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Bachelor Thesis

Role of methanogens in biogas production plants

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December 2012

Annotation

The aim of this work is to give an overview of the biogas production in anaerobic digesters and the methanogenic community involved in this process.

České Budějovice, December 2012   Lukas Penz

Acknowledgements

List of Abbreviations

AG	Aktiengesellschaft (Joint-Stock company)
BLASTn	Nucleotide-nucleotide Basic Local Alignment Search Tool
C	Carbon
CHP	Combined heat and power unit
CO_2	Carbon dioxide
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
FISH	Fluorescence *in situ* hybridization
H_2S	Hydrogen sulphide
IPC	Internal positive control
*mcr*A	Methyl-coenzyme M reductase α-subunit
MPN	Most probable number
N	Nitrogen
NGE	Naturgas Engerwitzdorf GmbH
NH_3	Ammonia
NH_4^+	Ammonium
OÖ	Oberösterreich (Upper Austria)
P	Phosphorus
qPCR	Quantitative real-time polymerase chain reaction
S	Sulphur
TGGE	Temperature gradient gel electrophoresis
T-RFLP	Terminal restriction fragment length polymorphism
VFA	Volatile fatty acid

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1. Introduction

Due to the increased demand for energy and the high dependence on fossil fuels, the need for renewable energy sources is certainly going to increase over the next decades. Therefore there is a great need for alternatives, and the reduction of fossil fuel-derived CO_2 emission is another vital aspect for which it is important to find sustainable solutions (Weiland, 2010).

One possible alternative to fossil fuels is the production of biogas by anaerobic digestion of wastes, residues and energy crops. This form of energy production has several advantages, such as the use of local resources, a reduction of the greenhouse gas emissions and it has been evaluated as a very effective form of bio energy production (Fehrenbach et al., 2008). As a direct result of this, the production of biogas and the construction of biogas plants have increased in the countries of the European Union over the past years. Moreover, the sector has also gained more and more economic recognition (Abraham et al., 2007).

In order to obtain the desired biogas a conversion of the organic material has to occur inside the biogas digester under set up conditions. This biological formation of methane (methanogenesis) is the last step in the degradation of biomass. Methanogenesis itself can be divided into three major pathways: the CO_2 reduction pathway, the acetotrophic pathway and the methylotrophic pathway (Ferry, 2010). The microorganisms involved in this process are called methanogens and belong to the domain of the *Archaea*.

Very little is known about the interaction of the microorganisms inside a biogas reactor. Therefore it is important to understand and describe the microbial diversity and growth dynamics inside the bioreactor in order to further optimize the conditions of biogas production (Weiland, 2010).

