Review

Microbial Community Analyses in Biogas Reactors by Molecular Methods

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Abstract

Successful biogas production is based on stable or adaptable microbial community structure and activity which depends on type of substrate used and several physico-chemical conditions in the bioreactor. Monitoring those and the dynamics of microbiota is important for planning and optimizing the biogas process, avoiding critical points and reaching the maximum methane yield. Methanogens are extremely difficult to study with culture-based methods. Molecular methods for microbial community structure analysis in biogas reactors, which offer qualitative and quantitative information on bacterial and archaeal species and their microbial community changes, and causes for process instability are surveyed in this review. For comparative studies semi-quantitative, rapid and cheap techniques like T-RFLP, DGGE and TGGE are used. More laborious and expensive techniques with high-throughput like semi-quantitative FISH and DNA microarrays and also quantitative techniques like qPCR and sequencing are used for phylogenetic analysis. Technique type adequacy for certain study depends on what information is needed and on several advantages and disadvantages every technique possesses.

Keywords: Biogas, microbial community, methanogenesis, molecular methods

1. Introduction

Renewable sources of energy represent a good replacement for fossil fuels such as coal, oil and natural gas, which quantity is limited and yet we are still heavily dependent on them. Anaerobic digestion of continuously generated organic waste is therefore a perfect way to reduce the organic pollutants in waste and wastewater, greenhouse gas emissions, leakages of methane into the atmosphere and to produce methane as an alternative energy source. Utilizing organic waste for biogas production is a cost-effective and environmentally friendly technology that produces carbon dioxide-neutral renewable energy which can be later used for generation of electricity, heating or as a direct fuel.

Anaerobic digestion is a multi-step bioprocess depending on interactions among bacterial and archaeal microbial communities and their substrate and product specificities. In the absence of terminal electron acceptors such as oxygen, metals, nitrate or sulfate, the methanogenic conversion of organic substances becomes the predominant pathway.[1] The main product of the process is biogas which consists of methane, carbon dioxide and trace gases such as hydrogen and hydrogen sulfide. There are several groups of microorganisms involved which make this process complex and sensitive, therefore it is a valid subject for control and optimization. Biogas production process has four main steps as follows: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Fig. 1). First, hydrolysis of complex organic materials into smaller units occurs by the excreted enzymes of hydrolytic and fermentative bacteria. Hydrolyzed substrates are then digested by acidogenic bacteria, resulting in short chain fatty acids and hydrogen. Alcohols and short chain fatty acids with more than two carbon atoms need to be further oxidized by acetogenic bacteria, resulting in acetate, carbon dioxide and hydrogen. In the last step methanogenic archaea convert acetate to methane and carbon dioxide.[2]

In anaerobic digesters about 70% of methane is produced via acetotrophic methanogenesis and the rest is generated via hydrogenotrophic methanogenesis. The filamentous acetotrophic methanogens are obligate anaerobes (*Methanosaeta* sp., *Methanosarcina* sp.), which produce methane from acetate with carbon dioxide as a byproduct. *Methanosaeta* sp. are favored at low acetate concentrations and they disappear at high concentrations of ammonium and sulfide, which can often be found in substrates like swine or cattle manure. *Methanosarcineae*

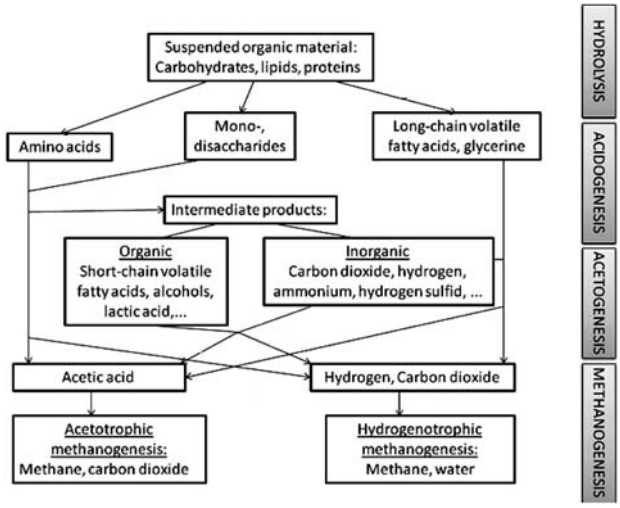

Figure 1: Schematic overview of the four main steps in the anaerobic digestion process[9,10]

are favored at high acetate concentrations, because their immobilization in flocks and granules protects them against toxic agents.[3] The hydrogenotrophic pathway includes production of methane mostly by *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanopyrales*, *Methanocellales* and species related to those of the genus *Methanoculleus* which produce methane from hydrogen as electron donor and carbon dioxide with water as a byproduct.[4–6] Recent studies applying modern microbial structure analysis techniques showed that the proportion of acetotrophic and hydrogenotrophic methanogenesis can vary in certain biogas production conditions. In batch fermentors operating under thermophilic conditions (60 °C), where sugar beet silage was used as a substrate and plant-litter compost of the hot rot-phase as an inoculum, the addition of the same compost induced a shift in microbial community structure where hydrogenotrophic *Methanobacteriales* dominated.[7] A screening study of 20 Swedish full-scale biogas reactors using different substrates (sewage sludge, industrial wastes, house-hold wastes, energy crops, manure) showed that aceticlastic methanogens dominated in reactors treating sewage sludge, while hydrogenotrophic methanogens dominated in reactors using mixtures of wastes as substrate.[8]

2. Understanding of Microbial Communities

With the increasing application of the anaerobic digestion process for biogas production there is a continuous and urgent need to improve our understanding of processes taking place in biogas reactors.[11–13] Biogas is produced in a complex process driven by different microbial species (Fig. 2). Bacteria, especially members of the classes *Clostridia* and *Bacilli*, play an important role in hydrolytic digestion of macromolecular substrates, while archaea are needed for methanogenesis. Therefore there are less archaea present (about 10–20%) in methanogenic sludge, as quantity and number of species are concerned, in comparison to the amount and diversity of bacteria (about 75–90%).[14,15] The exact amounts may vary, depending on the type of analysis used.

