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Creating connections between biotechnology and industrial sustainability

August 25 to 28, 2024
Costão do Santinho Resort, Florianópolis, SC, Brazil

XXIV SINAFERM ·· XV SHEB ·· XV ENZITEC   FLORIANÓPOLIS ·· 2024

INDUSTRIAL MICROBIOLOGY: PROSPECTING AND APPLIED MOLECULAR BIOLOGY

MICROBIAL DIVERSITY OF METHANOGENIC ARCHAEA IN ANAEROBIC DIGESTION USING TWO DIFFERENT PRIMERS FOR 16S rRNA GENE REGIONS

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ABSTRACT

The production of biomethane is a promising alternative in sustainable energy production. Anaerobic digestion is the process where microorganisms convert different biomasses using their metabolic pathways in a consortial manner. Biomethane is generated by archaea in the final stage of the chain. Identifying these microorganisms ensures the success of clean energy production. This study evaluates the microbial diversity of methanogenic archaea in anaerobic digestion reactors using two different primers for 16S rRNA gene regions. DNA samples from the reactors were used for next-generation sequencing with the Illumina HiSeq platform and subsequent taxonomic identification using bioinformatics tools. The study's results revealed that the U519F-806R primers were more efficient in identifying archaea, with higher abundances and diversities of phyla compared to the 341F-806R primers. Samples RA2, RB2, and RC2 showed up to 63 times higher archaea abundances than RA1, RB1, and RC1. The findings emphasize the importance of using a variety of primers to broaden the range of archaea identification in biogas reactors, which can optimize biomethane production and help replace current energy sources with sustainable and non-polluting options.

Keywords: Biomethane. Metataxonomy. Anaerobic digestion. Archaea. Renewable energy.

1 INTRODUCTION

With the worsening of climate change, biogas production emerges as a promising alternative in the search for sustainable energy solutions. With the growing global concern over reducing greenhouse gases, studies are seeking alternatives that enable a transition to renewable energy sources aimed at mitigating environmental and socioeconomic impacts. In this context, anaerobic digestion (AD) is an established technology in energy generation and the valorization of organic waste. Various substrates can be converted into biogas through the biotransformation by naturally involved consortia of microorganisms. The AD process depends on different groups of microorganisms that act in distinct stages of conversion.[1] Initially, groups of hydrolytic bacteria such as those from the genus *Bacillus* and *Clostridium* will break down macromolecules (carbohydrates, proteins, and lipids) into smaller compounds like amino acids and fatty acids.[2] In the second stage, these compounds will be used by other groups of bacteria like *Lactobacillus* and *Pseudomonas,* which will produce volatile fatty acids (VFAs), carbon dioxide (CO2), ethanol, and hydrogen (H2) in the acidogenesis phase. Subsequently, in the acetogenesis stage, the compounds formed in the previous phase will be utilized by *Syntrophobacter, Acetobacterium*, and other bacteria to generate acetate, H2, and CO2.[3] Finally, for the substrates' conversion to biogas to be completed, groups of archaea such as *Methanobacter* and *Methanosaeta* are necessary for methanogenesis. This stage consists of the conversion of acetate, H2, CO2, and methylated compounds into biogas through different metabolic pathways (hydrogenotrophic, acetoclastic, and methylotrophic).[4] However, understanding the composition of the microbial community and its dynamics varies greatly depending on the biomass used and the physicochemical conditions imposed in the process, requiring a focus on research that seeks a return on economic investment.

Archaea are groups of microorganisms extremely sensitive to oxygen and physicochemical variations in the environment where they grow, making it a complex challenge to keep their communities active and balanced within reactors and anaerobic digestion environments. To better guide these processes and ensure efficient waste utilization, a widely used tool is DNA sequencing of samples from different inocula and AD reactors to identify the microorganisms. By conducting a metataxonomic assessment of these microbial consortia, it is possible to evaluate the presence of the archaeal groups necessary for successful substrate conversion.[5] The most commonly used method for analyzing microbial consortia is metataxonomy, which, through the sequencing of the 16S rRNA gene and genetic alignment in specialized databases, provides a qualitative and quantitative analysis. The method was developed with great success for the differentiation of bacteria, but archaea belong to another domain of the classification of living organisms.[5] Molecular analyses tend to follow a standard of using primers that perform genetic sequencing; however, new primers need to be tested to ensure their use in identifying methanogens. In this context, the present study aimed to evaluate the microbial diversity of methanogenic archaea present in anaerobic digestion reactors using two different methodologies with distinct primers for regions of the 16S rRNA gene in microbial metataxonomy.