2. Biogas plants

2.1 Structure of a typical biogas plant

In figure 1 the typical structure of a biogas plant is depicted. However, some constituent parts can be added depending on the specific type of biogas plant. In this section the parts that are necessary for the biogas production itself will be shortly described, whereas the cleansing step and usage are discussed later.
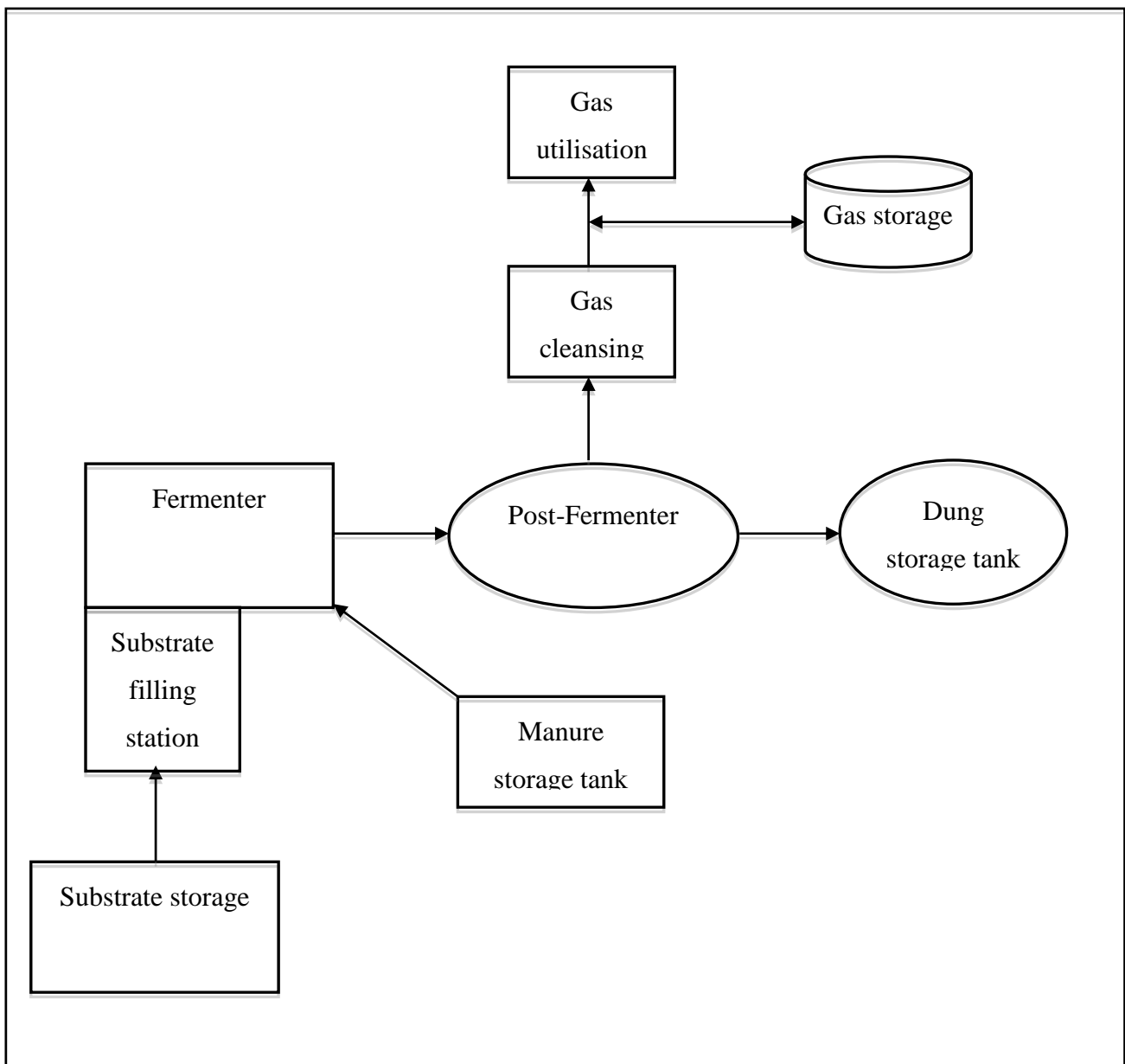
Figure 1: Constituent parts of a biogas plant. Adapted from Stifter, 2011.

2.1.1 Manure storage tank and substrate storage

In these two components the starting materials are stored. On the one hand the liquid manure from cattle and pig is stored and then pumped into the fermenter, on the other hand the so called co-ferment, mostly energy crops, is stored by ensiling and filled into the fermenter via the substrate filling station.

2.1.2 Fermenter

The fermenter, also often referred to as digester, is the core part of every biogas plant where the anaerobic digestion takes place. The fermenter is equipped with a stirring device to ensure proper mixing of the newly added and the old substrates. The mixing is also important to avoid deposits and also to maintain a constant temperature (Dobelmann 2004). In addition to assure a constant temperature the fermenter is usually equipped with a heating system. The required heat energy is often produced at the site of the biogas plant itself with a combined heat and power unit (CHP) (Dobelmann, 2004).

In general fermenters can be divided into horizontal and vertical ones. Vertical fermenters have the advantage of minimizing heat loss and lower cost of material. Horizontal fermenters have the advantage of avoiding the mixing of newly added substrate with already decomposed material at the other end of the fermenter (Jüngling, 1999; Öchsner & Knebelspieß, 1999).

2.1.3 Post-fermenter

Post-fermenters are used to maximize the biogas yields by also capturing the methane emissions caused by secondary fermentation. Moreover the post-fermenter is used as storage tank as the products of fermentation are usually not immediately used as dung (Dobelmann, 2004).

2.2 Production of biogas

The production of biogas follows the normal anaerobic digestion process with the only exception that the conditions are set up artificially. The process can be divided into four major steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Watter, 2009).

During hydrolysis and acidogenesis, complex polymers are attacked and broken down by hydrolysing and fermenting microorganisms. These microorganisms produce exoenzymes as for example amylase, lipase, cellulase and protease which degrade the polymers to acetate, hydrogen, carbon dioxide, alcohols and volatile fatty acids such as butyrate and propionate (Weiland, 2010; Watter, 2009). The bacteria involved in these processes are mostly strict or facultative anaerobes as for example Bacteriocides, Clostridia and Streptococci (Weiland, 2010).

