Development of efficient tools for monitoring and improvement of biogas production

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In tiefer Dankbarkeit und Liebe widme ich diese Dissertation meinen Eltern und meiner Schwester Miriam.

Contents

I. Introduction

Energy is the driving force behind modern human development

Over the last century human civilization has become more and more dependent on electrical energy. Since the beginning of the industrial age, the global energy demand continues to rise. The energy used worldwide is set to grow, primarily because of the economic and political growth of developing countries such as India, China, Africa, the Middle East, and Southeast Asia (International Energy Agency 2015). In a recent study, it was found that without any change in our current use of energy, the world energy demand will have increased by 50 - 80 % in 2020 compared to the energy level in 1990 (World Energy Council 2013).

Most of the energy needed worldwide currently originates from fossil fuels like coal, petroleum, and natural gas. Fossil fuels are carbon sources conserved during millions of years by anaerobic degradation processes. However, growing energy demand promotes the exhaustion of existing fossil energy resources. Beyond that, the extensive extraction of fossil combustible material is responsible for enormous amounts of CO_2 released into the atmosphere, leading to ongoing climate warming (Dincer 1998). Primarily in Europe, this effect led to an introduction of policies to find alternative materials to further develop renewable energy sources for environmental protection and to secure a sustainable energy supply. Renewable energy sources like bioenergy, hydropower, ocean energy, geothermal energy, solar and wind energy are characterized by natural replenishment on a human timescale and are thus CO_2 neutral. These resources provide energy by power generation, heating, and cooling of air as well as water and transport fuels. Currently, 14 % of the worldwide energy demand is provided by renewable energy sources (Demirbas 2009).

In Germany, in the past two decades, public awareness of the need for sustainable energy production has increased. This led to the government introducing the first decisive Renewable Energy Law in 2000 (EEG 2000), followed by amendments in the subsequent years (EEG 2004, EEG 2008, EEG 2012). The law financially triggered the decentralized construction of renewable energy sites such as photovoltaic, wind turbines, and biogas plants. Consequently, gross electricity production from renewable energy sources increased from 36.0 bil KW h^{-1} in 2000 to 195.9 bil KW h^{-1} in 2015 (Federal Ministry for Economic Affairs and Energy 2015).
The percentage of gross electricity production from renewable energy sources is divided into wind power (12.3 %), biomass (6.9 %), photovoltaic (6.0 %), hydropower (2.9 %), and also a small portion of household waste (0.9 %) (Arbeitsgemeinschaft Energiebilanzen e. V. 2016). Renewable energy from biomass is the key player in renewable-based electricity generation. In the year 2015, 25.5 % of the renewable-based electricity generation resulted from the metabolization of biomass (Federal Ministry for Economic Affairs and Energy 2015). Biomass-based electricity can be divided into electricity derived from biogas, biogenic fuels, and waste as well as sewage and landfill gas, the main proportion originates from biogas with 60.2 % (Federal Ministry for Economic Affairs and Energy 2015).

Renewable energy from biogas

In Germany, there are about 9,000 biogas plants operating with an installed electric capacity of 4.18 GW (Fachverband Biogas e.V. 2015). Therefore, biogas has become an essential factor for energy supply. Biogas is produced by microbial degradation of organic material and then turned into electric power and heat by combined heat and power units, or purified to biomethane and fed into the national gas grid. One major advantage of biogas when compared to solar or wind energy is that it offers the option of storing it temporarily as well as producing it seasonally thus, tailoring its availability to peak consumption times.

Downsides of biogas production include its relative inefficiency when compared to fossil fuels. Furthermore, energy crop cultivation can displace existing agricultural crops leading to regional monocultures in e.g. maize crop. To meet the increased demand of energy crops, formerly fallow land or grassland may be converted to arable land and thus, cause a loss of biodiversity. However, when applied sustainably and responsibly, biogas production represents a major and important source in renewable energy generation.

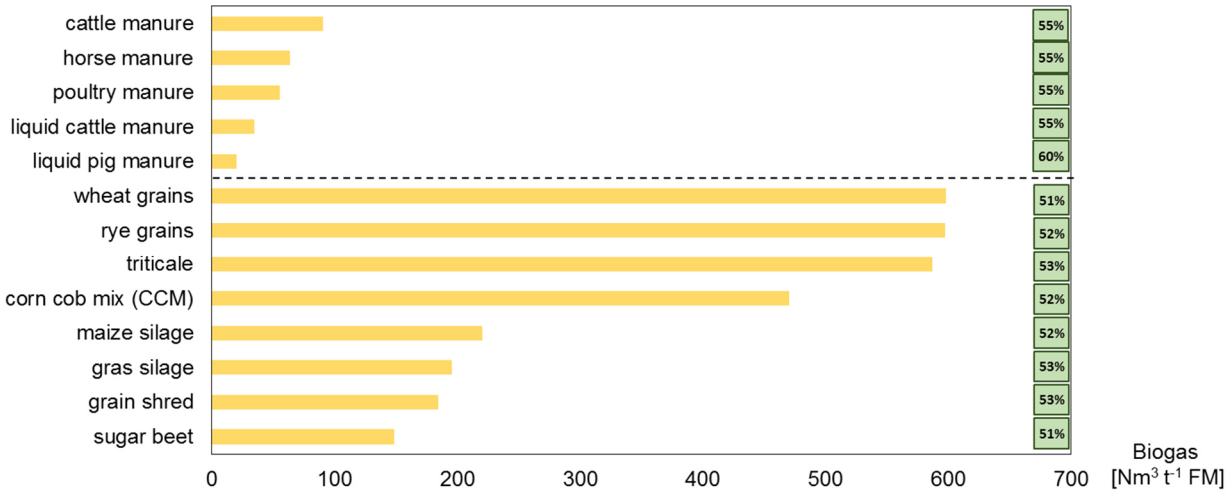

Figure 1: **Biogas yield and CH_4 proportion in different substrates fed into a biogas tank.** *Yellow bars*, biogas yield [Nm^3 t^{-1}FM]. *Green squares*, CH_4 proportion [%]. Modified from Fachagentur für nachwachsende Rohstoffe e.V. 2015.

The existing legislation promotes biogas production from agricultural animal and vegetable derived biomass. This is why mainly energy crops (52 %) and animal excrements like slurry and manure (43 %) are utilized for the production of biogas in Germany (Fachagentur für nachwachsende Rohstoffe e.V. 2015). Residue materials from industry and agriculture (2 %) as well as municipal biological waste (3 %) are utilized as well but currently play only a minor role. The organic substances have different microbial fermentative potentials, meaning the degradation of these raw materials results in various amounts of CH_4. Among the energy crops, maize silage contains a biogas yield of 220 Nm^3 t^{-1} fresh mass with an average CH_4 content of 52 % (Fig. 1). Within the animal excrements, poultry manure possesses the highest yield in biogas production (55 Nm^3 t^{-1} FM) and the CH_4 proportion is about 55 % (Fig. 1). Cattle manure is also often used to generate biogas with an average

CH_4 content of 55 % and a biogas yield of 90 $Nm^3\ t^{-1}$ FM (Fachagentur für nachwachsende Rohstoffe e.V. 2015) (Fig. 1).

For the production of biogas three areas intertwine that need to be in a perfect balance with each other, so that biogas production can occur without problems. Firstly, the choice of substrates plays a crucial role in biogas formation, as well as, secondly, the process technology that accompanies the process. In addition, it is essential that the microbial degradation of organic material can take place under optimum environmental conditions for the microorganisms involved.

Biogas can be produced in multi- as well as one-step systems (Weiland 1993). In Germany, predominantly one-step agricultural biogas systems are applied for biogas production (Weiland 1993, Lindorfer *et al.* 2008). For feeding, liquid substrates such as cattle manure or silage effluent are collected and homogenized in a collecting or mixing tank and afterwards a dosing unit is used to introduce solid substrates like crop silage into the biogas plant (Fig. 2). The digestion of the substrates takes place in the primary or main fermenter. This fermenter is gastight, heatable, insulated, and often equipped with weatherproof cladding. For stirring, digesters accommodate one to several agitators and they are covered by a single or double membrane roof for gas storage (Fig. 2). The main share of microbial degradation and biogas production takes place in this main fermenter. In a number of cases, the main fermenter is followed by a secondary tank, featuring the same layout as a primary tank except for a feeding mechanism. A storage tank is then used for storing the biodegraded digestate until field-spreading and additionally to collect biogas (Fig. 2).