To optimize biogas production and maximize methane yield, good understanding of the food web and interactions of microorganisms in the bioreactor is needed. Questions that are arising while setting or monitoring the bioprocess are as follows: (1) which microorganisms are present in a reactor and which are active and growing, (2) how many different types of microorganisms are there, (3) how these microorganisms behave under certain conditions. Many species may be present but only a few might be active at certain time. As only a fraction of microorganisms has been cultured to date, molecular methods are more suitable to use because they are fast, facilitate a high throughput, identify microorganisms that are yet uncultu-

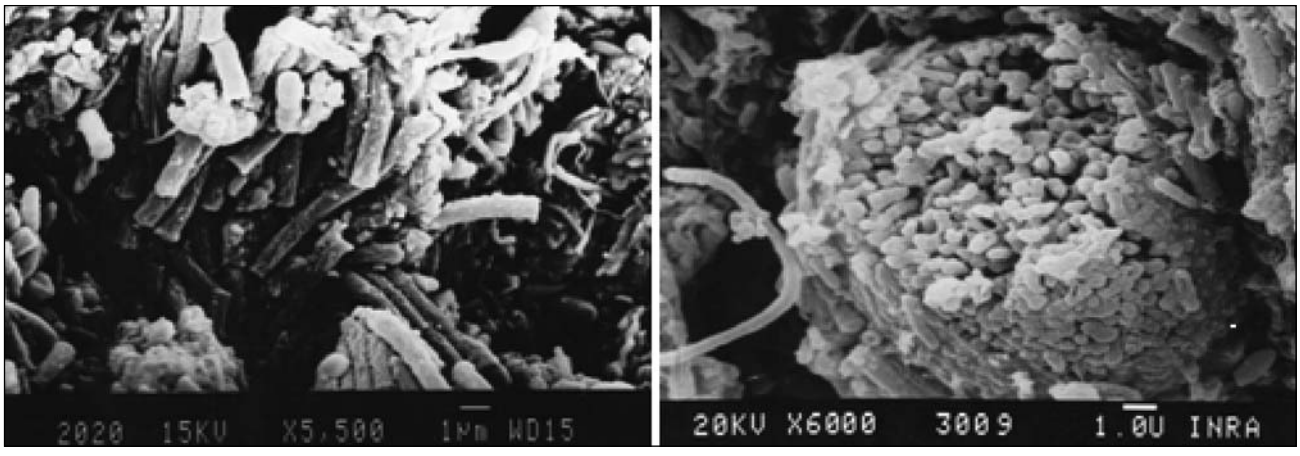

Figure 2: Electron micrographs of microbial communities in anaerobic methanogenic granules (SEM; magnification 5500X left and 6000X right; prepared by Marinšek-Logar)

red and enable quantification of microorganisms present in biogas reactor.[16] Therefore determination of microorganisms is based on DNA analysis, but the active population can only be detected based on the analysis of RNA.

Cases like substrate overload of biogas reactors, toxic compounds in substrate, substrate pretreatment or variations in microenvironmental physical conditions can rapidly result in microbial community changes and may lead to process breakdown.[17,18] pH or temperature have a direct influence on microorganisms. Temperature affects the activity of certain microbial groups and the structure of the community, influencing the rate and path of carbon flow during methanogenesis.[19] Substrate disturbances include changes in its composition or concentration and generation of toxic and inhibitory compounds which are produced during the substrate degradation (short chain fatty acids, ammonia, phenolic compounds).[2] All of these can affect the structure of the microbial community and therefore its activity, resulting in decrease of biogas production. Analysis of microbial community structure is essential for determining who and which activity is affected. Knowledge about the dynamics of microbial community structure and activity is essential for successful planning of the biogas process, monitoring its parameters and for reaching the main goal: process stability and maximum biogas yield.

2. 1. Effects of Substrate Overload on Methanogenic Microbial Community

In biogas production short chain fatty acids (SCFAs) are important intermediate products and also the main potential inhibitor as their concentrations during the process affect the fermentation efficiency. In the case of substrate overload SCFAs like acetic, propionic and butyric acid start to accumulate, because hydrolysis and acidogenesis run faster than acetogenesis and methanogenesis. Consequently the lowered pH value negatively affects the biogas process, as inhibitory effects of SCFAs are also pH-dependend.[20] Because the functional redundancy among different phylogenetic groups allows shifts in populations with no effects on the reactor function, the dynamic bacterial community often shows shifts in community structure even when the reactor is operating stably.[21,22] In contrary, archaeal communities are normally less dynamic than bacterial.[23] Shifts in archaeal communities are therefore due to the changes in operating conditions and process parameters such as SCFAs concentration.[24,25] High concentrations of SCFAs inhibit *Methanosaetaceae* and consequently *Methanosarcina* species dominate.[3] The inhibition by SCFAs is much stronger at lower pH values. At the pH value above 7.5, the concentration of SCFAs can be much higher (up to 4500 mg L^{-1}) before any inhibition occurs.[26] pH influences the ratio of the dissociated and undissociated species of SCFAs directly and the latter can have toxic effects on the microorganisms because they can diffuse through the membrane and cause irreversible damage by changing the intracellular pH value and disrupting the homeostasis.[27,28] That is why SCFA monitoring is a relevant measure for process stability as they represent a warning indicator for process imbalance.[2] Propionate is known to be more toxic than other SCFAs.[29] Thermophilic methane production at pH 7.2–8.2 from biogas plant sludge in lab-scale experiments was inhibited at 36 mmol $L^{-1}$of propionate and butyrate. Acetate concentration variations led to an inhibition beyond 36 mmol L^{-1}. Addition of formate did not cause inhibition of methane production until the concentration of 120 mmol L^{-1}. This result indicates that a high number of formate utilizing microorganisms (hydrogenotrophic methanogens like species of the order *Methanobacteriales*) is present in the biogas plant sludge.[30,31] No methane production was detectable at the highest concentration of formate (360 mmol L^{-1}) for the first 3 days only. Interestingly, after 2 additional days the recovery of methanogenic population occurred.[32]