In the third step of the process, the acetogenesis, the fatty acids are broken down into acetate and hydrogen by acetogenic bacteria. Examples of involved bacteria are *Acetobacterium woodii* and *Clostdridium aceticum* (Weiland, 2010).

During the last step of the process acetate, carbon dioxide and hydrogen or methanol are converted into methane and carbon dioxide, the so-called biogas. These conversions are carried out by a class of microorganisms called methanogens that are described in detail in a later section (chapter 3) of this work.

A close interaction of all the involved microorganisms is of utter importance for the biogas production. This is especially true for a well-balanced partial pressure of hydrogen. A too high concentration of hydrogen can hinder the metabolism of the acetogenic bacteria. Therefore it is important that the hydrogen is constantly being used up by the methanogens in order to avoid a breakdown of the whole process (Weiland, 2010).

Other factors which highly influence the biogas production are the process conditions at which the digestion process is carried out.

2.2.1 Oxygen

Most of the involved microorganisms, in particular the methanogens, are strict anaerobes and therefore oxygen exclusion is necessary for a functioning digestion process. Nevertheless, it is difficult to guarantee total anoxic conditions in a fermenter. One reason why the metabolism of the methanogens is not immediately inhibited if oxygen occurs, is the presence of facultative anaerobic bacteria that use the oxygen before it kills other microorganisms (Schattauer & Weiland, 2006).

2.2.2 Temperature

Most fermenters are operated at mesophilic (32-42°C) temperature conditions while only a minority is operated at thermophilic (50-57°C) temperature conditions (Schattauer & Weiland, 2006). It is of great importance, however, to have a constant temperature inside the fermenter in order to achieve a constant biogas production. It is therefore more recommendable to run digestion processes at mesophilic temperature conditions since mesophilic microorganisms are more tolerant to slight temperature fluctuations (Weiland, 2010). This is in contrast to the fact that chemical reactions generally proceed faster if temperatures are higher (Schattauer & Weiland, 2006). Consequently one could assume thermophilic temperature conditions should be favoured. It is important to acknowledge, however, that the risk using thermophilic conditions is higher and therefore these conditions are not applied very often.

2.2.3 pH

Similarly to temperature conditions, a pH interval must be obtained that is tolerated by the whole consortia of microorganisms involved in the digestion process. The optimal pH range for hydrolysing and fermenting bacteria is between 4,5 and 6,3. In contrast, methanogens and acetogens need a pH in the range of 6,5-8,5 with an optimal methane production at a pH interval of 7,0-8,0 (Schattauer & Weiland, 2006; Weiland, 2010). Fortunately, the hydrolysing and fermenting bacteria can also operate at a slightly higher pH value and are thereby only slightly inhibited. A typical pH interval at which a fermenter operates is therefore in the neutral region of around 6,8-7,5 (Schattauer & Weiland, 2006). In general, the pH value in the fermenter is regulated by the degradation process itself and held at a neutral interval by a buffer system. Nevertheless, it may happen that the buffer capacity is exhausted and that the pH drops to a value which inhibits the methanogens. Consequently, the pH value drops even further due to an accumulation of volatile fatty acids which are no longer broken down to methane and carbon dioxide. In this case, the addition of substrate to the fermenter must be

stopped in order to allow the methanogens to degrade the volatile fatty acids while no new acids can be generated due to a lack of substrate (Schattauer & Weiland, 2006; Weiland, 2010).

2.2.4 Nutrients

Incoming substrate does not only allow a high methane production but should also provide the necessary nutrients. In order to guarantee a sufficient supply of nutrients for specific microbial groups in biogas production, the substrate C:N:P:S ratio should be around 600:15:5:1 (Weiland, 2010). In addition, the presence of some trace elements is necessary. These include iron, nickel, cobalt, selenium, molybdenum and tungsten. For methanogens, the presence of nickel and cobalt which are necessary for the cofactor F_{430} and the corrinoid factor III are particularly important respectively (Weiland, 2010). One possibility to avoid the artificial addition of micronutrients is the use of energy crops and manure as substrate. Thereby the micronutrients are provided by the manure. However, an artificial addition of micronutrients can still upgrade the digestion process (Weiland, 2010).