2 MATERIAL & METHODS

Biological samples were obtained from three biogas production reactors containing equal amounts of vinasse for DNA extraction and subsequent microbial identification using the two different primers for each reactor. The 50 ml samples were transferred to sterilized Falcon tubes and centrifuged at 10,000 rpm for 5 minutes. The resulting pellet was separated for DNA extraction and subsequent purification. Genomic DNA was extracted using the Soil DNA Isolation Kit (NORGEN Biotek Corp), following the manufacturer's instructions. The extracted and quantified nucleotide material was used for next-generation sequencing via the Illumina HiSeq PE250 platform. The obtained DNA was quantified with a Qubit fluorometer (Thermo Fisher Scientific). DNA amplification was processed with primers 341F-806R (CCTAYGGGRBGCASCAG, GGACTACNNGGGTATCTAAT) for the V3 and V4 regions of the 16S rRNA gene (samples RA1, RB1, and RC1) and U519F-806R (CAGYMGCCRCGGKAAHACC, GGACTACNSGGGTMTCTAAT) for the V4 region (samples RA2, RB2, and RC2).[6] FastQC software was used to evaluate the quality of the sequenced nucleotides.[7] Data processing and subsequent analysis of the reads were performed using the QIIME2 (Quantitative Insights Into Microbiological Ecology) pipeline. The nucleotide sequences corresponding to the primers used in the amplification stage were trimmed using the q2-cutadapt plugin. Subsequently, chimeric sequences and other errors that may occur during DNA sequencing were filtered using the DADA2 (Divisive Amplicon Denoising Algorithm) algorithm.[1] Sequentially, the ASVs (Amplicon Sequence Variants), FASTA sequences, and relative abundance values were used for taxonomic assignment. The feature classifier plugin, using the classify-sklearn method, was employed to compare the obtained sequences with the public SILVA database.[2]

3 RESULTS & DISCUSSION

The microbial diversity assessment using primers 341F-806R resulted in a total of 142,016, 41,505, and 54,854 reads for samples RA1, RB1, and RC1, respectively. After the filtering process with DADA2, 112,907, 31,025, and 41,660 non-chimeric reads were obtained, respectively (RA1, RB1, and RC1). For the assessments using primers U519F-806R, a total of 204,424, 206,452, and 219,028 reads were obtained for RA2, RB2, and RC2, and 140,415, 137,031, and 145,197 non-chimeric reads were recovered after the filtering process.

The archaeal diversity for primers 341F-806R showed 1.22% of identified archaea and 98.77% of bacteria in the total microbial community for sample RA1, 0.85% archaea and 99.14% bacteria for RB1. Sample RC1 presented the lowest abundance of archaea at 0.74%. The archaeal diversity was 2.67 times higher with primer U519F-806R, where sample RC2 predominated with 53.9% archaea, followed by 31.6% for RA2 and 22.1% in RB2.