2. 2. Effects of Toxic Compounds on Methanogenic Microbial Community

During biogenic and abiogenic hydrolysis of the lignocellulose substrates weak acids, furan derivatives, cresols and phenolic compounds are generated which inhibit the acetotrophic methanogenesis.[33,34] These phenolic monomers have toxic effects on bacteria and archaea.[35] When phenol compounds are present in the methanogenic sludge, they inhibit the activity of archaea and consequently the activity of acetogenic bacteria. Concentration of SCFAs increases which results in decreased pH value and decreased rate of the whole process. During the hydrolysis of hemicellulose at high temperatures in acid substrate pretreatments oligosaccharides can dehydrate to toxic furfural, which inhibits microbial growth and respiration.[36,37] Fedorak and Hrudey[38] tested the effects of phenols on anaerobic process and their results showed that methane production was inhibited at phenol concentrations higher than 2000 mg L^{-1}. Recent experiments showed that cresols are more toxic to methanogenesis than phenols and hydrogenotrophic methanogenesis showed to be more sensitive than acetoclastic one. It is also known that suspended microbial cells are more sensitive to p-cresol than immobilized (granulated) biomass. Inhibition starts already at 125 mg L^{-1} for *o*-cresol and 100–240 mg L^{-1} for *p*-cresol in suspended methanogenic biomass.[39,40]

Heavy metals and alkali metals stimulate microbial growth and activity when in traces, but they are toxic in higher concentrations in substrate. In biogas production common problematic metals are copper, lead, cadmium, zinc, nickel, chromium as well as sodium, potassium, calcium and magnesium; they all cause dehydration of bacterial cells. Inhibition appears due to the toxic effects of heavy metals on SCFA-degrading microorganisms. The

most toxic are cadmium and copper, while lead and nickel are the least toxic.[41]

Also ammonium in form of ammonium ion or ammonia, byproducts of protein digestion and hydrolysis of urea, can be problematic for methanogenesis. Increased production of ammonia in biogas reactors can be caused by substrates with low C:N balance.[20] Ammonia inhibition depends on the presence of free unionized ammonia which is the result of changes in pH, temperature or both.[42–44] The fraction of total soluble nitrogen in the form of free ammonia is higher at high temperatures and pH, therefore ammonia, when present at those conditions, is more toxic than ammonium ion. Free ammonia inhibits methanogenesis at concentrations 100–1100 mg L^{-1}, however quite a few experiments showed that adaptation of the microorganisms to higher ammonia concentrations is also possible.[45–49]

2. 3. The Role of Hydrogen on Methanogenic Microbial Community

In normal biogas process conditions, acetogenic bacteria convert propionate and butyrate into acetate and hydrogen and their activity depends on the activity of methanogens removing hydrogen and producing methane. This way the metabolism of acetogenic bacteria is thermodynamically possible as their reactions are endergonic under standard conditions which means they only occur when hydrogen is kept below a certain concentration. Therefore the relationship between the SCFA-degrading bacteria and hydrogen-utilizing methanogens is defined as syntrophic and the process is called interspecies hydrogen transfer. The thermodynamics of SCFA degradation is therefore better at low hydrogen concentration.[50] Hydrogen concentration in the bioreactor is important as higher partial pressure of hydrogen results in less acetate and methane produced.

2. 4. Other Factors Causing Microbial Community Disorders

Sulfur-reducing bacteria can represent a competition to methanogenic archaea as they both are hydrogen consumers. Symbiosis of acetogenic bacteria with sulfur-reducing bacteria results in starvation of methanogens and methanogenesis is slowed down. Sulfur is reduced to hydrogen sulfide which, at higher temperatures, becomes toxic and inhibits the growth of methanogenic archaea. The toxicity is correlated with increased pH, as the inhibitory concentration of hydrogen sulfide in the pH range 6.4–7.2 is 250 mg L^{-1} and only 90 mg L^{-1} in pH range 7.8–8.0.[51]

Also other factors as light, tannins, herbicides, disinfection compounds, insecticides, surfactants, compounds with –CN group, long chain fatty acids, formaldehyde, chlorinating hydrocarbons and antibiotics inhibit the activity of methanogenic microbial community.[20]

3. Microbial Community Analysis Methods

Time consuming and laborious methods using expensive equipment were needed before coming of modern molecular methods. As granular methanogenic sludge is a complex ecosystem, species identification is a difficult task. Only presumptive identification of species is possible based on their morphology. Definite identification of the microbes present in such complex samples is possible only by specific probes like polyclonal or monoclonal antibodies.[52] Immunogold labeling, coupled with transmission electron microscopy, was first introduced already in 1971.[53] Bacterial associations in granules and biofilms were studied with specific immunological probes (polyclonal antibodies), developed for detecting *in situ* dominant species present in anaerobic digesters.[54] Cross-reactions of the probes within some genera are possible and represent one of the drawbacks for this technique.[55]

Methanogenesis, a limiting step, directly connected to the amount of methane produced, is an industrially interesting phase in the biogas production process. Several studies based on molecular biology have been made upon the structure of methanogenic communities in the biogas process as bio-molecular approach introduces useful bioindicators for early diagnosis of any unbalance in the microbial community.[56–58] Culture-independent molecular methods enable microbial community monitoring giving information about the quantity and identity of microorganisms, relating to different environmental conditions.[59,60] Microbial identification can be done using fluorescence *in situ* hybridisation (FISH), DNA microarrays or sequencing. The diversity and structure of microbial community can be determined by genetic fingerprinting techniques such as amplified ribosomal DNA restriction analysis (ARDRA), single strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (T-RFLP) and ribosomal intergenic spacer analysis (RISA). Microbial activity can be measured in batch tests as a specific methanogenic activity (SMA).[61–63] Culture-independent techniques are usually based on sequence divergences of the ribosomal small subunit RNA (16S rRNA). 16S rRNA is highly conserved in bacterial and archaeal species, but it also contains variable regions that yield a phylogenetic signal; therefore it is a most widely used target for phylogenetic identification.[64] Also, methyl-coenzyme M reductase (*mcr*) genes can be used as a phylogenetic marker. The *mcr*A gene is unique to the methanogenic archaea (except for methane-oxidizing archaea), encoding the α-subunit of methyl-coenzyme M reductase and offers a deeper investigation of methanogen population structure.[65–67] Molecular methods are able to get an insight into the microbial diversity in the biogas reactor, giving us qualitative and quantitative information

on bacterial and archaeal species and their changes in the microbial community caused by several factors discussed before.[16] Decision which technique is the most appropriate for certain study is made upon what information is needed and upon advantages and disadvantages certain technique possesses.