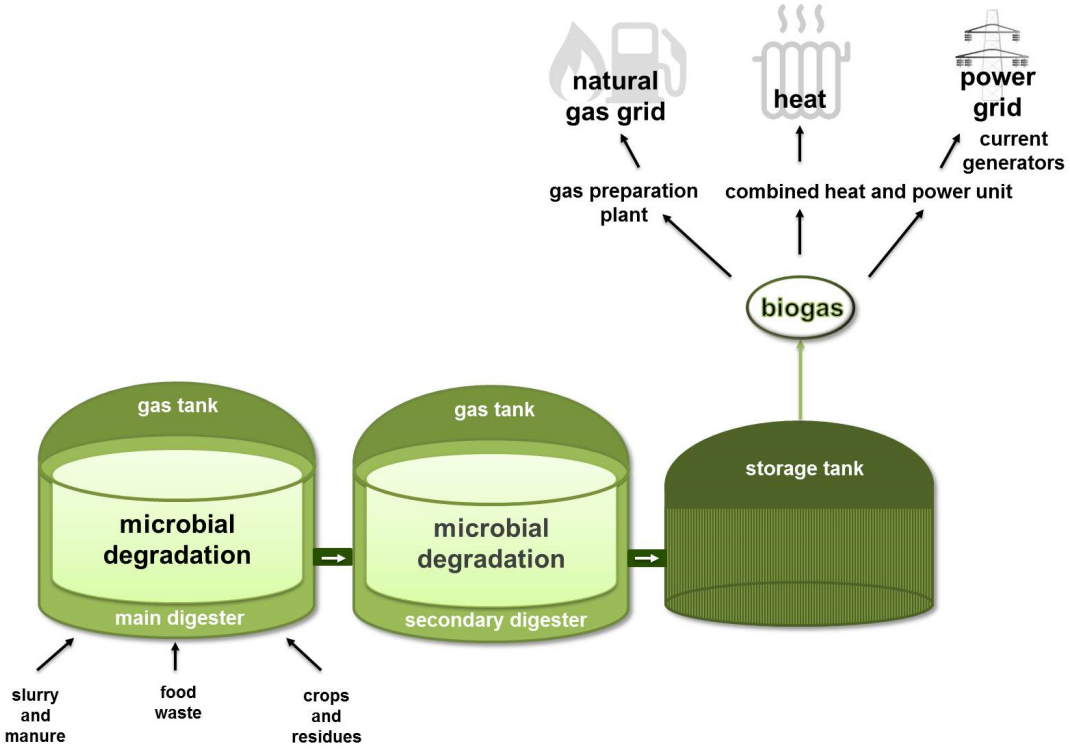

Figure 2: **Structure of an agricultural biogas plant fed with renewable resources.**

Biogas can be treated to remove CO_2 resulting in biomethane that can be fed into the gas grid or converted into electric power and heat in a combined heat and power unit (Weiland 2010, Lansche and Müller 2012). Biomethane can be used in various ways, as fuel for cars, trucks, and buses. Apart from generating electricity, combined heat and power units produce thermal energy which can be utilized in local and district heating systems.

The existing biogas plants in Germany differ in composition and structure, as well as in the organic materials used and the physico-chemical parameters, which play a crucial role in the synthesis of biogas. Thus, the composition of biogas is different. Biogas mainly consists of 45 – 70 % CH_4 and 30 - 45 % CO_2 (Rasi *et al.* 2007). Depending on the utilized substrate, the percentage of each component can change like measured in samples of biogases from a landfill, sewage treatment plant, and agricultural biogas plants (Rasi *et al.* 2007). Besides CH_4 and CO_2, biogas contains trace gases such as NH_3, N_2, H_2, volatile organic compounds, and different sulfur components, e.g. H_2S (0 - 5000 ppm) (Rasi *et al.* 2007).

Microbial degradation processes in a mesophilic agricultural biogas reactor

Degradation of different organic materials from agricultural plants and animal excrements is conducted by an anaerobic microbial degradation chain. The final products of this process are CO_2 and CH_4. The organisms' composition may vary depending on the type of plant, type of substrate, and on different process parameters. Nevertheless, the degradation scheme of renewable raw materials is always the same. In the first step, the hydrolysis, the polymeric constituents of different substrates in the form of proteins, lipids, and polysaccharides are hydrolyzed so that amino acids, fatty acids, and sugars are formed. In the next step, the primary fermentation, also called acidogenesis, these intermediates are metabolized by a variety of fermentative bacteria to short chain fatty acids and alcohols. In the acetogenesis syntrophic microorganisms synthesize acetate, CO_2 and H_2, so that in the final step of methanogenesis CH_4 can be produced. These four degradation steps will be further described in more detail (Fig. 3, see following sections Hydrolysis, Acidogenesis, Acetogenesis, Methanogenesis).

Hydrolysis

The process of hydrolysis describes a cleavage of chemical bonds in organic matter with the help of H_2O. A complex microbial consortium secretes hydrolytic enzymes which catalyze these reactions. Especially glycosidases, lipases, and peptidases are crucial classes of enzymes involved in the anaerobic hydrolysis of organic material. Glycosidases catalyze the cleavage of glucosidic bonds such as those found in many polymers like starch, cellulose, hemicellulose, and pectin. The more complex the respective substrate is the more difficult is the cleavage procedure. Especially the division of lignocellulose is highly complex but lignocellulose is the most abundant plant cell wall component of the biosphere (Glass *et al.* 2013). Due to the presence of lignocellulose in a variety of plant silages, which are used as substrates, it is increasingly found in agricultural biogas plants. Lignocellulose is mainly composed of cellulose, hemicellulose, and lignin (Saha 2003). Cellulases cleave cellulose molecules (Zhang and Lynd 2004). In some anaerobic cellulose-degrading microorganisms cellulases

are arranged in a multi-enzyme complex, so-called cellulosomes (Bayer *et al.* 1994, Schwarz 2001, Lynd *et al.* 2002). These consist of a scaffold protein with cohesin that interact with dockerin connected with linkers, sitting at the catalytic domains of the enzymes, ensuring a very efficient degradation of cellulose (Bayer *et al.* 2004, Ding *et al.* 2008). Cellulosomes could be identified in a variety of anaerobic bacteria, e.g. in some species of the genus *Clostridium* (*Clostridium* (*Cl.*) *cellulovorans* (Kosugi *et al.* 2001, Murashima *et al.* 2002, Park *et al.* 2001), *Cl. josui* (Jindou *et al.* 2002), *Cl. thermocellum* (Prates *et al.* 2001), *Cl. acetobutylicum* (Nölling *et al.* 2001) and *Cl. cellobioparum* (Lamed *et al.* 1987)), also in *Butyrivibrio fibrisolvens* (Berger *et al.* 1990) and *Ruminococcus* sp. (Miron *et al.* 2001, Ohara *et al.* 2000, Ding *et al.* 2001, Rincón *et al.* 2005). In mesophilic agricultural biogas plants, a number of different Clostridia spp. have been identified by amplicon sequencing and metagenome analysis of 16S DNA (Sun *et al.* 2013, Ziganshin *et al.* 2013, Jaenicke *et al.* 2011). Moreover, a whole series of uncharacterized microorganisms of the order Firmicutes persist, which might be involved in the process of hydrolysis (Kröber *et al.* 2009). Some *Actinomyces* are also identified in biogas reactors (Ziganshin *et al.* 2013, Qiao *et al.* 2013) which include for example the cellulose-degrading *Cellulomonas* sp. (Singh and Jain 1986, Dubey *et al.* 2014).

In addition to the breakdown of carbohydrates, hydrolytic degradation of lipids and proteins plays a major role in a biogas plant. Lipases catalyze the cleavage of ester bonds, as they are frequently present in lipids. Peptidases or proteolytic enzymes are capable of degrading peptide bonds. Especially animal excrements are very protein-rich, so a large number of proteases are required to cleave the proteins (Hahnke *et al.* 2015, Tiquia 2002). Despite these facts, it is difficult to describe the whole process reliably because hydrolysis of complex insoluble substrate depends on many different parameters such as particle size, production of enzymes, pH, and temperature.

Besides bacterial hydrolysis of polymers, fungi can cleave mainly vegetable polymers mechanically and enzymatically. Just recently, the life cycle of anaerobic fungi of the phylum *Neocallimastigomycota* has been elucidated (Gruninger *et al.* 2014). They play a central role in the degradation of plant materials in the gut of mammalian herbivores. Additionally, in a few biogas plants, these anaerobic fungi were also found (Dollhofer 2015). They contribute, although not essential, to hydrolytic cleavage of the biomass used for biogas production (Dollhofer 2015).

Acidogenesis

Hydrolytically produced monomers and oligomers of amino acids, sugars, and long chain fatty acids are converted into short chain fatty acids, but also short-chain alcohols in the next step of the anaerobic degradation chain, in the primary fermentation or acidogenesis (Fig. 3). Mainly carboxylic acids such as acetic acid, butyric acid, or propionic acid are formed, but also lactic acid, valeric acid, ethanol, H_2 and CO_2 can be produced. Clostridia, assigned to the phylum Firmicutes, are well equipped for this environment and thus, occur abundantly in biogas reactors (Sun *et al.* 2013, Jaenicke *et al.* 2011, Kröber *et al.* 2009). Members of the phylum Bacteroidetes are also frequently found in biogas reactors. Often they are the second most abundant group of the microbial community

(Hanreich *et al.* 2013, Kröber *et al.* 2009, Schlüter *et al.* 2008, Ziganshin *et al.* 2013). Bacteroidetes represent a metabolically heterogeneous group comprising species with a broad range of capabilities (Hahnke *et al*. 2015). In a biogas plant they are responsible for the hydrolysis of polysaccharides and proteins, as well as for the fermentation of sugars and production of VFA (Vos *et al.* 2011).

Acetogenesis

The next step in the anaerobic degradation chain is the secondary fermentation or acetogenesis (Fig. 3). This step of fermentation is directly linked to the final step of degradation, the methanogenesis. Under anoxic conditions, a complete breakdown of one molecule glucose as catalyzed by microbial consortia to CO_2 and CH_4 releases -390 kJ mol^{-1} free energy (Schink 1997). In contrast, aerobic metabolization of glucose provides -2870 kJ mol^{-1} free energy. Thus, there is only a small amount of energy available in methanogenic conversion which forces the involved microorganisms to a very efficient cooperation. The mutual dependence of partner bacteria with respect to energy limitation can go so far that neither one partner can operate without the other and that together they exhibit a metabolic activity that neither one could accomplish on its own (Schink 1997). Such cooperations are called syntrophic relationships. Syntrophy describes a special case of symbiotic cooperation between two metabolically different types of microorganisms which depend on each other for degradation of a certain substrate, typically for energetic reasons (Schink 1997). The term was used for the first time to describe the cooperation of fatty acid-oxidizing, fermenting bacteria with hydrogen-oxidizing methanogens (McInerney *et al.* 1979).

