lambda phage in the control DNA preparations without its addition, amount of such sequences was negligible compared with amount of lambda phage DNA added to the main preparations and could not influence the results.

The biogas plant “Luchki” (AltEnergo L.L.C.) is situated in the north of Belgorod oblast (Russia). Its architecture consists of the four main tanks with mixing and thermostat facilities. Fermenting mix from tanks 1 and 2 enters the tank 3, than the tank 4 and than is discarded and utilized (fig. 2). Tanks 1 and 2 are loaded with complex mix of swine manure, meat waste, silage, sugar beet pulp and other organic substrates. Operating temperature is 39 ºC.

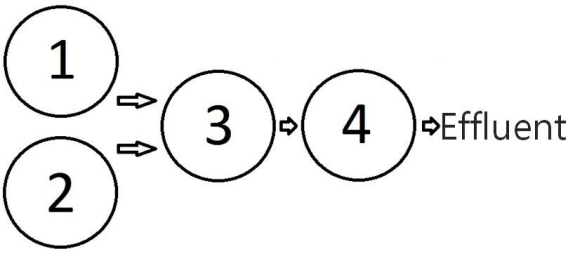

Fig. 2. The flow direction of the fermentation mixture between the four tanks at the biogas plant "luchki" during sampling.

III. RESULTS AND DISCUSSION

Molar concentrations of 16S rRNA genes of *Bacteria* and *Archaea* that were determined in the present study are shown in table I and in fig. 3. The most dense bacterial population we detected in the tanks 2 and 3. This should point the most intense processes of substrate decomposition and fermentation. The highest amount of *Archaea* is observed in the tank 3. Here we should observe the most active methanogenesis processes. The lowest levels of both *Bacteria* and *Archaea* are in the tank 4 and in effluent poured out from it.

The level of *Bacteria* varies from 615 pM of their 16S rRNA genes to 1.5 nM. *Archaea* level in all the cases is much lower and counts from 10 to 125 pM. *Archaea* percentages are from 1.6 to 8.3 % showing numeral dominance of *Bacteria*. These results are consistent with previous studies [5-8] and reflect biochemical balance of syntrophyc cooperation in methanogenic microbial communities [14]. In general, the processing steps of the substrate preceding the release of methane lead to the extraction of most of the energy from the compounds contained therein. Accordingly, when they occur, the largest part of the biomass of microorganisms present in the reactor is formed. Thus, at all technological stages of substrate fermentation, the biomass of bacteria, and, accordingly, their genomic DNA, dominates. This seems to be quite expected, given that the main direct precursors of methane, namely acetic acid, hydrogen and carbon dioxide [10], are the products of fermentation of those energy-rich compounds that are in the substrate, and only the waste of these processes get *Archaea* by syntrophic interaction with *Bacteria*.

TABLE I. MOLAR CONCENTRATIONS OF BACTERIAL AND ARCHAEAL 16S RRNA GENES IN THE SAMPLES FROM DIFFERENT TANKS AND EFFLUENT OF BIOGAS PLANT “LUCHKI” AND THE PERCENTAGES OF ARCHEAL ONES

	Tank 1	**Tank 2**	**Tank 3**	**Tank 4**	**Effluent**
Bacteria, pM	776	1 571	1 461	615	634
Archaea, pM	71	32	125	10	17
Archaea, %	8.3	2.0	7.9	1.6	2.6