3. 1. Amplification and Quantification of 16S RNA Genes

Starting the molecular microbial community analyses, first nucleic acids need to be extracted from a sample of digester biomass. Secondly, 16S rRNA genes are amplified by polymerase chain reaction (PCR). High temperatures separate the double-stranded DNA and universal primers, complementary to the conserved regions of 16S rRNA genes, are annealed to it. DNA polymerase generates a new double-stranded DNA. Cycling of this process results in an exponentional amplification of all 16S rRNA genes of the microbial community in the sample. Amplified 16S rRNA gene preparations are also known as 16S rRNA gene amplicons.[16]

Standard PCR method is unreliable for quantification of the DNA present in the sample. Quantitative PCR (qPCR), also called real-time PCR (rtPCR), is more suitable for purposes to reliably quantify the amount of DNA. It is similar to standard PCR but a compound that fluoresces when bound to double-stranded DNA (PCR product) is added to the reaction mixture. The level of fluorescence in a test sample is plotted against the number of PCR cycles using a logarithmic scale. The amount of DNA present in the sample can be quantified by reference to a standard curve derived from parallel amplification of known target copy numbers.[68] The intensity of the fluorescence signal is proportional to the amount of DNA in the sample. Primers can be designed to target all bacterial and archaeal phyla or a single phylum or species. For example, targeting the *mcrA* gene with real-time qPCR offers an evaluation of abundance of methanogenic archaea in time.[58] Klocke et al.[69] used qPCR to quantify major methanogenic archaeal groups within the two-phase leach-bed biogas reactor. Their results showed that the dominant species were related to hydrogenotrophic methanogen *Methanoculleus.* Karlsson et al.[70] studied the influence of the addition of trace elements on anaerobic digestion of food industry- and household waste in semi-continuous reactors. An increase in *Methanosarcinales* was determined by qPCR analysis targeting methanogens on the level of the order. Quantification of the levels of three mesophilic syntrophic acetate-oxidizing bacteria *Syntrophaceticus schinkii*, *Clostridium ultunense* and *Tepidanaerobacter acetatoxydans* by qPCR showed high abundance of *S. schinkii* and its stable gene copy number during the operational period. The additions of trace elements did not have any impact on the growth of this microorganism. There were also higher degradation rates observed and lower concentrations of SCFAs detected. These results together with qPCR results prove that adding trace elements in a bioreactor affects the ability of the microbial community to degrade SCFAs. Although qPCR method is fast and gives much information, it is unable to identify unknown species. For providing more detailed information on diversity and abundance of microbial community, qPCR method is useful in combination with DGGE or DNA microarrays which are semi-quantitative.[16]

3. 2. Fingerprinting Techniques

DNA fingerprinting is a tool for microbial community analysis where DNA fragments in a sample are compared. There is no phylogenetic identification. Techniques most often used are DGGE, TGGE, T-RFLP, SSCP and RISA.

3. 2. 1. DGGE and TGGE

DGGE technique separates complex mixtures of 16S rRNA gene amplicons of the same length but different sequences. The mixture of 16S rRNA amplicons is applied on a polyacrylamide gel with linearly increasing gradient of denaturant (formamide or urea). An electric current is applied and the amplicons migrate though the gel. First, the fragments travel according to their molecular weight and as they are exposed to the increasing concentration of denaturant, the DNA strands begin to denature. At their specific melting point their migration stops. Therefore separation of the fragments is the consequence of different melting temperatures according to their DNA sequence variations.[71] TGGE works in a similar manner to DGGE, but a linear temperature gradient is used instead of a denaturing gradient gel. DGGE and TGGE are suitable for species identification as sequence variation between species exists in analyzed regions. They are both fast and semi-quantitative techniques and are usually used for comparative purposes, for example to compare biogas processes at different operant conditions where microbial community at the start and by the end of the experiment is analyzed. The bands can be excised from the gel for further sequencing or probe hybridization.[16] Malin and Illmer[30] applied the DGGE technique to monitor the community shifts in anaerobic fermenter sludge. Microbial community composition was analyzed and two clusters appeared on the gel. They excised dominant bands from the gel, reamplified and sequenced them and most sequences were closely related to *Lactobacilli* and yet uncultured microorganisms. They concluded that DGGE technique is suitable for microbial community monitoring although community shifts are not readily detectable by DGGE-pattern analysis, therefore other alternative factors influencing the function of fermenter should also be investigated. Worm et al.[72] studied the influence of the lack of molybdenum, tungsten and selenium in the medium on

the activity and community structure of propionate degrading bacteria in a propionate-fed upflow anaerobic sludge blanket (UASB) reactor. Strong bands of *Syntrophobacter*-like bacteria appeared in DGGE profiles at the start of the experiment. As the fermentation continued and methanogenesis decreased due to the lack of the elements, the DGGE bands of *Smithella propionica* relatives, clones related to *Pelotomaculum propionicicum* and *Chlorobium phaeobacterioides* got more intensive. The methanogenic activity decreased and a competition for propionate arised between relatives of *Smithella propionica* and *Pelotomaculum* spp. and *Syntrophobacter* spp.