The classic example of syntrophic symbiosis is probably a culture of *Methanobacillus omelianskii*. This culture was isolated by Barker in 1940 (Barker 1940). It was assumed that this methanogenic organism is able to synthesize CH_4 from ethanol and acetate. However, there are actually two syntrophic partners living together. The fermentative bacterium ‘ S.’ catalyzes the oxidation of ethanol to acetate and H_2 (Eq. 1). The methanogenic archaeon ‘ M.o.H.‘ then reduces CO_2 to CH_4 by using the electrons from the previously produced H_2 (Bryant *et al.* 1967). It was found that the first reaction can occur and provide energy for strain ‘S.’ only if the H_2 partial pressure is kept low enough (<100 Pa) by the methanogen ‘M.o.H’. (Bryant *et al.* 1967). Decreasing the H_2 partial pressure and removing end products out of balance changes the thermodynamic conditions of the reaction. Under standard conditions this metabolization is an endergonic reaction ($\Delta G^{\circ\prime}$ = +9.6 kJ mol^{-1} ethanol) but becomes an exergonic overall reaction by coupling with the synthesis of CH_4 ($\Delta G^{\circ\prime}$ = -112 kJ mol^{-1} CH_4) (Schink 1997). Therefore, methanogens are essential to maintaining the low concentration of H_2 and making the reaction sufficiently exergonic to support energy conservation, cell maintenance, and growth for the syntrophic bacteria (Stams and Plugge 2009). This process plays an important role in anaerobic reactors in biogas plants. Syntrophic bacteria such as *Pelobacter* sp. (Schink 1985, Seitz *et al.* 1990), *Thermacetogenium phaeum* (Hattori 2008), *Thermoanaerobium brockii* (Ben-Bassat *et al.*1981) are able to completely oxidize ethanol to CH_4 only in syntrophy with hydrogenotrophic methanogens. Besides the conversion of ethanol, the oxidation of short-chain fatty acids, originating from the anaerobic degradation of biomass, is an endergonic reaction under standard conditions (P_{H2} 1 atm, substrate and product concentrations 1 M, temperature 298 K, pH 7.0 (Thauer *et al.* 1977)). Meaning

the oxidation of short-chain fatty acids cannot proceed without a syntrophic partner. A thermodynamic approach is necessary in order to understand the process in detail. Under standard conditions, the change of free energy for the oxidation of butyrate to acetate and H_2 is $\Delta G°´$ = +48.3 kJ mol^{-1} (Eq. 2) (Müller *et al.* 2010).

Eq. 1: Ethanol + H_2O → Acetate$^-$ + H^+ + 2 H_2 $\Delta G°´$ = + 9.6 kJ mol^{-1}

Eq. 2: Buytrate$^-$ + 2 H_2O → 2 Acetate$^-$ + H^+ + 2 H_2 $\Delta G°´$ = + 48.3 kJ mol^{-1}

Eq. 2: Propionate$^-$ + 2 H_2O → Acetate$^-$ + CO_2 + 3 H_2 $\Delta G°´$ = + 76.0 kJ mol^{-1}

Thus, all organisms oxidizing butyrate would need energy for their metabolism and therefore would not be viable independently. Butyrate is degraded by the process of β-oxidation by organisms of the family *Syntrophomonadaceae*, which belong to the order of Clostridiales (Zhao *et al.* 1993) and of the order Syntrophobacterales belonging to the Deltaproteobacteria (Müller *et al.* 2010). These include organisms such as *Syntrophomonas* (*S.*) *wolfei*, *S. erecta*, *S. curvata*, *S. zehnderi*, and also *Thermosyntropha lipolytica* (Müller *et al.* 2010). In the course of butyrate oxidation electrons are transferred to electron carrier molecules (e.g. NAD^+) (McInerney *et al.* 2008). These carrier molecules can be reduced during the oxidation of organic or inorganic compounds. In the above mentioned butyrate oxidation pathway these molecules are formed during oxidation of butyryl-CoA to crotonyl-CoA and of 3-hydroxybutyryl-CoA to acetoacetyl-CoA. Oxidation of butyryl-CoA to crotonyl-CoA is the energetically most difficult reaction in butyrate conversion (E_0' = -15 mV) (Thauer *et al.* 1977, Hauge 1956). Even at H_2 partial pressures of 1 Pa this is an endergonic reaction because the standard midpoint redox potentials of the reduced electron carriers are too high for the reduction of protons to form H_2 (-414 mV) (Thauer *et al.* 1977, Schink 1997). To drive this reaction, metabolic energy by reverse electron transport is required. Butyrate oxidizers utilize parts of the gained ATP by an energy conservation chain coupled to the cytoplasmic membrane to shift electrons to this redox potential (Thauer and Morris, 1984). Partner organisms, preferably methanogenic archaea, keep the H_2 partial pressure low, thus raising the redox potential of proton reduction to around -300 mV (Schink 1997); remaining ATP can be used for biosynthesis and growth. An even more positive standard free energy of $\Delta G°´$ = +76.0 kJ mol^{-1} is calculated for the oxidation of propionate (Eq. 3) (Müller *et al.* 2010). Organisms such as *S. fumaroxidans*, *S. wolinii*, *S. sulfatireducens* or *Pelotomaculum* (*P.*) *schinkii*, and *P. propionicum* as well as *Desulfotomaculum thermobenzoicum ssp. thermosytrophicum* are able to oxidize propionate using the methylmalony-CoA pathway in syntrophic association with methanogens (Müller *et al.* 2010). Similar to the oxidation of butyrate, electrons are transferred to electron carrier molecules (e.g. NAD^+ or Fd_{ox}). These reduced electron carrier molecules can be reoxidized via a membrane-bound electron transport chain and H_2, formate, and acetate are generated. Especially, the oxidation of succinate to fumarate and malate to oxaloacetate are thermodynamically difficult reactions during the propionate oxidation pathway. However, the reactions are favored by the methanogenic consumption of H_2 and formate. Accordingly, the process would stop without the methanogens. All these described processes occur in a biogas reactor and are sensitively dependent on the interaction

of acetogenic bacteria and methanogenic archaea, involved in the final step of the energy production from biomass.

Methanogenesis

Methanogenic archaea utilize a limited number of substrates: acetate, H_2 + CO_2, formate, and methylated compounds (Ferry 1993). In the central metabolism, referred to as methanogenesis, all substrates are converted to CH_4. Depending on the type of substrate three main types of methanogenic pathways can be distinguished: hydrogenotrophic methanogenesis based on the conversion of H_2 + CO_2, aceticlastic methanogenesis using acetate as substrate and methylotrophic methanogenesis where methylated compounds are metabolized. Hydrogenotrophic methanogens belong to the orders Methanobacteriales, Methanocellales, Methanococcales, Methanopyrales and Methanomicrobiales. Till now, there are only two genera discovered, able to grow on acetate: *Methanosaeta* and *Methanosarcina*. *Methanosaeta* strains depend on the presence of acetate (Jetten *et al.* 1992). In contrast, *Methanosarcina* spp. are the most versatile methanogens. Besides the conversion of acetate, H_2 + CO_2 as well as methylated compounds can be used as substrate. Thus, in the genome of most *Methanosarcina* sp. enzymes for all three types of methanogenesis are encoded (Deppenmeier *et al.* 2002).

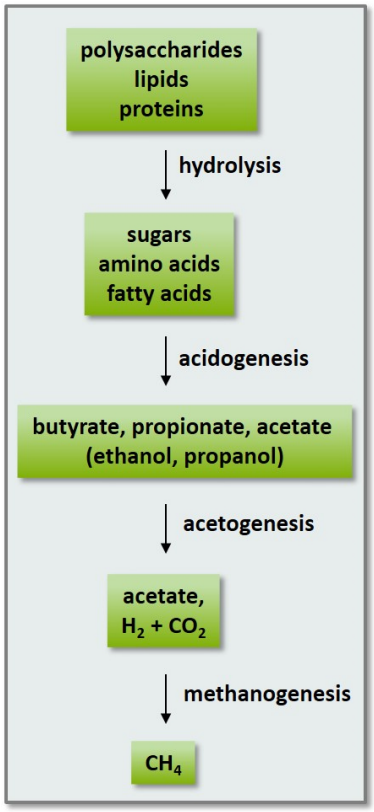

Figure 3: **Anaerobic degradation chain of organic material in a biogas reactor.**

There are only a few exceptions showing a mixture of the different metabolic pathways. One example is *Methanosphaera* (*Mp.*) *stadtmanae*, a human gut commensal (Fricke *et al.* 2006), growing on methanol and H_2. Another recently discovered example is *Methanomassiliicoccus* (*Mm.*) *luminyensis* isolated from the human gut (Dridi *et al.* 2012). The organism grows with H_2 and methanol or

methylated amines and belongs to the new order of Methanomassiliicoccales (Dridi *et al.* 2012, Brugère *et al.* 2014).

The presence of certain substrates defines the occurrence of methanogenic species. Due to the anaerobic degradation of biomass in a mesophilic biogas plant, mainly hydrogenotrophic and aceticlastic methanogenesis take place (Fig. 3). In most mesophilic agricultural biogas plants the methanogenic order Methanosarcinales can be found (Nettmann *et al.* 2008, St-Pierre and Wright 2013, Bergmann *et al.* 2010b, Ziganshin *et al.* 2013). So far, only two families are known in this order, *Methanosarcinaceae* and *Methanosaetaceae*. In many biogas reactors, those organisms are responsible for the degradation of acetate (Kern *et al.* 2016a, Kampmann *et al.* 2012, St-Pierre and Wright 2013, Nettmann *et al.* 2010). Hydrogenotrophic methanogens use electrons from H_2 to catalyze the stepwise reduction of CO_2 to CH_4. The diversity in hydrogenotrophic methanogenic orders is much higher. Mainly microorganisms belonging to Methanobacteriales and Methanomicrobiales could be identified in biogas reactors (Nettmann *et al.* 2010, St-Pierre and Wright 2013, Nettmann *et al.* 2008, Bergmann *et al.* 2010a, Ziganshin *et al.* 2013, Kröber *et al.* 2009, Jaenicke *et al.* 2011), dominated by organisms of the genus *Methanoculleus* (Nettmann *et al.* 2010, Kröber *et al.* 2009, Jaenicke *et al.* 2011).