2.2.5 Inhibiting factors

The digestion process is also influenced by inhibitory factors. Inhibitory factors can either be introduced into the system via the substrate or they can be intermediates which are generated during the digestion process (Schattauer & Weiland, 2006). Some examples for substrate related inhibitors are antibiotics, herbicides, salts and heavy metals. Already small amounts of these substances can inhibit the microbial degradation. However, it is difficult to define concentrations toxic to the microorganisms as they are able to adapt to new conditions (Schattauer & Weiland, 2006). Two inhibitory factors that are generated by the digestion process itself are ammonia (NH_3) and hydrogen sulphide (H_2S). The ammonia concentration increases at a higher pH and with higher temperatures as the equilibrium of ammonium and ammonia is thereby shifted towards ammonia (Maehnert, 2007). In contrast to NH_4^+ which acts as a nitrogen source, ammonia shows inhibitory characteristics already in small concentrations (Schattauer & Weiland, 2006). Hydrogen sulphide can act as an inhibitory factor in two ways. In its dissolved form it acts as a strong cell toxin whereas it can precipitate essential trace elements as sulphides too (Schattauer & Weiland, 2006).

Furthermore, sufficient moisture (min. 50% water) has to be available in order to allow microbial growth (Watter, 2009).

2.2.6 Substrates

"All types of biomass can be used as substrates for biogas production as long as they contain carbohydrates, proteins, fats, cellulose and hemicelluloses as main components" (Weiland, 2010). This citation might indicate that a larger variety of substrates is used in the biogas production. However, substrates are used with the aim of maximizing the methane content of the produced biogas. This is of importance as the methane yield of the different substrates depends on their composition. Carbohydrates and proteins are converted at a higher rate than fats but provide a lower biogas yield (Weiland, 2010). Therefore, so-called energy crops are chosen according to their net energy yield per hectare and in most cases a mixture of renewable primary products such as grass- or maize silage and manure is used (Stifter, 2011; Weiland, 2010). The advantages of energy crops such as maize and rye-whole crop silage are well-known harvesting practices and an easy storage by ensiling. The process of ensiling can be regarded as a pre-treatment of the substrate by which the pH value is considerably lowered to inhibit the growth of detrimental microorganisms.

Another important fact concerning ensiling is the compacting of the silage and the sealing by means of plastic wraps to avoid aerobic degradation before usage. Moreover, the substrate should be added to the fermenter continuously in order to avoid fluctuation in the process conditions (Weiland, 2010).

2.3 Composition of biogas and its utilisation

Biogas which is produced by the processes described on the previous pages is generally obtained in the following composition (Table 1). The two major components are methane and carbon dioxide. Other components include hydrogen, water, hydrogen sulphide, nitrogen, oxygen and trace amounts of dust particles (Stifter, 2011; Schattauer & Weiland, 2006).

Components	Concentration
Methane (CH_4)	50-75 Vol%
Carbon dioxide (CO_2)	25-45 Vol%
Water (H_2O)	2-7 Vol%
Hydrogensulfide (H_2S)	20-20000ppm
Nitrogen (N_2)	< 2 Vol%
Oxygen (O_2)	< 2 Vol%
Hydrogen (H_2)	< 1 Vol%

Table 1: Average compostion of biogas (Schattauer & Weiland, 2006)

Before the biogas can be used it has to be cleaned. This includes the desulfurization, drying and the removal of carbon dioxide. One popular possibility of performing desulfurization is the oxidation of H_2S in the presence of *Sulfobacter oxydans*. The bacterium converts the H_2S into elementary sulphur and sulphurous acid (Weiland, 2010). This can easily be achieved by the injection of air into the headspace of the fermenter. Desulfurization is particularly important due to possible corrosion. The drying of the gas can be achieved by cooling the gas and consequent condensation of the water which is collected and channelled away (Watter, 2009). Some possible methods for the removal of carbon dioxide are the use of scrubbers or the use of adsorption at activated carbon or molecular sieves (Weiland, 2010). The cleaned biogas can now be used to generate electricity in a combined heat and power unit (CHP) or it can be upgraded and injected into the grid (Weiland, 2010).

2.4 Naturgas Engerwitzdorf GmbH (NGE) – case study of a local biogas plant

The biogas plant in Engerwitzdorf, Upper Austria, is a project realized by a cooperation of Naturgas Engerwitzdorf GmbH, OÖ Ferngas AG and erdgas OÖ. The aim of the project was to provide an energy supply from renewable resources which can be cultivated in the local surroundings. After three years of intensive planning the biogas production was finally started in November 2010.