3. 2. 2. T-RFLP

The T-RFLP technique is based on fragmentation of the 16S rRNA gene amplicons by restriction endonucleases to analyze possible changes in the microbial community structure from the start to the end of the biogas production process on lab-, pilot- or full-scale level. The similarity of T-RFLP profiles can be assessed statistically to evaluate significant differences in the structure of microbial communities and analyzed using hierarchical clustering algorithms.[73–75] Therefore T-RFLP gives information about the diversity, structure and dynamic of complex microbial community in anaerobic reactors (Fig. 3).[76] The main disadvantages are PCR bias and low resolution, but on the other hand it is fast, cheap and semi-quantitative, and as T-RFLP does not allow phylogenetic identification, it is often combined with 16S rRNA clone library analysis.[16,77]

For example, Figure 3 represents results of the T-RFLP analysis of the microbial community. Results showed significant shifts in the initial microbial community structure during 21 days of anaerobic digestion in samples with brewery spent grain and brewery wastewater supplemented up to 250 mg L^{-1} of *p*-cresol as an potential inhibitor of biogas production. On day 21 negative control and samples with brewery spent grain formed two distinct clusters at 39% dissimilarity for archaeal microbial community, which showed a high response to the addition of brewery spent grain.

McKeown et al.[78] were following the microbial community structure development in a cold (4–15 °C) anaerobic bioreactor treating industrial wastewater inoculated with mesophilic biomass. They used 16S rRNA gene clone libraries, qPCR and T-RFLP analyses to observe the bacterial and archaeal community shifts following start-up and during temperature decreases from 15 to 9.5 °C. Results showed that the relative abundance of *Methanosaeta*-like (acetoclastic) methanogens decreased and a psychrophilic hydrogenotrophic methanogenic community developed, where acetogenic bacteria and *Methanocorpusculum*-like (hydrogenotrophic) methanogens dominated. Genetic fingerprinting therefore allowed them to conclude that a well-functioning psychroactive methanogenic community can

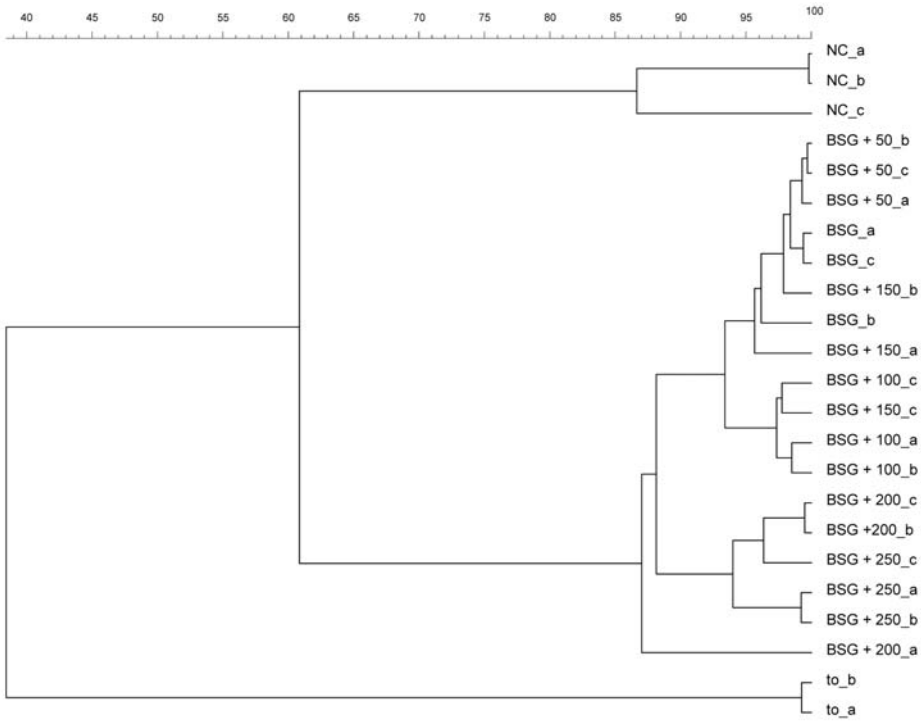

Figure 3: An example of T-RFLP analysis of archaeal communities. Pearson correlation dendrogram of archaeal T-RFLP fingerprints from BMP test with brewery spent grain and different concentrations of *p*-cresol (up to 250 mg L^{-1}) as a potential inhibitor. t_o – initial state; NC – negative control at t_{21}; BSG – sample with brewery spent grain at t_{21}; BSG + 50-250 – sample with brewery spent grain and appropriate concentration of p-cresol at t_{21}; a,b,c – parallels. (prepared by Fanedl, to be published)

be established also in psychrophilic cultivation of mesophilic biomass which represents a potential of low-temperature anaerobic digestion technology.

Zupančič et al.[79] successfully used T-RFLP technique to monitor the influence of excess of brewery yeast to brewery wastewater in UASB reactor on the microbial community in biogas plant. The results showed that the differences in archaeal community structure were small and not significant (up to 7%) but there were larger shifts detected in bacterial community (32% dissimilarity between the start of the biogas experiment and the end on day 189). The separate cluster of bacteria showing a strong response of bacterial community to the addition of waste yeast was detected. Bacterial community successfully adapted to the new substrate as the biogas production was efficient and stable. Dependency of bacterial community structure in anaerobic granules on substrate type has been previously proved by Nelson et al.[80]

3. 2. 3. SSCP

The single-strand conformation polymorphism (SSCP) technique is useful for detecting point mutations and DNA polymorphisms. It has been applied to study genetic diversity as it is fast, simple and offers large-scale screening.[81] First, genomic DNA is digested with restriction endonucleases. Digested DNA is then denaturated in alkaline solution and separated with electrophoresis on a neutral polyacrylamide gel. Then it is transferred to a nylon membrane where hybridization of single-stranded DNA fragments with probes (RNA copies, synthesized on each strand of the DNA fragment) occurs to detect the mobility shift, caused by the nucleotide substitution. SSCPs are useful genetic markers as they are allelic variants of true Mendelian traits, like restriction fragment length polymorphisms (RFLPs). Compare to RFLP analysis, SSCP analysis has the advantage of detecting DNA polymorphisms and point mutations at a variety of positions in DNA fragments.[82] Kampmann et al.[83] investigated the methanogenic community in biogas reactors digesting liquid manure, casein, starch, cream and other defined substrates. They used SSCP analysis and subsequent sequencing of the DNA bands to identify the key methanogenic microorganisms and to monitor the stability of the methanogenic community, while qPCR was used to quantify methanogenic *Archaea*. SSCP analysis revealed a stable community of few hydrogenotrophic methanogens, one species closely related to *Methanospirillum hungatei* and the other one distantly related to other methanogens related to *Methanopyrus kandleri*. Acetoclastic methanogens were identified only in the samples from the bioreactors fed with acetate and methanol, while all samples included different hydrogenotrophic methanogens. They suggested that ammonia concentrations in the manure of the laboratory biogas reactor were high enough to inhibit the growth of the acetoclastic methanogens.