Hydrogenotrophic methanogenesis

In the following section the focus will be on CH_4 formation from H_2 + CO_2. Microorganisms of the orders Methanomicrobiales, Methanococcales, and Methanobacteriales perform hydrogenotrophic methanogenesis in a biogas reactor.

The series of reactions is initiated by the formylmethanofuran dehydrogenase (Fig. 4, reaction 1). The enzyme catalyzes the formation of *N*-carboxymethanofuran from methanofuran (MFR) and CO_2 and subsequently the reduction to formyl-MFR (Bartoschek *et al.* 2000). Reducing equivalents for this reductive process derive from Fd_{red}, provided by a bifurcation process explained below. Afterwards, the formyl group is transferred to tetrahydromethanopterin (H_4MPT) (Fig. 4, reaction 2) (Breitung and Thauer 1990, Shima *et al.* 1996, Kunow *et al.* 1996) and stepwise reduced to methyl-H_4MPT (Fig. 4, reaction 3 – 5). The first intermediate is methenyl-H_4MPT synthesized by the methenyl-H_4MPT cyclohydrolase (Fig. 4, reaction 3) (te Brömmelstroet *et al.* 1990a, Pomper *et al.* 1999, Vaupel *et al.* 1996). In the next enzymatic reaction, the methylene-H_4MPT dehydrogenase converts methenyl-H_4MPT to methylene-H_4MPT (Fig. 4, reaction 4) (te Brömmelstroet *et al.* 1991a, te Brömmelstroet *et al.* 1991b). The produced intermediate is further reduced to methyl-H_4MPT by the methylene-H_4MPT reductase (Fig. 4, reaction 5) (te Brömmelstroet *et al.* 1991b, te Brömmelstroet *et al.* 1990b, Ma and Thauer 1990a, Ma and Thauer 1990b). The electrons transferred in the last two reactions are provided by the methanogenic cofactor F_{420} ((N-L-lactyl-γ-L-glutamyl)-L-glutamic acid phosphodiester of the 7,8-didemethyl-8-hydroxy-5-desazariboflavin-5´-phosphate). F_{420} is reduced with the help of molecular H_2 by the F_{420}-reducing hyrogenase localized in the cytoplasm (Fig. 4, reaction 6) (Vaupel and Thauer 1998, Sorgenfrei *et al.* 1997). In the next step, the methyl-H_4MPT-CoM methyltransferase catalyzes the transfer of the methyl-moiety to coenzyme M (HS-CoM) thereby producing methyl-S-CoM (Fig. 4, reaction 7) (Gottschalk and Thauer 2001). This enzyme reaction is an exergonic process coupled to

Na^+ ion translocation out of the cell, resulting in the generation of an electrochemical Na^+ gradient (Becher *et al.* 1992a, Becher *et al.* 1992b, Gottschalk and Thauer 2001). This gradient is subsequently used for ATP synthesis by an A_1A_0 ATP synthase (Fig. 4, reaction 9) (Becher and Müller 1994, Deppenmeier *et al.* 1996, Perski *et al.* 1982).

Methyl-S-CoM is the central intermediate and in the final step of methanogenesis the methyl group is reduced to CH_4 (Fig. 4, reaction 8) (Ermler *et al.* 1997, Ellermann *et al.* 1989, Ankel-Fuchs and Thauer 1986, Ankel-Fuchs *et al.* 1986). This reaction is catalyzed by the methyl-CoM reductase, which reduces the methyl group to CH_4, while HS-CoM is combined with coenzyme B (HS-CoB) resulting in the formation of the heterodisulfide (CoM-S-S-CoB) (Fig. 4, reaction 8). CoM-S-S-CoB is the terminal electron acceptor of the metabolism and is reduced to HS-CoM and HS-CoB by a bifurcation reaction of a multi enzyme complex consisting of a [NiFe] hydrogenase (Mvh) and a heterodisulfide reductase (HdrABC) (Fig. 4, reaction 10) (Thauer *et al.* 2008). The Mvh/HdrABC complex is able to use electrons derived from H_2 oxidation for the exergonic reduction of CoM-S-S-CoB and the endergonic reduction of Fd_{ox}. This process is called electron bifurcation. The resulting Fd_{red} is used for CO_2 fixation, previously described, thus, the circle of hydrogenotrophic methanogenic metabolism is closed. Additionally, a Na^+/H^+ antiporter is located in the cytoplasmic membrane of hydrogenotrophic methanogens (Fig. 4, reaction 11). This transporter functions in both directions and has a function of pH homeostasis.

Overall, only one catalytic step in the hydrogenotrophic metabolism translocates ions across the membrane that can be used for the generation of ATP, the methyl group transfer by methyl-H_4MPT-CoM methyltransferase (Gottschalk and Thauer 2001). For the synthesis of ATP from ADP and orthophosphate (P_i) at least -50 kJ mol^{-1} are required (Thauer *et al.* 1977). The standard free energy change that is associated with the reduction of CO_2 with H_2 to CH_4 is -131 kJ mol^{-1} (conditions: 25 °C, pH 7, H_2 and CO_2 and CH_4 in the gaseous state at 10^5 Pa, all other compounds at a concentration of 1 M (Thauer *et al.* 1977)). At H_2 partial pressures of 1-10 Pa, as they occur in most natural habitats of methanogens, the free energy change that is associated with the CO_2 reduction to CH_4 is only -40 kJ mol^{-1}, which is not even sufficient for the synthesis of one ATP molecule (Hoehler *et al.* 1998, Conrad and Wetter 1990, Thauer *et al.* 2008). Energy conservation in these is only possible by a chemiosmotic mechanism that involves the generation of an electrochemical ion gradient across the cytoplasmatic membrane as catalyzed by the methyl-H_4MPT-CoM methyltransferase and ATP synthesis as catalyzed by an A_1A_0 ATP synthase (Mayer and Müller 2014).

In addition to H_2 + CO_2, formate is a common substrate for about half of all methanogens. But it is not used by any *Methanosarcina* spp.. Methanogenesis from formate involves oxidation of the substrate to produce CO_2 and a reduced electron carrier. All other reactions are identical to the process of hydrogenotrophic methanogenesis. Formate oxidation is catalyzed by a formate dehydrogenase. The enzyme has been isolated from *Methanobacterium* (*Mb.*) *formicicum* and *Methanococcus* (*Mc.*) *vannielii* (Barber *et al.* 1983, Jones and Stadtman 1981). In the course of the oxidation of formate coenzyme F_{420} is reduced. CO_2 enters the carbon dioxide reduction pathway outlined above and $F_{420}H_2$ serves as an electron donor for the reduction of methenyl-H_4MPT and methylene-H_4MPT (Sparling and Daniels 1990, te Brömmelstroet *et al.* 1991a, te Brömmelstroet *et al.* 1991b).