The biogas production falls into the agenda of NGE, a company owned by four innovative farmers. They sell the produced biogas to OÖ Ferngas AG that operates a gas treatment plant and provides the upgraded biogas to the grid.

The production of the biogas is achieved by a mixture of energy crops and manure. In the year 2010 the crops used were maize, millet, sunflower and whole plant silage (rye, triticale). The manure was either from pigs or cattle. The two components are mixed in the fermenter which is operated at mesophilic temperature conditions of 40°C. As has already been mentioned earlier, these conditions offer a good environment for the involved microorganisms and also tolerate slight fluctuations in the process conditions. In order to guarantee a constant supply of organic material, the farmers have to add 25 to 30 tons of biomass, which is stored by ensiling, each day. The substrate is then used in the fermenter for 45 days. This shortened storage time is only possible because a post-fermenter is also used.

Desulfurization is achieved with the aid of liquid oxygen and the sulphur is bound to the biogas slurry at the bottom of the mixture. This effect makes the biogas slurry a good fertilizer which is used on the fields again. The operators of the biogas plant are obligated to provide biogas with a methane content of more than 50%. In case this value should not be reached, the biogas is once again conducted through the whole facility (Stifter, 2011).

From this short summary of biogas production it can be seen that this form of energy production is environmentally friendly. The energy crops are locally harvested and the biogas slurry can be distributed on the same fields again. The biogas plant produced 135-140 m^3 of clean gas per hour in the first half of the year 2011 and an increase of this value is planned for the coming years, as the plant is designed to provide energy for 4000 households (Stifter, 2011).

3. Methanogen community in biogas plants

3.1 Specific aspects of methanogens
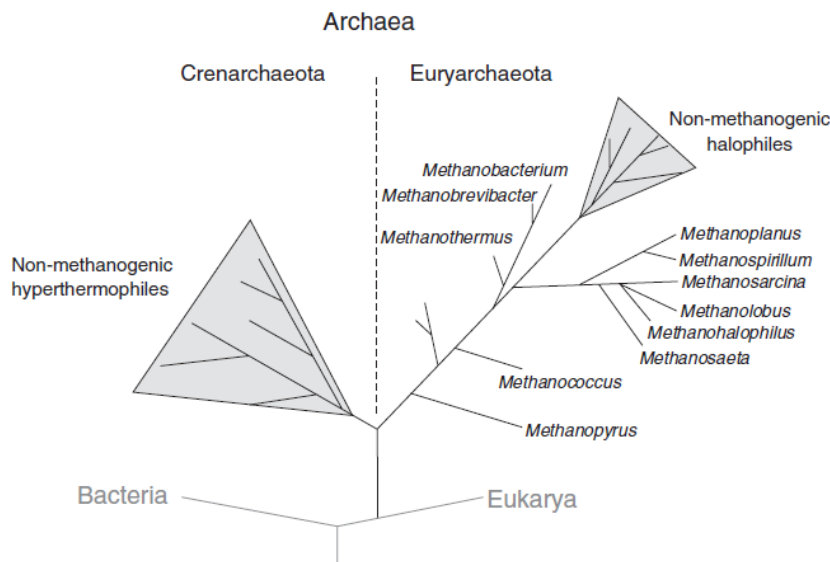
Figure 2: Phylogenetic tree of *Archaea* with selected genera of methanogens (Sowers 2009).

Methanogens belong to the domain of the *Archaea* (Figure 2) and can be divided into five orders Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales and Methanopyrales (Sowers, 2009; Madigan et al., 2009). A possible sixth order, the Methanocellales, has recently been discussed (Ferry, 2010). The methanogens are extremely diverse and range from psychrophilic species to thermophilic species, from acidophilic species to alkaliphilic species and from halophilic species to non-halophilic species. An example for an acidophilic and thermophilic methanogen is the *Methanotorris igneus* with optimum growth conditions at a pH of 5,7 and 88°C. By contrast, the *Methanobacterium subterraneum* has its optimal growth conditions at a pH between 7,8-8,8 and temperatures between 20-40°C. However, most methanogens are mesophiles and non-halophilic and grow at a neutral to slightly alkaline pH (Madigan et al., 2009; Sowers, 2009). The most unifying characteristic of methanogens is that they all produce methane (Sowers, 2009).