3. 2. 4. RISA

Ribosomal intergenic spacer analysis (RISA) is based on the variation of the DNA length and sequence diversity of the region between 16S and 23S rRNA genes. RISA is a commonly used freshwater bacterial community analysis technique as it is successful in detecting community shifts, therefore it is also suitable for analyzing sludge samples from biogas reactors.[84] Boulanger et al.[85] used automated ribosomal intergenic spacer analysis (ARISA) for following the dynamics of methanogenic communities using archaeal domain. Several shifts in archaeal populations were found due to various inoculum to substrate ratios and these results show a great impact of the inoculum on further community structure.

3. 3. Species Identification Techniques

Probe hybridization techniques and sequencing are used for detection of gene expression and identification of microbes present in a biogas digester. *In situ* hybridization combined with PCR method enables also the examination of genes with low levels of expression.[86] First probes which hybridize with nucleic acid sequences were a radiolabeled DNA or 28S RNA. Later they were replaced with non-isotopic, fluorescent dyes which are safer, offer better resolution, do not need additional detection steps and are of different emission wavelengths, enabling detection of several target sequences within a single hybridization step. Fluorescent *in situ* hybridization (FISH) and DNA microarrays are probe hybridization techniques. FISH technique detects nucleic acid sequences by a fluorescently labeled probe that hybridizes specifically to its complementary target sequence within the intact cell.[87] Based on hybridization of specific oligonucleotide probes targeting specific taxonomic groups or species, these techniques are used for phylogenetic identification and quantification of species in a sample. The spot pattern of fluorescence can determine species profile, metabolic activities or expressed enzymes. Microarrays offer analysis of multiple microorganisms at a time, in contrast to qPCR analysis.[88] The populations dynamics in mesophilic anaerobic digesters can be studied with rRNA-based oligonucleotide probes for methanogens designed by Zhao et al.,[89] Raskin et al.,[90] Stams,[91] Harmsen et al.,[92] Hansen et al.,[93] Zheng and Raskin[94] and McMahon et al.[95]

For taxonomic identification to species level, sequencing is applied. It requires information from the full-length 16S rRNA gene and that can only be practically sequenced from a clone library insert. To identify the microorganism, the sequence is compared to a database, for example a specialized database The Ribosomal Database Project, which is specified for ribosomal RNA genes. To delineate the species taxonomic rank, a sequence divergence range of 0.5–1% is used and a 97% cut-off point is also used to define operational taxonomic units.[96] Sequencing can be performed directly on the 16S rRNA amplicon

or by excision of bands from DGGE, TGGE or T-RFLP gels, where reamplification of bands with PCR is needed.

3. 3. 1 FISH

FISH is a technique for detection and separation of microbial groups with different functions using order-level probes. It is suitable for studying microbial gene expression under different growth conditions and the influence of metabolites and chemicals on the different pathways at the level of single cell. Simultaneous visualization, identification and localization of individual cells are possible.[97] FISH is a rapid, semi-quantitative technique with no PCR bias, suitable for analyzing large number of environmental samples, though it is limited when carried beyond the limits of oligonucleotide probes. FISH is unable to identify unknown species as it is dependent on probe sequences.[16] Fluorescently labeled 15–25 bp long oligonucleotide probes are used to hybridize with target 16S rRNA gene sequences. Fixation of denaturated DNA in a hybridization solution with cross-linking agents or precipitating agents is a crucial step to ensure optimal results.[98] Added fluorescently labeled probes are incubated overnight in a hybridization solution at high temperatures (usually 65–75 °C). Combinating FISH with flow cytometry gives a high-throughput technique for analysis of mixed microbial community. Using flow cytometry, fluorescence can be detected and quantified when hybridization occurs, resulting in identification of the target species.[99] Whole microbial cells can also be fixed and then hybridized with specific probes on a glass slide where cells can be visualized by epifluorescence or confocal laser scanning microscopy.[75] Further on, FISH can be combined with microautoradiography (FISH-MAR), which links phylogeny and ecosystem function by *in situ* association of a certain phylotype to substrate uptake. An environmental sample is incubated with a radioactive substrate and fixed on a matrix for FISH analysis and microautoradiography. The analysis of both images shows the phylotype of bacteria which have incorporated the radioactive substrate.[88] The knowledge of its substrate preferences leads to a better optimization of the bioprocess. Ariesyady et al.[100] studied propionate degradation by syntrophic propionate-oxidizing bacteria coupled with hydrogen removal via methanogenesis by hydrogenotrophic methanogens. The level of uptake of propionate was high for the *Smithella* sp. and low for the *Syntrophobacter* spp., while the only MAR-positive archaeal cells were *Methanosaeta* cells. Kubota et al.[101] used an upgraded two-pass tyramide signal amplification FISH (two-pass TSA-FISH) to understand the *in situ* physiological activity of microorganisms. This TSA technique involves deposition of dinitrophenyl (DNP), followed by application of horseradish peroxidase-conjugated anti-DNP, and then incubation with fluorophore-labeled tyramide which produces more than 10-fold stronger signal.[102] A key enzyme for methanogenesis, methyl coenzyme M reductase (*mcr*), in *Methanococcus vannielii* was targeted. The fluorescence was spotty in the detected cells, which indicates that the target enzyme is very localizated. Therefore the number of mRNA copies for this enzyme is different among the cells, which reflects the *in situ* physiological activity of these cells. Quantitative determination of the spots found of interest is possible by connecting FISH technique with one of the fingerprinting techniques (usually with DGGE or T-RFLP) or even with sequencing.[103–105]