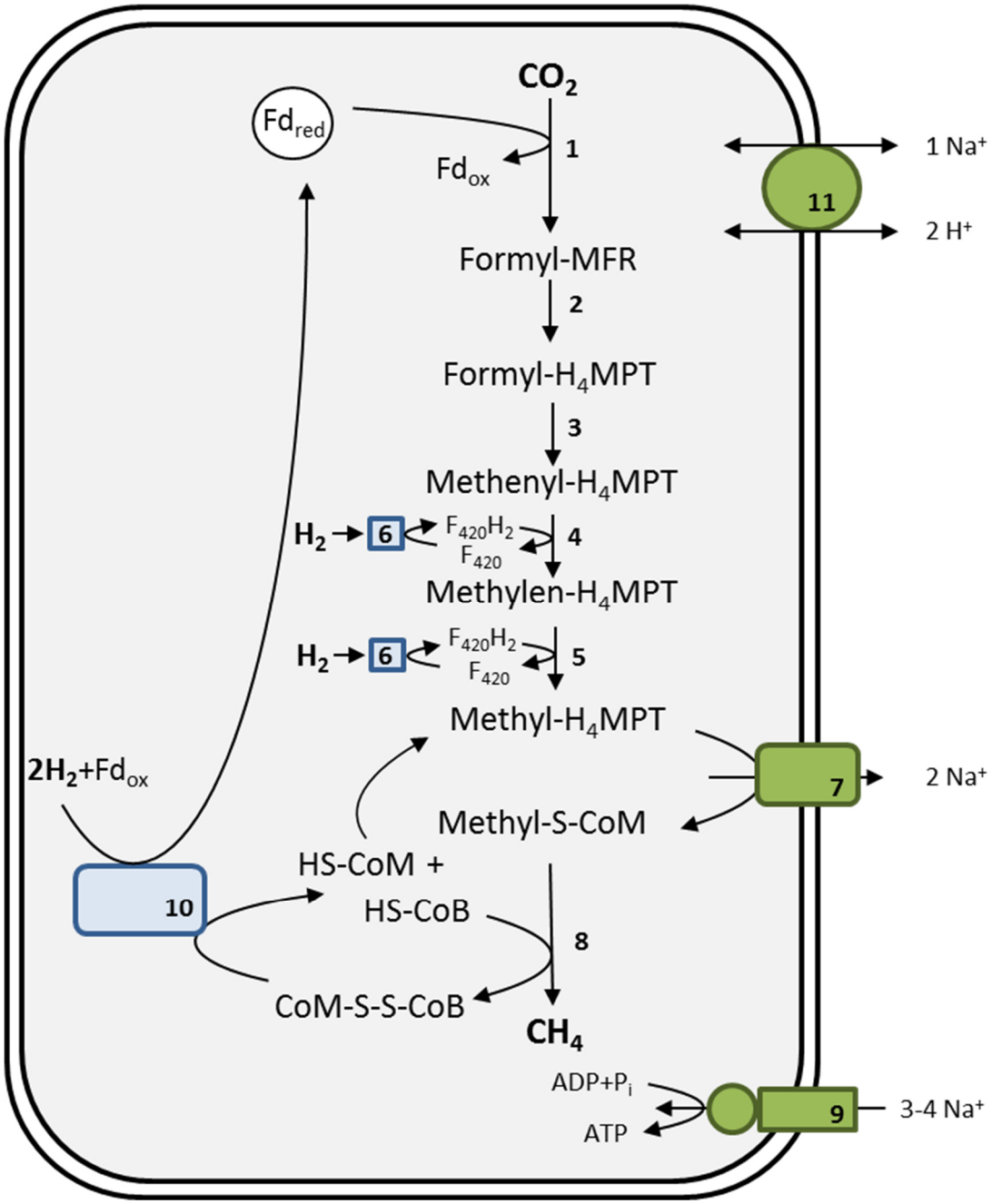

Figure 4: **Scheme of hydrogenotrophic methanogenesis coupled with an energy conservation system.** This pathway exists in all methanogens without cytochromes (Methanomicrobiales, Methanococcales, Methanopyrales, and Methanobacteriales). *Green*, membrane-bound enzyme complexes. *Blue*, cytoplasmic enzyme complexes. 1, formylmethanofuran dehydrogenase. 2, formylmethanofuran: tetrahydromethanopterin formyltransferase. 3, methenyl-tetrahydromethanopterin cyclohydrolase. 4, methylene-tetrahydromethanopterin dehydrogenase. 5, methylene-tetrahydromethanopterin reductase. 6, F_{420}-reducing hydrogenase. 7, methyl-tetrahydromethanopterin-coenzyme M methyltransferase. 8, methyl-coenzyme M reductase. 9, A_1A_0 ATP-Synthase. 10, multienzyme complex of [NiFe] hydrogenase and heterodisulfide reductase. 11, Na^+/H^+ antiporter. Fd, ferredoxin. H_4MPT, tetrahydromethanopterin. HS-CoB, coenzyme B. CoM-S-S-CoB, heterodisulfide. Modified from Thauer *et al.* 2008.

Aceticlastic methanogensis

Besides the consumption of H_2 and formate by hydrogenotrophic methanogens, the degradation of acetate is essential, which is also formed as end-product of acetogenesis. Acetate plays a key role in a biogas reactor because syntrophic bacteria can degrade short chain fatty acids and alcohols only at low acetate concentrations (Schink 1997). If methanogens are impaired in their activity, there is an accumulation of propionic acid and other carboxylic acids (for example, butyric acid or valeric acid) which ultimately leads to breakdown of the biogas plant. Therefore, acetate conversion by methanogenic archaea is essential in the anaerobic microbial degradation process. The metabolization of acetate is called aceticlastic methanogenesis that can only be performed by members of the order Methanosarcinales (Deppenmeier and Müller 2007). CH_4 formation based on acetate is connected to a change in the free energy of only -36 kJ mol^{-1}. Hence, aceticlastic methanogens must possess efficient energy-conserving systems to cope with the thermodynamic limitation (Deppenmeier 2002). Despite the fact that two thirds of the global CH_4 production originates from the methyl group of acetate (Metje and Frenzel 2007), only two genera, *Methanosarcina* and *Methanosaeta,* are able to metabolize this substrate for growth. Both genera are found in biogas reactors of agricultural biogas plants (Kern *et al.* 2016a, Kampmann *et al.* 2012, St-Pierre and Wright 2013, Nettmann *et al.* 2010). Thus, it is important to study aceticlastic methanogenesis in detail. In all aceticlastic methanogenic species acetate is activated to acetyl-CoA connected to the consumption of ATP. In *Methanosarcina* strains the activation starts by an ATP-dependent phosphorylation of the carboxyl group of acetate by an acetate kinase (Fig. 5, reaction 1) (Latimer and Ferry 1993, Buss *et al.* 1997). Subsequently, a phosphotransacetylase converts the resulting acetyl-phosphate to acetyl-S-CoA (Fig. 5, reaction 2) (Rasche *et al.* 1997, Latimer and Ferry 1993). In *Methanosaeta* spp. acetate activation is performed by an acetyl-CoA synthetase forming acetyl-S-CoA, AMP, and pyrophosphate (PP_i) from acetate, coenzyme A (HS-CoA), and ATP (Jetten *et al.* 1989). PP_i is hydrolysed by a soluble pyrophosphatase to drive the reaction (Jetten *et al.* 1992). Consequently, for the activation of one mole acetate in *Methanosaeta* spp. two equivalents of ATP are required.

In the next step, acetyl-CoA is cleaved into its carbonyl and methyl moiety by the action of a CO-dehydrogenase/acetyl-CoA synthase (CODH/ACS) in *Methanosarcina* and *Methanosaeta* spp. (Fig. 5, reaction 3) (Raybuck *et al.* 1991, Abbanat and Ferry 1991). The carbonyl group of acetyl-CoA is oxidized to CO_2 and the electrons are used for Fd_{ox} reduction. The methyl group is transferred to H_4SPT. Subsequently, a methyl-H_4MPT-HS-CoM methyltransferase catalyzes the transfer of the methyl group to HS-CoM, coupled by an Na^+ ion extrusion across the cytoplasmic membrane, resulting in the generation of an electrochemical ion gradient (Fig. 5, reaction 4) (Gottschalk and Thauer 2001). The methyl group of methyl-S-CoM is further reduced to CH_4, catalyzed by the methyl-CoM reductase (Fig. 5, reaction 5). The electrons for this reaction derive from HS-CoB, causing the formation of the mixed disulfide from HS-CoM and HS-CoB, the CoM-S-S-CoB. CoM-S-S-CoB is the terminal electron acceptor of an anaerobic respiratory chain and is reduced by electrons of Fd_{red}. The pathway of Fd_{red} oxidation differs in members of the genus *Methanosarcina*. *Methanosarcina* (*Ms.*) *mazei* and *Ms. barkeri* for example use the energy-converting [NiFe] hydrogenase (Ech hydrogenase) for the oxidation of Fd_{red} (Fig. 5, reaction 6) (Künkel *et al.* 1998, Meuer *et al.*, 1999). In contrast *Ms.*

acetivorans is a hydrogenase-negative strain and the oxidation of Fd_{red} is catalyzed by the Rnf complex (Fig. 5, reaction 8) (Ferry and Lessner 2008). The Ech hydrogenase transfers electrons from Fd_{red} to protons to form H_2. Additionally, Welte *et al.* (2010) demonstrated that the Ech hydrogenase acts as proton pump in the course of Fd_{red} oxidation and contributes to the electrochemical ion gradient.

Molecular H_2 formed by the Ech hydrogenase diffuses out of the cell and is oxidized by a membrane-bound methanophenazine-reducing hydrogenase (Vho/Vht) (Fig. 5, reaction 7) (Deppenmeier *et al.* 1992). The active site of Vho/Vht is exposed towards the periplasm, thus, two protons are released to the extracellular side of the membrane when H_2 is oxidized (Fig. 5, blue box) (Ide *et al.* 1999). The electrons are transferred to methanophenazine, an electron carrier located in the cytoplasmic membrane (Beifuss *et al.* 2000). The second Fd_{red} oxidizing enzyme is the Rnf complex found in *Ms. acetivorans* (Fig. 5, reaction 8). This enzyme complex catalyzes the electron transport from Fd_{red} to methanophenazine coupled to a Na^+ transport across the cytoplasmic membrane (Ferry and Lessner 2008).

In addition to already described membrane-bound enzyme complexes, the aceticlastic methanogens possess a heterodisulfide reductase (HdrDE) associated to the cytoplasmic membrane (Fig. 5, reaction 9) (Heiden *et al.* 1993, Heiden *et al.* 1994, Künkel *et al.* 1997, Smith and Ingram-Smith 2007). This enzyme complex catalyzes the reduction of the final electron acceptor, CoM-S-S-CoB. The electrons required for this reaction originate from Fd_{red}, generated during aceticlastic methanogenesis. Methanophenazine, localized in the membrane, transports the electrons to the catalytic center of HdrDE. In addition, the enzyme is able to transport protons across the cytoplasmic membrane.

Ms. mazei and *Ms. barkeri* are able to transfer at least five protons per acetate molecule across the membrane via the membrane-bound hydrogenases and HdrDE (Ide *et al.* 1999, Welte *et al.* 2010). Additionally, two Na^+ ions are translocated by the methyl-H_4MPT-HS-CoM methyltransferase (Becher *et al.* 1992b). In *Ms. acetivorans* two protons are translocated by HdrDE. In addition, the translocation of three Na^+ ions is conducted by the Rnf complex and two Na^+ ions are pumped by the methyl-H_4MPT-HS-CoM methyltransferase across the cytoplasmic membrane (Ide *et al.* 1999, Schlegel *et al.* 2012, Becher *et al.* 1992b). The resulted electrochemical Na^+/H^+ gradient can be used for ATP synthesis by an A_1A_0 ATP synthase (Fig. 5, reaction 11) (Pisa *et al.* 2007, Schlegel und Müller 2011).

In contrast to *Methanosarcina* strains, two ATP equivalents are required for the activation of one acetate molecule in *Methanosaeta* species. As described above during CH_4 formation in *Methanosaeta* spp. Fd_{ox} is reduced and CoM-S-S-CoB is produced. Fd_{red} is used as electron donor by a membrane-bound electron transport chain and CoM-S-S-CoB is reduced as terminal electron acceptor of the anaerobic respiratory chain (Welte and Deppenmeier 2011a). However, in the genome of *Methanosaeta* spp. neither genes encoding an Ech hydrogenase nor genes encoding a Rnf complex could be found (Smith and Ingram-Smith 2007). Nevertheless, all genes encoding the $F_{420}H_2$ dehydrogenase without the $F_{420}H_2$-oxidizing subunit FpoF were found in the genome of *Methanosaeta* strains (Welte and Deppenmeier 2011a).