At the phylum level for archaea, Euryarchaeota appeared with abundances of 0.09%, 0.080%, and 0.086% for RA1, RB1, and RC1, respectively (Fig 1). Sample RC2 showed the highest abundance of this phylum at 6.73%, followed by 1.75% in RA2 and 1.1% in RB2 (Fig 2). The methanogenic archaeal phylum Halobacterota was observed with 1.07% in sample RA1, followed by 0.73% for RB1 and 0.63% for sample RC1, and was identified in greater abundance in samples RC2, RA2, and RB2 respectively (7.57%, 6.13%, and 3.5%). Nanoarchaeota appeared with 0.03% in both RA1 and RB1, while sample RC1 had the lowest abundance at 0.01%. This archaeal phylum was the most abundant in samples RC2, RA2, and RB2, with frequencies of 19%, 15.5%, and 12.6%, respectively. The second most predominant archaeal phylum in the samples with primers U519F-806R was Thermoplasmatota, with 16.6% (RC2), 6.69% (RA2), and 3.83% (RB2). In samples RA1 and RB1, this phylum had an abundance of less than 0.01% and was absent in RC1. The last archaeal phylum identified was Crenarchaeota, which was present only in RC2 (4.02%), RA2 (1.52%), and RB2 (1.07%).
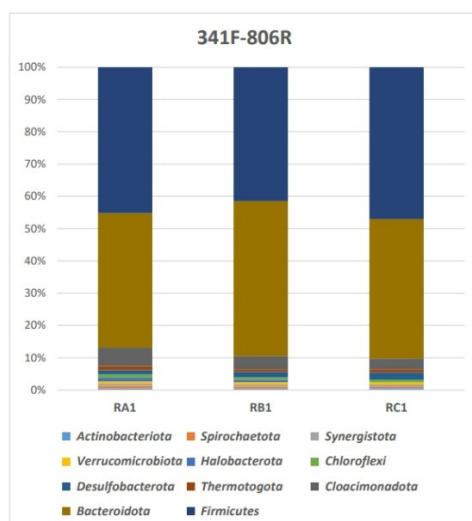
Figure 2 Diversity of Phyla with Primers U519F-806R (RA2, RB2 and RC2 DNA samples from the reactors were used for next-generation sequencing with the Illumina HiSeq platform and taxonomic identification using bioinformatics tools).
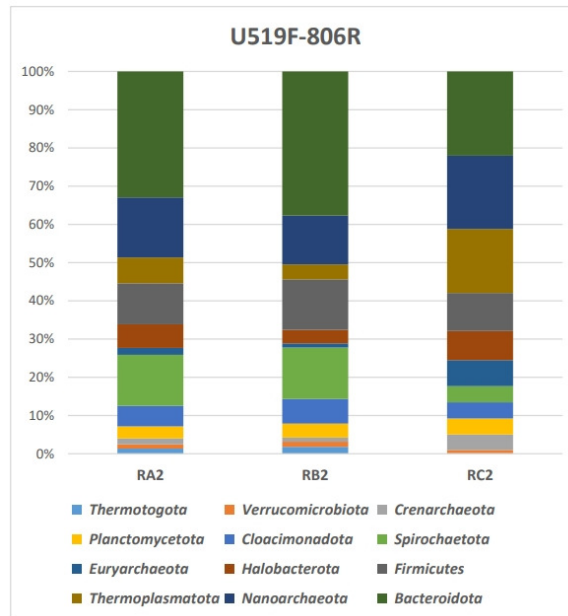
At the genus level, six different genera of archaea were identified in samples RA1, RB1, and RC1, and 16 genera in samples RA2, RB2, and RC2. Among the most abundant identified groups was the genus Woesearchaeales (phylum Nanoarchaeota), with 19.01% (RC2), 15.5% (RA2), and 12.6% (RB2). Woesearchaeales is not identified as a group of methanogenic archaea and its functions are little known.[8] However, this genus is described as a microorganism that has syntrophic relationships with Methanomassiliicoccales, which is part of methylotrophic methanogenesis.[9] In sample RC2, this phylum was not present, whereas in samples RA1 and RB1, it showed an abundance of only 0.03% in both samples. The second most abundant genus was Candidatus Methanoplasma with 15.3% (RC2), 5.7% (RA2), and 3.4% (RB2). In sample RA1, Candidatus Methanoplasma showed an abundance of less than 0.01% and was absent in samples RC1 and RB1. This genus, belonging to the phylum Thermoplasmatota, can convert a wide range of methyl compounds, hydrogen, and carbon dioxide into methane.[10]

Candidatus Methanofastidiosum is a genus of methanogenic archaea that uses the hydrogenotrophic metabolic pathway and also acetate and propionate as a carbon source for methane production.[11] It was identified with 6.7% in sample RC2, 1.73% in RA2, and 1.08% in RB2. This microorganism was also present in RA1 (0.09%) and RB1 (0.08%). The genus Bathyarchaeia is described as a microorganism with highly diverse metabolic patterns, participating in methane production through hydrogenotrophic, methylotrophic, and acetoclastic pathways and was observed only in samples sequenced with primer U519F-806R, with an abundance of 4.02% in RC2, 1.52% in RA2, and 1.07% in RB2.[12] Bathyarchaeia is also capable of degrading macromolecules such as proteins, cellulose, aromatic compounds, and chitin, assisting in the hydrolysis step of anaerobic digestion.[12]