3. 3. 2. DNA Microarrays

DNA microarrays, known also as DNA chip technology, are a fast, semi-quantitative technique for phylogenetic identification of bacterial and archaeal species. It is based on the hybridization between extracted DNA sample or 16S rRNA amplicons, which are fluorescently labeled, and complementary oligonucleotide probes that are immobilized on a glass slide. When hybridization occurs, fluorescence can be detected using a laser. DNA microarrays can detect microbial DNA present at a level of 0.00025% of the DNA sample;[106] therefore this technology is high-throughput and enables screening of microbial structure and activities.[75,107] It is mainly used to compare the microbiota between different populations, though it has a few disadvantages like possible cross hybridization and PCR bias.[16,108] Franke-Whittle et al.[109] designed an ANAEROCHIP microarray targeting methanogens from anaerobic digesters with 103 probes and combined this chip together with a cloning approach for investigating the methanogenic community sampled from a thermophilically operated continuously stirred tank reactor anaerobic digestion plant. Hybridization of chips with DNA from an anaerobic sludge showed strong signal of dominating *Methanoculleus*, while signals for *Methanosarcina, Methanobacterium, Methanobrevibacter* and *Methanosphaera* were weaker. They also determined the same microbial community structure by 16S rRNA gene cloning, sequencing and by restriction digestion and the results were identical to those from the chip. This confirmed that the ANAEROCHIP microarray is a reliable tool for studying methanogenic communities in the sludge. Further on, Novak[110] used the ANAEROCHIP microarray for analyzing the changes in methanogenic community in the biogas plant due to the changes in substrate. His results showed that there were no significant changes in microbial structure when brewery spent grain was used as a feedstock nor when cyanide was used. However the microarray showed that the archaeal group of *Methanosarcina* is correlated to the increased cyanide concentrations, as it is resistant to higher concentrations of ammonia and acetate. Also a microarray for detecting bacterial communities in anaerobic ecosystems was developed and named COMPOCHIP.[111] It contains 369 gene probes specific to microbes involved in the degradation process of organic

waste. COMPOCHIPs were used for analysis of different types of composts (green compost, manure mix compost and anaerobic digestate compost). Detected differences in microbial communities were also supported by parallel DGGE technique.

Although microarray technology is yet not offering molecular testing on-line, nanotechnology has a great potential designing microchips based on microfluids with biosensors for real-time monitoring of the abundance level of certain microorganisms and the diversity and activities of the microbial community associated with specific operational parameters.[112]

3. 3. 3. Sequencing Using the Sanger Method

Sanger sequencing is suitable for phylogenetic identification, though it is very laborious and expensive, also PCR and cloning biases are possible.[16] For phylogenetic analyses it is based on the sequencing of cloned full-length 16S rRNA gene amplicons with chain termination by dideoxynucleotides.[113] The key principle insists in incorporation of terminator dideoxynucleotides during the replication, resulting in stop of strand elongation. The mix of randomly terminated DNA fragments is separated by capillary gel electrophoresis and as the terminators are fluorescently labeled, emitting different wavelength each, the sequence can be read by a laser. The results are presented in a chromatogram and compared to databases.[16] Ács et al.[114] tested how casein and pig blood as a sole substrate added to continuously stirred tank reactor anaerobic digester influence the methanogenic microbial community. T-RFLP and Sanger sequencing of *mcrA* and 16S rRNA genes combined with capillary gel electrophoresis revealed that substrate composition influences archaeal community and their activity although archea are not directly active in decomposition of protein-rich substrates. The results showed that the archaeal community adapted to the new substrate in 5 weeks and that *Methanoculleus* species play a dominant role in the mesophilic anaerobic digester.

The diversity and composition of microbial community can also be examined by 16S rRNA clone libraries and ARDRA analysis. Yan et al.[115] investigated a mesophilic lignocellulolytic microbial consortium, which was previously proven to be successful for enhancing the biogas production. They amplified the 16S rDNA gene, cloned it into a vector and transformed it into *Escherichia coli* TOP 10. The 16S rDNA gene inserts in the *E. coli* transformants were amplified using vector universal primers. PCR products were analyzed by restriction digestion, and the restriction fragments were separated on agarose gels and grouped based on DNA fingerprinting. The representative cloned fragments were then sequenced. Results showed that *Firmicutes*, *Bacteriodetes*, *Deferribacteres* and *Proteobacteria* dominate in 16S rDNA clone library. Also *Lentisphaerae* and *Fibrobacteraceae* were detected.

3. 3. 4. Next-Generation Sequencing

As an improvement of Sanger's method of sequencing, the next-generation sequencing techniques were developed which are not applied to cloned amplicons but to total community DNA directly. They are expensive and laborious, but fast and they offer phylogenetic identification also of unknown species. Many DNA templates can be sequenced in parallel using commercial technologies available like 454 Pyrosequencing® (Roche Diagnostics GMBH Ltd, Mannheim, Germany) which uses beads, Illumina® (Illumina, San Diego, CA, USA) which uses slides and SOLiD™ (Life Technologies, Carlsbad, CA, USA) which uses solid surfaces.[16] Pyrosequencing is a very high-throughput method, being able to sequence 500 million bases, at 99% or better accuracy, in a single run.[116] Also bacteria in low concentrations present in a sample can be detected due to the advantage of the next-generation sequencing that more samples can be sequenced in parallel.[117] Apart from phylogenetic identification, pyrosequencing also provides quantitative data. Therefore it is mainly used to compare the microbial communities between different states, for example from the start to the end of the biogas process or at different stages of it or to detect the effect of variations, added to the process, on the microbial communities. Schlüter et al.[118] used 454-pyrosequencing to determine the dominance of methanogenic species related genus *Methanocelleus* and prevalence of hydrolytic clostridia in agriculture biogas plant.