3.2 Methanogenic diversity in biogas reactors

As the production of biogas is highly dependent on the microbial community in the reactor the study of its diversity is of growing interest. Nettmann et al. (2009) performed a study on ten agricultural biogas plants operated at a mesophilic temperature and with renewable primary products. The study revealed that the Methanomicrobiales is the predominate order in nine of ten analysed reactors. The most abundant genus was the *Methanoculleus* (Nettmann et al., 2009). Kröber et al. (2009) also found out that most methanogens in a biogas plant operated on maize silage and liquid manure belong to the genus *Methanoculleus*. Other studies also revealed the Methanomicrobiales as most abundant order, however in these studies the dominate genus was the *Methanocorpusculum* (Klocke et al., 2007; Rastogi et al., 2008). As most species in the order Methanomicrobiales grow by CO_2 reduction with hydrogen as electron donor (Sowers, 2009), it can be assumed that this is the main metabolic pathway in these reactors.

However, also other orders are present in agricultural biogas plants. According to Nettmann et al. (2009) members of the order Methanobacteriales can be found in seven of ten biogas reactors. Moreover also members of the genus *Methanosaeta* are present in seven of ten reactors (Nettmann et al., 2009). The presence of this genus introduces the acetotrophic pathway for the generation of methane. Nevertheless, according to Nettmann et al. (2009), the majority of methanogens in agricultural biogas reactors is expected to use the CO_2 reduction pathway for growth. This is in contrast to other literature sources in which the acetotrophic pathway is described as the dominate metabolic pathway (Ahring, 2003).

Certainly it is of great interest now to know which parameters influence the methanogenic diversity inside the reactor. On the one hand the used substrates and the hydraulic retention time seem to have little influence on the microbial community (Karakashev et al., 2005; Nettmann et al,. 2009). On the other hand abiotic factors, as for example oxygen, temperature, pH, total ammuniom content and the amount of volatile fatty acids (VFA) are expected to play an important role (Nettmann et al., 2009). According to Rastogi et al. (2008) the ambient temperature has a great influence on the methanogenic community in biogas reactors, although constant temperature is of high priority in any biogas fermenter. The study revealed that there is a seasonal shift in the microbial community from summer to winter, resulting in a

much lower microbial diversity during winter times. In areas with seasonal temperature variations the genus *Methanocorpusculum*, order Methanomicrobiales, is often found, as it optimally grows at mesophilic temperatures, however they are also psychrotolerante (Simankova et al., 2003). Furthermore the total ammonium concentration influences the methanogenic community to a large extend (Gavala et al., 2003; Nettmann et al., 2009). A high total ammonium concentration can inhibit the growth of strictly acetotrophic methanogens (Nettmann et al., 2009). Nevertheless the inhibitory mode of action of ammuniom on the microbial consortium is still under discussion and needs further investigation.

4. Methods for the detection of methanogens

4.1 Microscopic and microbiological methods

One possible method to detect methanogens is the most probable number (MPN) determination. Ludvigsen et al. (1999) used MPN on methanogens as this serial-dilution method is especially applicable on specific physiological types by varying the growth medium (Spiegelmann et al., 2005). This of course also provides a limitation to this method as it might happen that the growth medium does not support all the methanogens present (Burlage et. al., 1998). Another drawback of this method is that it is a time consuming assay due to long incubation times which might take up to six months (Ludvigsen et al., 1999).

Another possible method to detect methanogens is epifluorescence microscopy. This method is applicable on methanogens as they contain the autofluorescence cofactor F_{420} which fluorescence blue-green when excited (Burlage et al., 1998). Nevertheless, the method has the drawback that the F_{420} concentrations vary a lot within the methanogens, even to such a low level that it is no longer detectable (Gorris & Drift, 1994). This means that a non-fluorescence microbe is not automatically to be classified as a non-methanogen (Burlage et al., 1998). Regardless of this disadvantage the method assisted in the qualitative identification of methanogens in natural habitats (Burlage et al., 1998).

4.2 Molecular methods

4.2.1 Quantitative real-time PCR (qPCR)

qPCR is a powerful method to detect methanogens, as it allows a precise quantification of the analysed target, which is in contrast to conventional PCR (Zhou et al., 2011). The quantification in qPCR is performed in real-time by the use of dsDNA binding dyes or fluorescently labelled probes and the measurement of their fluorescent signal. The intensity of the signal is then compared to a reference to quantify the target (Tolvanen & Karp, 2011). Bergmann (2012) used qPCR in a study on the methanogenic *Archaea* of biogas reactors, as it is a very sensitive method (Lebuhn et al., 2004) that allows high throughput capacity (Vandesompele et al., 2002) and offers a wide dynamic range of quantification (Klein, 2002).