The $F_{420}H_2$ dehydrogenase is a $F_{420}H_2$-oxidizing membrane-bound enzyme complex found only in

members of the order Methanosarcinales. In *Methanosarcina* spp. during growth on methylated compounds the protein uses $F_{420}H_2$ as electron donor and reduces the electron carrier methanophanzine in the cytoplasmic membrane. In the course of one reaction cycle the enzyme complex can transfer two protons across the membrane contributing to the generation of an electrochemical ion gradient. In contrast, it was demonstrated that $F_{420}H_2$ is not oxidized by the membrane fraction of *Methanosaeta* (*Mt.*) *thermoacetophila* (Welte and Deppenmeier 2011a). However, the genes encoding the $F_{420}H_2$ dehydrogenase without the $F_{420}H_2$-oxidizing subunit FpoF are highly transcribed in *Mt. thermoacetophila* during growth on acetate (Welte and Deppenmeier 2014). This finding led to the hypothesis that the $F_{420}H_2$ dehydrogenase encoded in the genome of *Methanosaeta* spp. could be involved in the membrane-associated oxidation of Fd_{red}. The subunit FpoI of the $F_{420}H_2$ dehydrogenase contains iron-sulfur clusters and a C-terminal extension with an accumulation of lysine residues. Lysine, as basic amino acid, could facilitate the interaction with the acidic Fd_{red}. The electrons could be further transferred to iron-sulfur clusters in FpoI and thus, reach the active side of the enzyme complex for methanophenazine reduction. The redox potential difference of Fd_{red} as electron donor (-500 mV, Thauer *et al.* 2008) and methanophenazine (-165 mV, Tietze *et al.* 2003) as electron acceptor could enable the translocation of three protons across the cytoplasmic membrane. Taking into account all protons translocated by the $F_{420}H_2$ dehydrogenase and the HdrDE as well as the Na^+ ions extrusion across the cytoplasmic membrane by the methyl-H_4MPT-HS-CoM methyltransferase an electrochemical ion gradient could be generated. In total seven ions would be translocated during the breakdown of one acetate molecule which could be used by a bi-functional A_1A_0 ATP synthase for ATP synthesis. Estimating a stoichiometry of three ions per ADP phosphorylation, two ATP molecules and one extra translocated ion would be provided from one acetate molecule (Welte and Deppenmeier 2014). Considering the fact that two ATP molecules are required for the activation of one acetate molecule, it can be estimated that three mole acetate are needed to phosphorylate one extra mole of ADP to ATP (Welte and Deppenmeier 2014). This is the minimal energy quantum to sustain life but because of the low acetate concentrations needed for growth, *Methanosaeta* strains are able to occupy ecological niches with low amounts of acetate as found in some biogas plants (Jetten *et al.* 1992).

Biogas formation is a highly complex process in reactors of a biogas plant and various prokaryotes are involved, but how can the different classes, genera and species be identified and quantified? In the following chapters, various known approaches to identify and quantify the microbial community are discussed.

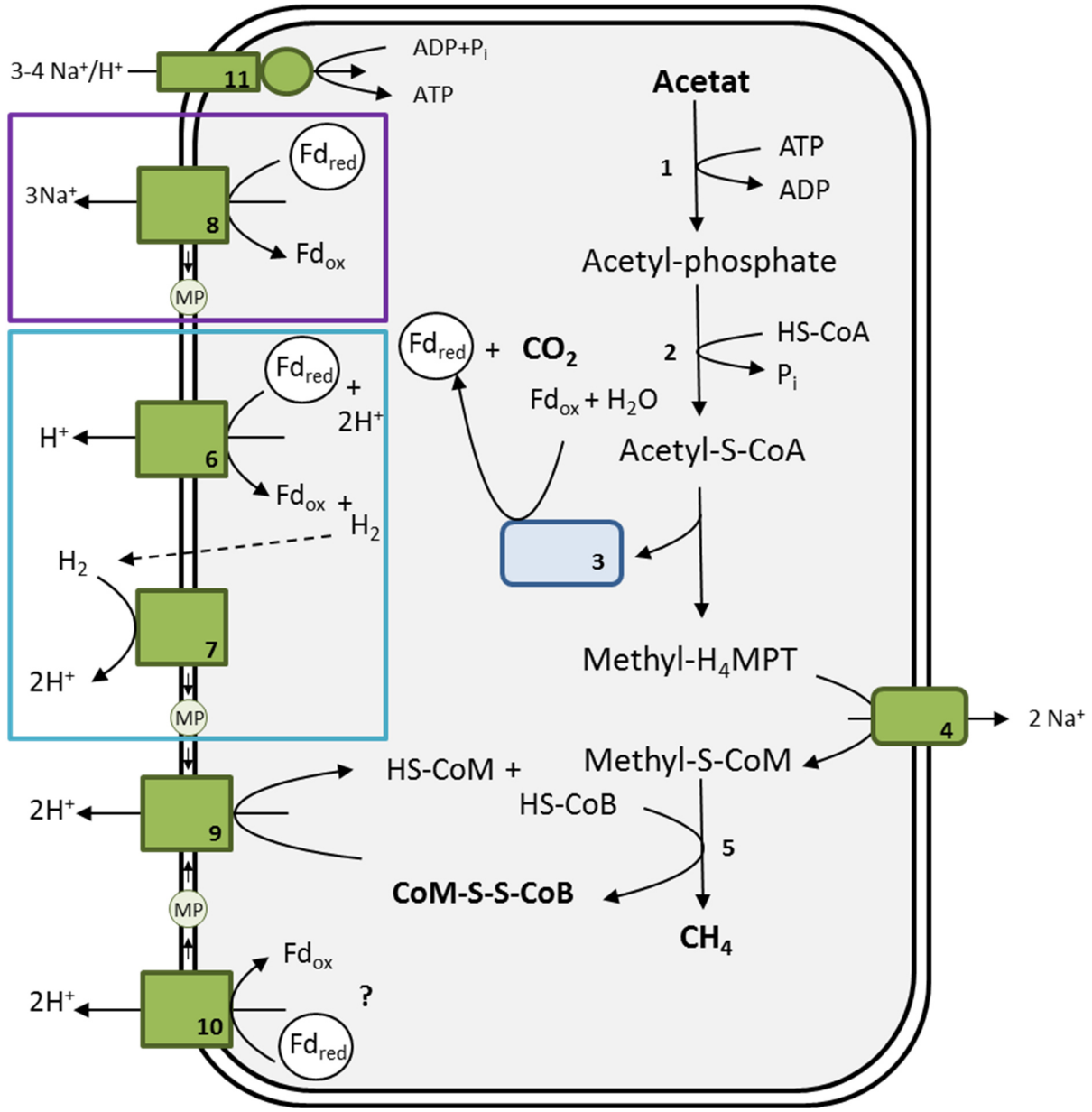

Figure 5: **Schematic presentation of the aceticlastic methanogensis of *Methanosarcina* sp. coupled with an energy conservation system.** The enzyme complexes shown in the *blue box* are from *Ms. mazei* and *Ms. barkeri* whereas the complex in the *puple box* is found in *Ms. acetivorans*. *Green*, enzyme complexes localized to the cytoplasmic membrane. *Blue*, cytoplasmic enzyme complexes. 1, acetate kinase. 2, phosphotransacetylase. 3, CO-dehydrogenase/ acetyl-CoA synthase. 4, methyl-tetrahydromethanopterin-coenzyme M methyltransferase. 5, methyl-coenzyme M reductase. 6, Ech hydrogenase. 7, F_{420}-non reducing hydrogenase. 8, Rnf complex. 9, heterodisulfide reductase. 10, $F_{420}H_2$ dehydrogenase. 11, A_1A_0 ATP-Synthase. Fd, ferredoxin. H_4MPT, tetrahydromethanopterin. HS-CoB, coenzyme B. CoM-S-S-CoB, heterodisulfide.

Identification and quantification of the microbial community in a biogas plant

The biological community in a complex habitat such as a biogas reactor, which is fed with various plant and animal organic matter, is difficult to describe to a detailed extent. More than 30 years ago, a detection method was developed based on species-specific regions of the 16S rDNA or 16S rRNA, which allows an organism-specific characterization (Woese and Fox 1977, Amann *et al.* 1995, Weisburg *et al.* 1991). Thus, this detection method allows to obtain more information about the community structures in anaerobic processes like biogas production (Oude Elferink *et al.* 1998, Yu *et al.* 2005, Karakashev *et al.* 2005, Klocke *et al.* 2008). Regions of the 16S rDNA in the genome are unique for each species which enable a species-specific detection. After the generation of PCR products various sequencing methods can be used (e.g. 454 pyrosequencing and illumina sequencing) to analyze those regions. Moreover, genomes of different associated species can be analyzed by metagenomic sequencing. The first successful isolation of DNA from thermophilic anaerobic digesters and the corresponding metagenome sequencing was done by Healy and coworkers (1995). Since 1995, hundreds of metagenomes from different types of biogas reactors have been published based on metagenome sequencing (Sundberg *et al.* 2013, Liu *et al.* 2009, Li *et al.* 2013, Kröber *et al.* 2009, Schlüter *et al.* 2008, Demirel *et al.* 2008, Supaphol *et al.* 2011, Klocke *et al.* 2007). The major outcome of this approach was that the different characteristics of the diverse reactors like temperature, substrate composition, pH value, hydrogen carbonate buffer capacity, total content of volatile fatty acids, or retention time affect the microbial composition of the metagenome (Demirel *et al.* 2008, Kröber *et al.* 2009, Sundberg *et al.* 2013).

