Studies of biogas-producing microbial communities by methagenomics approach

Ph.D. Thesis

Written by:

Roland Wirth

Supervisors:

Prof. Dr. Kornél L. Kovács

Dr. GergelyMaróti

University of Szeged, Department of Biotechnology

Hungarian Academy of Sciences BRC Institute of Biophysics

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Introduction

The contemporary human society is based on non-renewable fossil fuels (oil, coal, natural gas), which represents more than 80% of our energy consumption. The rapid growth of the human population, improvement of living standards and the parallel increase of industrial demands all call for more energy. The inevitable exhaustion of economically and energetically exploitable fossil fuels will not meet the energy needs of consumers. Because the increasing price of their recovery, fossil fuels gradually lose their competitive edge. This tendency is accompanied with the alarming phenomenon of climate change, which is the result of burning fossil fuels and releasing excess amounts of greenhouse gases to the atmosphere threatening the living conditions in our planet. Therefore, worldwide attention is paid to the replacement of the current energy carriers with alternative, renewable ones with rapid technology development. These alternatives have a common character, i.e. the energy comes from renewable sources and the raw materials for energy generation is reformed at the rate of utilization. One of the most promising alternatives is biogas.

The simplest and most common use of biogas is to burn it producing simultaneously heat and electricity. The fermentation residue can substitute artificial synthetic fertilizers, therefore it is an excellent soil nutrient replacement. The main component of the biogas is methane, depending on the fermentation substrate it makes up to 50-75% of the gas, in addition it contains carbon dioxide (28-48%) and other gases (~1-2%) for example hydrogen sulfide and nitrogen compounds. The fossil “natural gas” comprises usually more than 90% of methane. This value can be achieved in the case of biogas, if carbon dioxide is removed from the raw gas. After gas cleaning the heat value of biogas may be equivalent to natural gas, thus the gas can be fed to the gas network.

It has long been known that biogas production also occurs spontaneously in marshes, landfill sites, where anaerobic environment can develop. The biogas formation process takes place under anaerobic conditions, but facultative anaerobes are also involved in the breakdown of organic materials. The anaerobic conversion of various materials is a complex chain of biochemical process which is carried out by a diverse microbial community the composition of this community depends on various environmental factors. The events leading to biomass decomposition follow each other in precise order, each step is carried out by a specific microbial group. The different types of microbes are interdependent and operate in harmony. The conversion of complex organic molecules into methane can only be successful

if the bacteria develop specific community in which each species can make a living, and leaves behind a product for the following group of microbes as food. Biogas production is therefore a complex microbiological process, the microorganisms constitute a complex food chain where biogas is the end product. In such communities all microbial species compete for survival, the species, which are able to adapt to the changing circumstances will outlive and become dominant.

Presently, prices of renewable energy carriers is not yet competitive with fossil fuels. Therefore the development of increasingly more economical solutions and the improvement of efficiency will support the faster deployment, so they are of decisive importance. Understanding the details of biogas production includes learning about the microorganisms involved in the process and knowledge about their roles allows the development of more efficient and stable systems. Metagenomics –analysis of the total genetic material of the microbial community- is a novel opportunity to expand our knowledge of microbiology of such complex communities. Application of metagenomic methods can lead to the development of more efficient, more economical biogas producing microbial communities. Next Generation Sequencing (NGS) and the latest Third Generation Sequencing (TGS) technologies are available today to facilitate metagenomic studies. The majority of modern biogas plants operate with corn silage as a substrate. This is a good biogas substrate but the price of corn silage is constantly increasing and an additional disadvantage is that energetic biomass production competes with food and fed production for the same agricultural land. Alternative biomass sources grown on marginal lands or areas not suitable for agricultural activities, such as photosynthetic microorganisms, offer available alternative for biogas generation. Therefore there is a growing interest in various algae species worldwide, especially in so-called microalgae species. Microalgae are a diverse group of microscopic, autotrophic organisms. The microalgal biomass utilization for biogas production has several advantages over the traditionally used corn silage. Some species are able to double their biomass in 24 hours and develop more biomass than any terrestrial plant. They need no herbicides and pesticides. Algae can grow in a variety of aqueous environments, but their cultivations require less water than terrestrial plants for the production of the same amount of biomass. An additional advantage of microalga biomass is the higher methane content of the generated biogas, i.e. 60-75% methane content from alga biomass and 50-55% from maize silage, respectively. Algae utilize CO_2 as carbon source, hence they may absorb carbon dioxide from biogas combustion while additional biomass for biogas generation is formed. Theoretically, such closed circuit can make the biogas production technology a completely

zero-emission process. In addition microalgae can be used as a source for biodiesel, biohydrogen and other valuable compounds before fermenting their biomass to biogas. With a proper combination of the various processes the efficacy and economy of biomass utilization is improved.

Aims

The aims of this study were to investigate the anaerobic consortium of a biogas plant experimental fermenter by using a novel next-generation sequencing technology. This was planned to supplement and validate the results of the next generation sequencing technologies already present in the scientific literature.

Studies are being carried out in our research team in connection with hydrogen production by microalgae, i.e. a *Chlamydomonas sp.* and *Scenedesmus sp.* culture, and their syntrophic bacteria. One of my goals was to compare the biogas quality and quantity produced from the algae mixture left over the after the biohydrogen generation step, with corn silage and cofermentation. The fermentation characteristics and their stability were followed in continuously stirred and fed laboratory biogas reactors and metagenomic analysis the microbial communities of the biogas digesters were carried out. These allowed the optimization of the use of these alternative substrates for biogas production.

Scenedesmus obliquus microalga, cultivated under photoautotrophic conditions, had significantly smaller syntrophic bacterial contamination. With this substrate the aim was to examine the fermentation stability, the generated biogas quality and to monitor the microbial community of the fermenters. The data were compared with the previous experiments when algae mixture and its syntrophic bacteria were used as substrate.