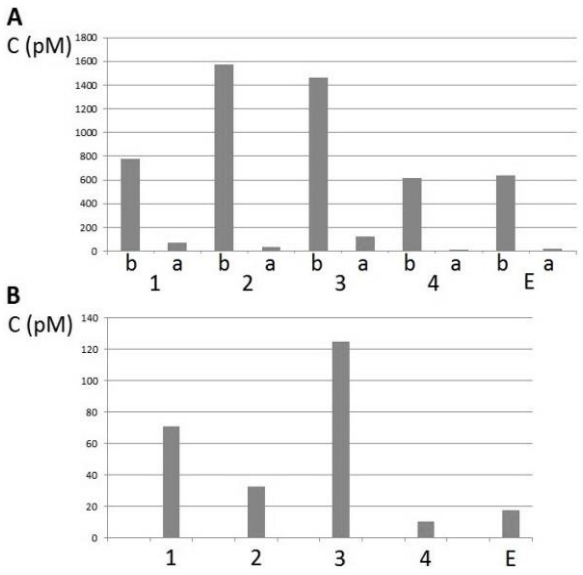

Fig. 3. A: molar concentrations of 16S rRNA genes of *Bacteria* (b) and *Archaea* (a) in different tanks (1, 2, 3, 4) of the biogas plant and in the drained effluent (E). B: the same for *Archaea* only.

The ratio of representatives of two prokaryotic domains of life (fig. 4) is different in two bioreactors for primary fermentation (1 and 2) that are fed with different substrate compositions, this is not surprising because of the differences in the amount and the nature of resources and accordingly in the tasks facing the microflora in both cases.

In the tank 2 *Archaea* concentration is twice lower and *Bacteria* concentration is twice higher than in tank 1. Tank 3 is fed with fermenting mix from tanks 1 and 2 equally, but the composition of microbiota in it tends to show *Bacteria* concentration higher than average between tanks 1 and 2. So digestion and fermentation processes performed by *Bacteria* are increasing on the way to the tank 3. The same time *Archaea* concentration increase in the tank 3 strongly showing the result of the substrate fermentation that is leading to higher availability of its products that are used by *Archaea* for active production of methane and for upscale of their biomass. The main part of methane is produced in the tank 3 and the process lowers strongly in tank 4 needed to gather the rest of the gas. Microbiota of the effluent is predictably similar to that of the tank 4.

Comparing with fig. 3 it becomes obvious that despite the overall similarity between diagrams 3B and 4 only absolute quantitative data can provide satisfactory information about microbiota dynamics during the fermentation process occurring in the plant.

The results show that by the time the fermented mixture is drained, both the content of *Bacteria* and the content of *Archaea* lower in it. Thus, the nutrient potential of the substrate has time to be depleted.

IV. Conclusion

The results are consistent with technological partition of the biomethanation process in the plant and confirm applicability of the method used. As shown in the previous sections, the quantitative data obtained correspond to the literature [5-8] and lend themselves to theoretical explanation.

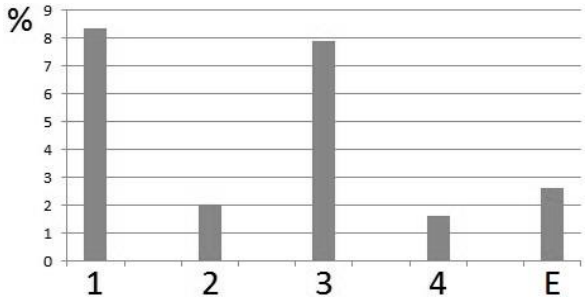

Fig. 4. *Archaea* percentages in different tanks (1, 2. 3, 4) of the biogas plant and in the effluent (E).

One of the main conclusions from the obtained data is the practical insufficiency of determining taxon ratios for the assessment of processes in a biogas plant. *Archaea* percentage depends not only on density of their population but also on the density of the population of *Bacteria*. Paying attention to trophic dependence of methanogenic *Archaea* on functions provided by *Bacteria* we still cannot achieve any strict relation between their populations. Bacterial communities in a biogas plant would be very different in the presence of different substrate compositions, this would lead to different efficiency of syntrophic interactions with methanogens [15, 16]. This all provides necessity to base on absolute quantitative data resuming microbiota composition in the biogas plant on different stages of fermentation. Development of a convenient method for estimating the absolute numbers of microorganisms belonging to different taxa in complex microbial communities, performed in our study, allows us to obtain information of this kind for a variety of practical applications.

Acknowledgment

The study was conducted with the support of a grant of the Department of Agriculture and Environment Restoration of Belgorod oblast of Russia received in 2019.

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