Metagenomics, also known as community genomics, is capable of determining the association between a given microbial pattern and physical conditions in a biogas reactor at the time of sampling. It enables a view into genetic diversity and functions of the microbiota. Metagenomics can also be used to explain whether the state of microbial community is a cause or effect of given conditions in a bioreactor.[16] Metagenomic approach is based on sequencing all the DNA fragments in the sample.[119] After isolation, DNA is first randomly fragmented and inserted into appropriate vectors. Then DNA cloning and transformation of suitable host is performed, constructing a metagenomic library. For construction of metagenomic libraries vectors (plasmids, cosmids, fosmids or BAC vectors, depending on the length of the insert) are commonly cloned into host cells of E. coli, although several other host strains are in use as well. After constructing the library, clones need to be screened. Sequence-based screenings are based on nucleotide sequences and are used to investigate microbial diversity by analysis of conserved rRNA gene sequences. Sequence-driven strategy can also be used for direct evaluation of shotgun sequencing-derived datasets. Microbiome shotgun sequencing therefore involves massive parallel sequencing of the whole community DNA and is computationally intense. The ability to assemble sequences recovered from shotgun libraries from complex microbial communities decreases with the increased complexity of the community. The metagenomic

output is then collected and shared in public databases.[120] The results from metatranscriptome analysis should be carefully interpreted as the metatranscriptome data usually do not represent the whole complexity of transcripts synthesis by a microbial community. Zakrzewski et al.[14] used a metagenome sequencing approach for analyzing structural composition and gene content of a microbial community from anaerobic fermenter of the biogas plant. They detected transcriptionally active members and their functions within the microbial community by 16S ribosomal sequence tags of the metatranscriptome dataset. This way it is possible to gather information about the metabolically active part of the community. The microbial community consisted of 24% archaea and 76% bacteria. On the other hand, high-throughput 16S rDNA amplicon sequencing showed that there are about 87% of bacteria and only about 2.4% of the sequences were classified as archaea. The results showed that members of the *Euryarchaeota* and *Firmicutes* play the dominant role in the active part of the community, followed by *Bacteroidetes*, *Actinobacteria* and *Synergistetes*. Transcripts for enzymes involved in methanogenesis were one of the most abundand mRNA tags. Therefore key transcripts for the biogas production can represent a valuable marker for biogas fermenter performance stability which can be further on used for optimizing the biogas process. Jaenicke et al.[15] analyzed metagenome from a production-scale biogas fermenter with Roche's GS FLX Titanium technology and taxonomic profiling based on 16S rRNA-specific sequences and an Environmental Gene Tag (EGT) analysis. About 70% of EGTs were classified to bacteria and about 10% to archaea.

4. Conclusion

Composition, dynamics and metabolic activity of microbial communities co-operating in biogas production process is very complex however the knowledge on composition of the microbial communities and interactions among the microorganisms involved is still insufficient. Molecular methods and techniques help us improve our understanding of the processes in biogas reactors on functional metabolic and interactions levels. They enable identification and enumeration of microorganisms present in the anaerobic sludge (qPCR, FISH, sequencing) and information about their activity, growth or inhibition under certain microenvironmental conditions (DGGE, TGGE, T-RFLP). General community flexibility monitoring helps to diagnose anaerobic processes under dynamic conditions.[121] Substrate overload, inflow and generation of toxic compounds, different types of substrate pretreatments and variations in physical and chemical conditions (pH, temperature, SCFA, ammonia, phenols, heavy metals and others) can decrease the efficiency and possibly lead to a biogas process breakdown and consequently high economical loss in large full-scale biogas reactors. Avoiding those critical points is possible by careful monitoring of the biogas process. Combining the monitoring of standard chemical and technological parameters with monitoring of the structure and metabolic activity of microbial communities, responsible for biogas production, is needed for optimizing the sustainable biogas production processes and keeping them on high production level.

5. Abbreviations

T-RFLP, terminal restriction fragment length polymorphism; DGGE, denaturing gradient gel electrophoresis; FISH, fluorescent *in situ* hybridization; qPCR (rtPCR), quantitative (real-time) polymerase chain reaction; SCFA, short chain fatty acid; ARDRA, amplified ribosomal DNA restriction analysis; SSCP, single strand conformation polymorphism; TGGE, temperature gradient gel electrophoresis; (A)RISA, (automated) ribosomal intergenic spacer analysis; SMA, specific methanogenic activity; UASB reactor, upflow anaerobic sludge blanket reactor; FISH-MAR, FISH with microautoradiography; TSA-FISH, tyramide signal amplification FISH; DNP, dinitrophenyl

6. References

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Povzetek

Pogoj za uspešno proizvodnjo bioplina sta stabilna ali prilagodljiva mikrobna združba in njena aktivnost, ki je močno odvisna od tipa uporabljenega substrata in številnih fizikalno-kemijskih pogojev v bioreaktorju. Za načrtovanje in optimizacijo bioplinskega procesa je potrebno spremljanje teh dejavnikov in dinamike mikrobiote. Le tako se je možno izogniti kritičnim točkam v procesu in doseči največji izplen metana. V preglednem članku so opredeljeni možni vzroki za nestabilnost bioplinskega procesa in različne molekularne metode za analizo mikrobne združbe v bioplinskih reaktorjih. Metanogene mikroorganizme je namreč izredno težko proučevati z gojitvenimi metodami. Molekularne metode ponujajo kvalitativne in kvantitativne informacije o zastopanosti bakterijskih in arhejskih mikrobnih vrst in o njihovih morebitnih spremembah znotraj mikrobne združbe. Za primerjalne študije so primerne polkvantitativne, hitre in cenovno ugodne tehnike kot so T-RFLP, DGGE in TGGE. Za filogenetske analize pa se uporabljajo zahtevnejše tehnike kot so FISH, DNA mikročipi, kvantitativni PCR ter sekvenciranje. Gre za dražje tehnike, s katerimi pridobimo veliko število rezultatov naenkrat. Primernost posamezne tehnike za določeno študijo je odvisna od narave informacije, ki jo potrebujemo ter od številnih prednosti in slabosti same tehnike.