Sc. obliquus microalga possess a thick cell wall which hinders the fermentation efficacy. This is a generally occurring problem in algal biomass utilization and several pretreatment technologies were proposed to break up the recalcitrant cell wall. The heat-thaw and the microwave treatments were tested. In this experiment the goal was to investigate the biogas production effectiveness of microalgal biomass after various pretreatments.

Methods

Custom designed continuously fed and automatically operated five liter pilot biogas fermenters were used in most experiments. Batch type fermentations were also carried out according to the VDI (Verein Deutscher Ingenieure), which is an internationally accepted

standard to determine the biogas potential of a particular substrate. In both systems the composition of biogas was determined by gas chromatography. The fermentation parameters were followed by measuring pH, total organic acid, buffer capacity, ammonia level. redox potential and temperature inside the reactors. The carbon and nitrogen content of the various biomass samples were determined in an automatic carbon and nitrogen measuring instrument ,minor elemental composition was measured using an analytical workstation based on spectrophotometric assays. The mixed microalgae and their syntrophic bacteria were cultured in TAP (Tris-acetate-phosphate) media under non-sterile conditions. *Scenedesmus obliquus* microalga was grown by Első Magyar Algatechnika Kft., under photoautotrophic conditions in a 2,5m^3 large-scale tubular fotofermenter. The microbial composition of the substrates and the biogas reactors were investigated by next generation DNA sequencers. High quality DNA sequence reads were generated using Applied Biosystems SOLiD sequencing equipment, operating on ligation based sequencing. Reads were assembled into contigs before phylogenetic and functional analysis by CLC Bio Genomics Workbench 4.6 program. Ion Torrent PGM (Life Technologies), which was employed in the studies related to biogas generation from algae, operates using a different sequencing strategy. The reads obtained by Ion Torrent PGM were directly used without contig assembly for phylogenetic analysis. Evaluation of metagenomic data were made by the online available MG-RAST software package.

Results

1. In the biogas plant simulation experiments the parameters were set to mimic the industrial biogas plants. Applied Biosystems SOLiD next generation sequencer was used for metagenomic investigations, this technique has not been used for similar microbial community studies before. The microbial system in the biogas fermenter was well organized and the various strains seemed to depend on each other. The results established that the members of Firmicutes and Bacteroidetes phyla play important role in the breakdown of cellulosic biomass and secondary fermentation process. Within the Firmicutes phylum the representatives of the *Clostridia* genus are abundant, many of these bacteria possess celullolytic and hydrogen producing capability. Their role in the efficient utilization of cellulose containing biomass is likely significant. In the Archaea domain the Methanomicrobiales order was dominant within this taxonomic group the hydrogenotrophic *Methanoculleus marisnigri* was outstanding in profusion. The results of

these studies correlated well with previous metagenom studies carried out by 454 type next generation sequencers using a distinct DNA sequencing technology. Thus of the various next generation sequencing approaches for the investigation of such complex microbial communities was validated and the results appeared reliable and reproducible.

2. The use of mixed microalgal-bacterial biomass for biogas production was examined. that the spontaneously formed microalga-bacteria mixture developed under non-sterile culture conditions had low C/N (carbon/nitrogen) ratio, which led to a biogas yield lower than that of corn silage although the biogas from microalgae consistently produced higher methane content. The system worked steadily for two mounts, but in microalgae fermentations a non-detriemental ammonium accumulation was observed in time. Cofermentation of microalga mixture and corn silage showed a balanced operation. The metagenomic results revealed that the syntrophic bacteria introduced together with the non-sterile alga biomass displaced or masked the bacterial community of the anaerobic reactors. In the Archaea domain the Methanosarcinales order dominated the utilization of the algal biomass.

3. *Scenedesmus obliquu*s was cultivated under photoautotrophic conditions. This microalga had higher C/N ratio than the microalgae mixture used in the previous set of experiments. Significant increase of ammonium content was not observed during the fermentation of this biomass. The amount of generated biogas is similar to that of the algae mixture, and the gas composition was similar as well. The semi-continuous steady biogas reactors lasted at least for three months. Because of the *Sc. obliquus* thick cell wall the biogas generating microbes require additional time to digest this biomass. Although there was no biomass input during the last month, a significant amount of biogas was still produced by the alga-fed reactors. Therefore longer retention time is needed for efficient biogas production from microalgae than the conventionally applied retention times based on maize silage fermentation. The methane concentration in the biogas was also high in cofermentation like in the previous set of experiments. These results may be related either to the relative majority of Clostridiales order in cofermentation or to the more balanced C/N ratio of the cofermentation biomass. In the Archaea domain, the Methanosarcinales order dominance was observed, which supported the diversity of metabolic pathways in the process.

4. *Sc. obliquus* has a thick and hemicellulose-rich cell wall. Several pretreatment strategies have been tested for cell wall disruption. Repeated heat-thaw treatments, autoclaving and microwave indiced cell wall destruction were tested in our experiments. Autoclaving and the microwave treatment turned out as most effective pretreatment methods although

unbroken cells were observed after both treatments under microscope. The disrupted microalgae cell contents were utilized quickly and effectively by the microbial community of the biogas digester, both the biogas quality and quantity exceed the maize silage control. The effect did not last long as after consuming the easily accessible substances had to degrade the cell wall materials.

5. According to the data presented the metagenomic approach in the assessment of the biogas producing microbial community have been validated and was proven reproducible. Microalgal biomass was a promising candidate to replace corn silage in the biogas industry, but the alga strain cultivated its pretreatment technology strongly influence the efficiency. The most preferred application seems to be cofermentation with plant biomass.

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