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

Viktoriia Iatsenko  
Belgorod State National Research University  
Belgorod, Russia  
viktorya-99@mail.ru

Anastasiya Nechayeva  
Belgorod State National Research University  
Belgorod, Russia  
nechayeva@list.ru

Violetta Klyueva  
Belgorod State National Research University  
Belgorod, Russia  
klyueva@bsu.edu.ru

Dmitriy Ohrimchuk  
L.L.C. “AltEnergo”  
Belgorod, Russia  
ockhrimchuk88@rambler.ru

Irina Batlutskaya  
Belgorod State National Research University  
Belgorod, Russia  
bat@bsu.edu.ru

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 °C (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.
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 °C for 5 min and 40 cycles of three steps at 95, 60 and 72 °C 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.

\[
C_1 = C_2 \frac{\lambda_{aq}^2}{L_3} \frac{A_3}{A_1} \frac{L_1}{L_2}
\]

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 \( \lambda \) 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 °C.

**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 syntrophic 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 ARCHAEOAL 16S rRNA GENES IN THE SAMPLES FROM DIFFERENT TANKS AND EFFLUENT OF BIOGAS PLANT “LUCHKI” AND THE PERCENTAGES OF ARCHAEOAL ONES**

|       | Tank 1 | Tank 2 | Tank 3 | Tank 4 | Effluent |
|-------|--------|--------|--------|--------|----------|
| **Bacteria, pM** | 776 | 1571 | 1461 | 615 | 634 |
| **Archaea, pM** | 71 | 32 | 125 | 10 | 17 |
| **Archaea, %** | 8.3 | 2.0 | 7.9 | 1.6 | 2.6 |
Advances in Biological Sciences Research, volume 7

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 fermenting (1 and 2) that are fed with different substrate compositions. 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.

REFERENCES

[1] M. Carlini, E.M. Mosconi, S. Castellucci, M. Villarini and A. Colantoni “An Economical Evaluation of Anaerobic Digestion Plants Fed with Organic Agro-Industrial Waste”, Energies, vol. 10, № 8, 2017, pp. 1-15.
[2] V. Paolini, F. Petracchini, M. Segreto, L. Tomassetti, N. Naja and A. Cecinato “Environmental impact of biogas: A short review of current knowledge”, Journal of Environmental Science and Health, vol. 53, № 10, pp. 899-906, 2018.
[3] F.R. Bengelsdorf, U. Gerischer, S. Langer, M. Zak and M. Kazda “Stability of a biogas-producing bacterial, archaeal and fungal community degrading food residues”, FEMS Microbiology Ecology, vol. 84, № 1, pp. 201–212, 2013.
[4] R. Wirth, E. Kovács, G. Marót, Z. Bagi, G. Rákely and K.L. Kovács “Characterization of a biogas-producing microbial community by short-read next generation DNA sequencing”, Biotechnology for Biofuels, vol. 5, № 1, 41, 2012.
[5] I. Bergmann, E. Nettmann, K. Mündt, M. Klocke, “Determination of methanogenic Archaea abundance in a mesophilic biogas plant based on 16S rRNA gene sequence analysis”, Canadian Journal of Microbiology, vol. 56, № 5, pp. 440-444, 2010.
[6] I. Bergmann, K. Mündt, M. Sontag, I. Baumstark, E. Nettmann, M. Klocke, “Influence of DNA isolation on Q-PCR-based quantification of methanogenic Archaea in biogas fermenters”, Syst Appl Microbiol, vol. 33, № 2, pp. 78-84, 2010.
[7] Y.S. Kim, M. Westerholm, P. Scherer, “Dual investigation of methanogenic processes by quantitative PCR and quantitative
microscopic fingerprinting”, FEMS Microbiol Lett, vol. 360, pp. 76–84, 2014.

[8] J. Lambrecht, N. Cichocki, Th. Hübschmann, Ch. Koch, H. Harms and S. Müller, “Flow cytometric quantification, sorting and sequencing of methanogenic archaea based on F420 autofluorescence”, Microb Cell Fact, vol. 16, № 180, pp. 1-15, 2017.

[9] S. Theuerl, J. Klang and A. Prochnow “Process Disturbances in Agricultural Biogas Production—Causes, Mechanisms and Effects on the Biogas Microbiome: A Review”, Energies, vol. 12, № 3, 365, pp. 1-20, 2019.

[10] F. Enzmann, F. Mayer, M. Rother and D. Holtmann “Methanogens: biochemical background and biotechnological applications”, AMB Express, vol. 91, № 1, pp. 1-22, 2018.

[11] P.G. Kougias and I. Angelidaki “Biogas and its opportunities—A review”, Frontiers of Environmental Science & Engineering, vol. 12, № 14, pp. 1-12, 2018.

[12] S. Broedersa, I. Huberb, L. Grohmannc, G. Berbend, I. Tavernierse, M. Mazzara, N. Roosensa and D. Morisset “Guidelines for Validation of Qualitative Real Time PCR Methods”, Trends in Food Science & Technology, vol. 37, № 2, pp. 115-126, 2014.

[13] K. Bayer, J. Kamke and U. Hentschel “Quantification of bacterial and archaeal symbionts in high and low microbial abundance sponges using real-time PCR”, FEMS Microbiology Ecology, vol. 89, № 3, pp. 679-690, 2014.

[14] L. Zhang, K.-Ch. Loh, J.W. Lim and J. Zhang “Bioinformatics analysis of metagenomics data of biogas-producing microbial communities in anaerobic digesters: A review.” Renewable and Sustainable Energy Reviews, vol. 100, pp. 110-126, 2019.

[15] Sun L., Liu T., Müller B. and Schnürer A. “The microbial community structure in industrial biogas plants influences the degradation rate of straw and cellulose in batch tests”, Biotechnol Biofuels, vol. 9, № 128, pp. 1-20, 2016.

[16] A. Rabii, S. Aldin, Y. Dahman and E. Elbeshbishy “A Review on Anaerobic Co-Digestion with a Focus on the Microbial Populations and the Effect of Multi-Stage Digester Configuration”, Energies, vol. 12, № 6, 1106, pp. 1-25, 2019.