The methanogenic archaeal genus Methanoculleus was identified with the highest frequency in sample RC2 (7.46%), followed by RA2 (6.05%), RB2 (3.45%), and only 0.25% and 0.21% in RB1 and RA1, respectively. Methanoculleus is known for using hydrogen and carbon dioxide in the methanogenesis process, indicating that all metabolic pathways were present among the evaluated samples.[13] Another group of methane-producing archaea present in lower abundance (0.1%) in RC2, (0.08%) in RA2, and (0.04%) in RB2, and showing higher frequency in RA1 (0.85%) and RB1 (0.48%) was Methanosaeta. This common group of archaea is identified as methane producers through electron transfer with propionate-oxidizing microorganism groups.[14]

The samples showed a significant number of unidentified genera. For primers U519F-806R, this number was 17.5% (RB2), 16.1% (RA2), and 15.5% (RC2). For primers 341F-806R, the frequencies of unidentified sequences were also very similar, with 15.8% in sample RA1, 15.1% in RC1, and 14.2% in sample RB1. Lastly, the samples still had a large portion of uncultivated microorganisms, with significant frequencies especially in samples RB1 (24%), RC1 (20%), and RA1 (19%). In the samples with primers specific to the V4 region, the frequency of uncultivated microorganisms was significantly lower, being 7% in sample RB2 and 6% in samples RC2 and RA2.
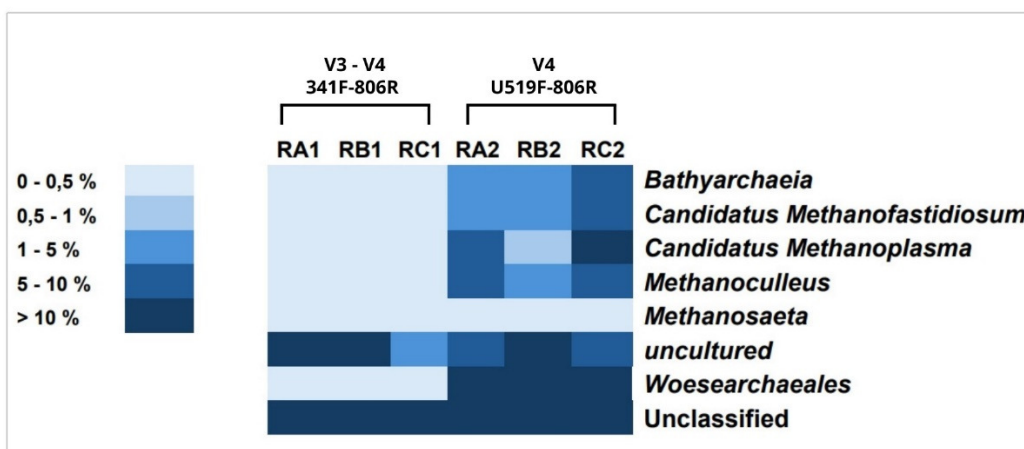
Figure 3 Heat Map for Archaea Diversity for Primers 341F-806R e U519F-806R (RA, RB and RC - DNA samples from the reactors were used for next-generation sequencing with the Illumina HiSeq platform and taxonomic identification using bioinformatics tools).

4 CONCLUSION

The metataxonomic analysis conducted in this study revealed greater efficiency in identifying archaea using primers U519F-806R, highlighting the importance of exploring a wider diversity of primers in the DNA sequencing process to identify these groups of microorganisms in biogas and biomethane production reactors. The use of more than one type of primer proved to be an efficient strategy for a broader identification of groups such as Woesearchaeales, which, although not methanogenic, may play an important syntrophic role. The identification of a variety of methanogenic archaeal genera in different abundances across samples underscores the need to better understand the reactor conditions that favor these different microorganisms. Applying different primers in the characterization of these anaerobic digestion ecosystems can help optimize biogas production, contributing to the transition to sustainable energy sources.

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ACKNOWLEDGEMENTS