The basis of the 16S gene analytic methods and metagenomic sequencing is the isolation and purification of the total DNA from a habitat as the initial step for metagenomic analysis. One major difficulty associated with the metagenomic approaches is related to the effectiveness of DNA isolation. The efficiency can vary depending on the method applied which can have influences on the results of the microbial community analysis (Theiss *et al.* 2016). Additionally, purified DNA is often contaminated with polyphenolic compounds (Streit and Schmitz 2004). These compounds are difficult to remove but they interfere with enzymatic modifications of the isolated DNA, needed for sequencing. Consequently, the construction of environmental DNA libraries is problematic. Furthermore, it is difficult to compare community structures determined by PCR analysis of 16S genes using different primer pairs because of low specificity of the used primers for the amplified DNA sequences (Fischer et al. 2016). Despite these deficiencies, it is possible to identify a large number of organisms in full-scale operating biogas plants. All analyzed reactors exhibit a lot of differences as described above. However, some similarities are found in the microbial composition of biogas reactors. Members of the phylum Firmicutes and especially various species of the class of Clostridia can be found in any type of reactor as well as methanogenic archaea, which are crucial for the production of CH_4.

Microbial characterization by enrichment cultures

Genome-based analysis enables the identification of individual organisms in a biogas reactor, but does not allow conclusions about their metabolic activity. Much is known about the basic metabolism in different types of anaerobic digestion processes, but little is known about the microbes responsible

for these processes (Weiland 2010). Only a few percent of bacteria and archaea have been isolated so far. The isolation of various species from biogas sludge and enrichment cultures offers the opportunity of carring out biochemical studies on the metabolism and the catalytic properties of these organisms during degradation of organic matter. Enrichment of archaeal and bacterial cultures from complex environments like biogas reactors might be successful in some cases but most microorganisms are nonculturable, because the imitation of environmental conditions in a laboratory proves a challenge.

In a few cases, however, enrichment cultures of reactor material were carried out successfully. Kern *et al.* (2016a) published an isolation of an archaeal strain from a mesophilic biogas reactor, *Ms. flavescens* sp. nov. E03.2^T a novel strictly anaerobic, non-motile, sarcina-like, coccal methanogenic archaeon. This strain grows autotrophically on H_2 + CO_2 and is also able to utilize acetate, methylamines, and methanol. The genome of strain E03.2^T was completely sequenced and in combination with phenotypic (sarcina-like structures) and physiological (methanogenesis from H_2 + CO_2, or acetate, or methylated compounds) characteristics it was concluded the organism belongs to the genus *Methanosarcina* (Kern *et al.* 2016a).

Mladenovska and Ahring (2000) also isolated *Methanosarcina* strains from full-scale operating thermophilic biogas plants. These biogas plants were fed with a mixture of animal manures or with industrial organic wastes. All isolates exhibited significantly higher growth rates and higher acetate binding affinities compared to the type strain *Ms. thermophila* TM-1^T. In addition, isolates from the tanks treated with a mixture of animal manures showed a higher affinity to acetate compared to isolates from biogas plants fed with industrial organic wastes (Mladenovska and Ahring 2000). Kern *et al.* (2016a) were able to enrich hydrogenotrophic strains from the same biogas plant, from which they isolated *Ms. flavescens* sp. nov. E03.2T. In addition, a new hydrogenotrophic species could be identified, belonging to the genus *Methanobacterium* which was named *Mb. aggregans* sp. nov. E09F.3T (Kern *et al.* 2015). This organism is characterized by forming large aggregates consisting of intertwined bundles of chains.

Methanobacterium sp. could also be isolated and characterized in other types of biogas plants. Strain *Mb. thermoformicicum* CB12, for example, was isolated from a thermophilic reactor (Zhao *et al.* 1986). The organism has characteristic properties of a thermophilic, hydrogenotrophic methanogenic strain like an optimal growth temperature of 56 °C but *Mb. thermoformicicum* CB12 is growing faster than all currently known thermophilic species belonging to hydrogenotrophic methanogens (Zhao *et al.* 1986).

Besides methanogens, which could be isolated from biogas reactors, bacterial microorganisms were enriched and pure cultures isolated. Ruan *et al.* (2014) have isolated a bacterial strain from a large-scale agricultural anaerobic digester, which is able to metabolize sugars and belongs to the genus *Kurthia*. The strain *Kurthia huakuii* LAM0618^T belongs to the phylum Firmicutes and might be involved in the hydrolytic step in a biogas reactor (Ruan *et al.* 2014). Additionally, type strains from the most prevalent of all known anaerobic, cellulolytic bacteria, *Clostridium* (*Cl.*) *thermocellum* strains, can be found in almost all biogas reactors and utilize cellulose and cellobiose (Koeck *et al.* 2014). Cellulase activities of all isolates could be measured to characterize the different strains and to get information on the hydrolytic state of the analyzed reactor (Koeck *et al.* 2014).

Enzymatic characterization of microbial communities

The isolation of bacterial and archaeal strains to characterize the microbial diversity in biogas sludge is challenging and difficult in general, especially, because many microorganisms are nonculturable and thus, cannot be grown in the laboratory. The isolation of some pure cultures gives the opportunity to characterize the metabolisms of single strains. However, it is not possible to analyze the metabolic capacity of all microorganisms involved in the four steps of anaerobic degradation (hydrolysis, acidogenesis, acetogenesis, methanogenesis). The metabolic capacity of a microbial community is a new parameter also referred to as Metabolic Quotient which is based on qPCR and RT-qPCR analysis (Munk *et al.* 2012). This quotient was introduced for the determination of methanogenic activity and as a warning system of process acidification. Thus, the metabolic capacity or the metabolic potential of a microbial community describes the metabolic activity or the overall capability to degrade substrates. A possible approach to analyze the metabolic capacity of the microorganisms involved in anaerobic degradation in biogas sludge could be the determination of enzymatic activities of each metabolic group of microorganisms. Enzyme activities of the first step of anaerobic degradation of biomass, the hydrolysis, and the second step, the acidogenesis, could already successfully be measured (Parawira *et al.* 2005, Kim *et al.* 2012, Gabris *et al.* 2015). In a new approach the measurement of a key enzyme of the last degradation step in biogas sludge, the methanogenesis, has also been shown (Refai *et al.* 2014a, **Chapter 1**).

Parawira and coworkers (2005) have measured the activities of several hydrolyzing enzymes such as amylases, pectinases, and proteases as well as different types of cellulases. The different enzyme activities were measured in the supernatant of centrifuged mesophilic biogas sludge samples, fed with solid potato wastes. Kim *et al.* (2012) noticed that the temperature during the anaerobic digestion process is crucial for the catalytic conversion by hydrolytic enzymes. In a thermophilic biogas reactor, the activities of amylase, protease, and lipase were significantly higher than in an equivalent process at mesophilic temperature (Kim *et al.* 2012). The hydrolysis of organic material is the first step of the anaerobic degradation process in a biogas reactor. Consequently, hydrolytic enzyme activities may also affect subsequent degradation steps (acidogenesis, acetogensis and methanogenesis).

Besides hydrolytic enzymes, acidogenic key enzymes were analyzed to evaluate the metabolic potential in this degradation step. Gabris *et al.* (2015) measured activities of the acetate kinase, the butyrate kinase and the butyryl-CoA: acetate-CoA transferase in cell-free extract from sludge samples of three different mesophilic agricultural biogas plants. While the activity of the butyrate kinase (<0.02 U mg^{-1} protein) was low in all three reactors, significantly higher activities were measured for the acetate kinase and the butyryl-CoA: acetate-CoA transferase (0.54 U mg^{-1} protein and 5.73 U mg^{-1} protein) (Gabris *et al.* 2015).

Moreover, in the course of this study a new approach for determining the activity of a key enzyme of CH_4 formation in biogas sludge was published to contribute to the characterization of the metabolic potential of methanogens which are responsible for the last degradation step in biogas plants (Refai *et al.* 2014a, **Chapter 1**). The Hdr, a key enzyme in the energy metabolism of methanogenic archaea,

catalyzes the specific reduction of the terminal electron acceptor (CoM-S-S-CoB). The genes of this key enzyme are encoded in the genome of all methanogenic species described so far. Therefore, the specific CoM-S-S-CoB reduction rate can be used for the determination of the metabolic potential of the methanogenic degradation step. The measurement of the Hdr was performed in cell-free extract of mesophilic biogas sludge (Refai *et al.* 2014a, **Chapter 1**).