However, samples from biogas reactors are rich in humic acids which act as inhibitors on DNA polymerase (Dionisi et al., 2003) and therefore their removal is of great importance. In order to test the quality and purity of the extracted DNA, spiking and recovery experiments, as for example the addition of an internal positive control (IPC) should be carried out (Bergmann, 2012). Moreover, a major limitation for the quantification is the possible difference in the gene copy number of different targeted methanogens when targeting 16S rRNA genes (Zhou et al., 2011). However, Steinberg and Regan (2009) used the methyl-coenzyme M reductase α-subunit (*mcr*A) genes as target in their qPCR study on methanogens and thereby overcame the limitations arising when targeting the 16S rRNA genes. Further advantages of the *mcrA* gene are, that it is exclusive to methanogens and methanotrophic *Archaea*, highly conserved and shows congruent phylogeny to the 16S rRNA gene (Steinberg & Regan, 2009).

4.2.2 Denaturing gradient gel electrophoresis (DGGE)

DGGE is a commonly used method for community characterizations that is based on the melting behaviour of DNA fragments in a special polyacrylamid gel (Spiegelman et al., 2005). The gel contains a linear gradient of denaturant, for example urea or formamide, which causes the denaturation of the DNA fragment according to its GC content and nucleotide sequence. It is thereby possible to achieve a separation based on single base pair differences of the fragments, provided that the gel is well calibrated (Zhou et al., 2011). Apart from the high resolution, DGGE is able to provide a good comparison of methanogenic communities as it is possible to compare the different band patterns of the gel. Wang et al. (2010) used this approach of comparing DGGE profiles to analyse the microbial community in a biogas reactor. They analysed the change in the archaeal community while changing the ratio of the added substrates, namely grass silage and cow manure. Thereby Wang et al. (2010) demonstrated that the archaeal community is only affected to a minimal extend by changes in the substrate composition. Moreover, Wang et al. (2010) state that they regard DGGE a potent approach to analyse the connection of bioreactor performance and microbial community structure. Furthermore, to ensure analysis of the whole diversity of the methanogenic community the primer selection in the DGGE experiment is very important. Hwang et al. (2008) constructed methanogenic profiles by DGGE in anaerobic sludge digestion and found that when using universal archaeal primers several methanogens involved in the degradation process were not detected. In order to provide insight into the whole methanogenic community it is therefore necessary to use primers targeting lower taxonomical levels, as for example at the order level (Hwang et al., 2008). Drawbacks of DGGE are its lower sensitivity

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compared to T-RFLP (Wang et al., 2010) and that the interpretation of very complex community samples can be difficult due to an overload of bands (Spiegelman et al., 2005). A possible modification of DGGE is temperature gradient gel electrophoresis (TGGE) in which the gradient of denaturants is replaced by a temperature gradient, yet following the same separation principles.

4.2.3 Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP is an analysis method based on the separation of labelled DNA fragments according to their size (Moeseneder et al., 1999). The label is introduced during PCR amplification via one labelled primer and the restriction digestion is performed by restriction endonucleases. The actual separation of the fragments is carried out by gel electrophoresis or by capillary electrophoresis (Spiegelman et al., 2005; Tolvanen & Karp, 2011). Wang et al. (2010) also used T-RFLP for their investigation on the microbial community of a biogas reactor under the influence of varying substrate composition. Thereby the finding of the DGGE analysis, the fact that the archaeal population is only little affected by the substrate composition, could be confirmed. An advantage of T-RFLP is, however, the better resolution in complex microbial consortia (Wang et al., 2010; Schwarz et al., 2007). In another study conducted on the methanogenic population in a bioreactor by T-RFLP, Padmasari et al. (2007) analysed the methanogens while changing the loading rate of the bioreactor. They found out that methanogens belonging to the order Methanomicrobiales and Methanosarcinales were dominant throughout the analysis. However, their relative abundances were fluctuating due to different concentrations of acetate and hydrogen. An increase in hydrogen production inside the bioreactor was accompanied by an increasing abundance of *Methanomicrobiales spp.* and a fall in the amount of *Methanosarcina spp.* (Padmasari et al., 2007). In addition, Padmasari et al. (2007) tested their results obtained by T-RFLP by conducting a variance component analysis of selected peak areas and thereby verified their results and demonstrated that T-RFLP is a rapid, robust and reproducible method to analyse methanogens. In general, T-RFLP is a very sensitive method that allows a high-throughput due to automated detection and provides an immediate analysis (Spiegelman et al., 2005), whereas its main limitations are problems related to incomplete or non-specific digestion (Padmasari et al., 2007; Justé et al., 2008).

4.2.4 Fluorescence in-situ hybridization (FISH)

FISH is a technique where fluorescently labelled probes are introduced to target cells to hybridize to specific genes (Tolvanen & Karp, 2011; Zhou et al., 2011). After the hybridization, usually to the cell ribosomes, the analysis can be performed by epifluorescence microscopy (Tolvanen & Karp, 2011). A great advantage of this technique is that the methanogens can be identified *in vivo* and also the morphology, the spatial arrangements and their association to other organisms can be analysed (Amann et al., 1995; Madigan et al., 2009). Nettmann et al. (2010) carried out a study on six full-scale biogas plants in which the archaeal community was also analysed by FISH. FISH revealed that in three of the six biogas plants methanogens belonging to the order Methanomicrobiales were dominant with 60-64% of archaeal cell counts (Nettmann et. al., 2010). In one of the analysed biogas plants methanogens belonging to the family *Methanosaetaceae* were detected with the highest percentage of 64% of archaeal cell counts (Nettmann et al., 2010). The findings are in accordance with the results obtained by qPCR, which was performed at the same time (Nettmann et al., 2010). In another study conducted by FISH in an anaerobic digester for treating palm oil mill effluent, Tabatabaei et al. (2009) demonstrated the dominance of the family *Methanosaetaceae* in this bioreactor with *Methanosaeta concili* being the most common species. Conclusively, FISH is a good estimate of the dominant species of methanogens (Tabatabaei et al., 2009) and its drawbacks are related to the presence of the autofluorecent F_{420} in methanogens (Zhou et al., 2011).

4.2.5 Metagenomics

Metagenomics implies the study of the genes of a whole community in an environmental sample (Cardenas & Tiedje, 2008). The new high-throughput technologies are so called sequence-by-synthesis methods which allow the determination of the genome sequence with the advantage of avoiding the bias underlying the cloning procedures (Cardenas & Tiedje, 2008). The currently most popular example for such a technology is pyrosequencing (Cardenas & Tiedje, 2008). Schlüter et al. (2008) used the ultrafast 454-pyrosequencing technology to analyse the microbial community of a biogas reactor sample and found methanogens belonging to the genus *Methanoculleus* being most abundant. This result was obtained by the mapping of the metagenome reads to the reference genome of *M. Marisnigri JR1* by BLASTn. Moreover, Schlüter et al. (2008) demonstrated that genes for all enzymatic functions needed in the CO_2 reduction pathway of methanogenesis are present in the analysed sample. This is of interest as the genus *Methanoculleus* comprises species which use the CO_2

reduction pathway for methane production (Sowers, 2009) and thereof the dominance of species belonging to the *Methanoculleus* was confirmed (Schlüter et al., 2008). Furthermore, these findings are supported by another study performed by Krause et al. (2008) using massive parallel pyrosequencing. The analysis was carried out on a similar biogas reactor and the same results were achieved. Most methanogens found belong to the genus *Methanoculleus* and consequently the major pathway is the CO_2 reduction pathway. Concluding, the major problem related to next-generation sequencing is no longer the feasibility of the sequencing, but the enormous effort of data analysis (Cardenas & Tiedje, 2008).

5. Conclusion

In conclusion, it can be expected that the impact of biogas plants on the energy market will further increase in the next years. Since the production of biogas is highly dependent on the microbial community involved in the anaerobic digestion process, it is important to extend our knowledge of these microorganisms. Especially the methanogenic community has to be monitored carefully aiming at optimizing the methane yields. In order to achieve this goal the process conditions like temperature, pH, nutrient supply and the substrates have to be well selected to allow the involved microorganisms to prosper. This is however only possible when the methanogenic community is studied in great detail. Modern analysis tools as for example DGGE, qPCR, t-RFLP and pyrosequencing are used to deepen the understanding of methanogens. However, these microorganisms are to a large extent unexplored and subsequent investigations are necessary in order to obtain further knowledge in this field.

6. References

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