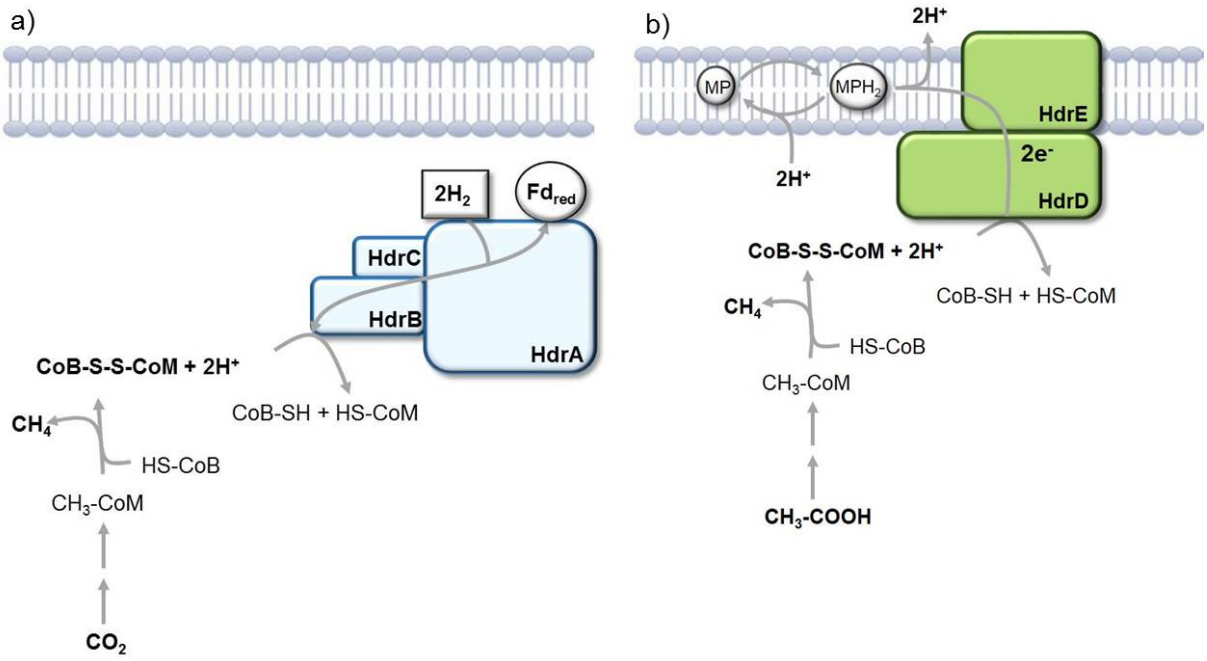

Figure 6: **Scheme of heterodisulfide reductases in methanogenic archaea and their localization in the cells.** a) The heterodisulfide reductase of hydrogenotrophic methanogens is localized in the cytoplasm. The enzyme complex consists of three subunits (HdrA, HdrB, HdrC) and is able to use the electrons from 2 H_2 for a bifurcation, the reduction of ferredoxin (Fd_{red}) and the heterodisulfide (CoB-S-S-CoM → HS-CoB + HS-CoM). b) In aceticlastic methanogens the heterodisulfide reductase consists of two subunits (HdrD, HdrE) and is localized in the cytoplasmic membrane in the cell. The electrons for the reduction of the CoB-S-S-CoM originate from the conversion of actetate and are transferred from the methanophenazin (MPH_2) localized in the membrane to the catalytic center in subunit HdrD.

In the enzyme assay electrons from reduced methyl viologen, an artificial electron donor, were used for the reduction of the CoM-S-S-CoB. The analysis of Hdr activity offers the opportunity not only to define the entire metabolic potential of methanogens, but to differentiate between the two occurring metabolic types of methanogens. In hydrogenotrophic methanogens the heterodisulfide reductase HdrABC forms a complex with the hydrogenase Mvh in the cytoplasm of the cells (Fig. 6a). In aceticlastic methanogens the heterodisulfide reductase HdrDE is localized in the cytoplasmic membrane (Fig. 6b), so that activity measurements in the cytoplasm and cytoplasmic membrane fractions characterize the metabolic activity of both methanogenic pathways (Refai *et al.* 2014a, **Chapter 1**). It was found that in a mesophilic biogas plant, which was fed with maize silage, dry chicken, and cattle manure, a ratio of two third hydrogenotrophic and one third aceticlastic methanogens are present (Refai *et al.* 2014a, **Chapter 1**). So far, quantification of these organisms in such a complex community as a biogas reactor is done by sequence analysis of 16S rRNA, or other

marker genes like the gene coding for the subunit A of the methyl-CoM reductase (Bergmann *et al.* 2010b, Hanreich *et al.* 2013). The quantification of methanogenic cells via Hdr activity is specific and much faster than gene sequence analysis. Moreover, enzyme activities are highly sensitive to changes in process conditions.

Monitoring the biogas production process

Monitoring of biogas production is necessary to ensure successful operation of biogas reactors and to detect process imbalances at an early stage for the prevention of process incidents. The anaerobic digestion process itself is a highly complex system involving many interacting groups of microorganisms. Several prokaryotes are sensitive to a number of operating factors which can influence the efficiency of the production process. The physico-chemical factors affecting biogas production are mainly based on operating conditions and substrates fed into the biogas reactors (e.g. consistency, stirring power, liquid content). Operating conditions, including pH value, temperature, loading rate, and retention time, can directly influence the microbial community structure. Effects from feedstock, including overall composition, dry mass, and organic dry mass, or toxic and inhibitory compounds, can adversely affect the microbial degradation of organic matter. Sometimes, toxic compounds are not initially present in the feed but are generated in the reactor during degradation processes (e.g. H_2S or ammonia/ammonium (NH_3/NH_4^+) concentrations).

A large number of parameters have been studied with the aim to characterize the biogas formation process and to search for early-warning systems for the detection of developing process incidents. The common indicators for monitoring the biogas production process are the quantity and composition of the feedstock, biogas yield, gas composition, fermentation temperature and pH value, volatile fatty acid (VFA) concentration, hydrogen carbonate buffer capacity, H_2, and ammonia (NH_3) concentrations as well as micronutrient concentrations (Boe *et al.* 2010, Cadena Pereda *et al.* 2010, Michaud *et al.* 2002). Hydrogen carbonate buffer capacity and VFA concentrations as well as H_2 and NH_3/NH_4^+ concentrations can be used as warning parameters indicating process imbalances (Hawkes *et al.* 1994, Boe *et al.* 2010, Weiland 2008, Marchaim and Krause 1993). However, alternative parameters such as measurement of microbial enzyme activities and community structure can also be used to monitor biogas formation as already described in **Chapter 1** (Parawira *et al.* 2005, Kim *et al.* 2012, Gabris *et al.* 2015, Refai *et al.* 2014a).

Monitoring parameters

Monitoring the feedstock input is essential because changes in the amount of feeding and raw material composition can cause process instabilities (Drosg 2013). In agricultural biogas plants, the organic raw materials are usually quite similar in their composition (energy plant silage, poultry-, pig-, cattle- or horse manure, cereal grains). Changes in the composition of feeding can be caused by different sources of supply. For an optimal production process a balanced substrate composition is crucial. Besides the type of substrate, proportion of plant fibers, water content, nitrogen content, particle size, and gas yield of the individual substrates, as well as frequency of feeding play an important role. High frequency of feeding intervals (about 48 feeds per day) can lead to high biogas

yields and stable process conditions. This may avoid interference of stirring power and accumulation of VFA.

Biogas yield

Biogas production is probably the most common parameter used for long-term monitoring of anaerobic degradation. The amount of biogas produced provides information on the status of the overall process. However, biogas volume detection is one of the most challenging parameter in the monitoring process of biogas production because there is a great dependency on the amount of produced biogas and the substrate composition as well as retention time and organic loading rate (Angelidaki *et al.* 1999, Deublein and Steinhauser 2008, Fantozzi and Buratti 2009). Depending on the substrate more or less biogas may be produced (Fig. 1). Changes in the amount of produced biogas cannot be used as reliable parameter indicating process imbalances because any increase in volumetric loading or retention time can raise biogas production. On the other hand, a decrease in biogas production often occurs not before the process is severely inhibited or has already broken down, thus, it is not an effective early warning indicator for process imbalances (Switzenbaum *et al.* 1990, Moletta *et al.* 1994). Nevertheless, the measurement of biogas yield is very simple and widely used to monitor anaerobic degradation in biogas reactors, especially in agricultural biogas plants with constant feeding and constant process conditions.

CH_4 content

As already described, biogas mainly consists of CH_4 and CO_2. The partial pressure of these two gasses is usually constant in a reactor during stable process conditions for a given carbon substrate. Thus, biogas composition, especially the partial pressures of CO_2 and CH_4, might be useful parameters for process monitoring. However, the CH_4 content also depends on substrate composition, loading rate, temperature, and pH value (Amon *et al.* 2007, Liu 2003, Mshandete *et al.* 2006). Ahring and coworkers (1995) tested the use of the CH_4 production rate and the CH_4 yield as process indicators. They showed that the CH_4 yield can reflect process imbalances but changes in this parameter were relatively small (Ahring *et al.* 1995). Similar to biogas production, response in CH_4 production is significant only when the process imbalance is well developed (Switzenbaum *et al.* 1990). In summary, the overall anaerobic degradation process is already severely disrupted when a decrease in CH_4 yield occurs.

pH value

The pH value is another parameter simply measurable in a biogas reactor. The pH can be analyzed either manually or automatically in an online monitoring process and is mainly influenced by the hydrogen carbonate buffer capacity. Moreover, the concentration of VFA and the NH_3/NH_4^+ concentration can change the pH in a biogas reactor. In addition, the feedstock pH affects the pH value in a biogas reactor. Normally, the pH is neutral to alkaline in a biogas plant (pH 6.5 – 8.5). This is important and decisive for the microbial community and their functionality in anaerobic digestion. However, the pH response has low sensitivity in a well-buffered system like a biogas reactor and is not

recommended to indicate process imbalances (Björnsson *et al.* 2000). Angelidaki and Ahring (1994) observed different pH values within 0.5 units in biogas reactors when the process was inhibited by high NH_3 concentrations and VFA accumulation. In this case, even strong process incidents had no decisive influence on the pH value. Accordingly, measurable pH changes indicate an ongoing process instability where the degradation process is impaired and the microbial community sustainably damaged (Nielsen and Ahring 2006). Thus, the pH value cannot be used as a reliable parameter indicating process imbalances.

Hydrogen carbonate buffer capacity

The parameters described so far can characterize a biogas production to a certain extent but, due to the limitations mentioned above, they might not be suited to reliably indicate process instability at an early stage. The buffer capacity is a better alternative for displaying process imbalances (Jantsch and Mattiasson 2004). In a biogas reactor the hydrogen carbonate/carbonate buffer system is mainly responsible for the regulation of pH homeostasis with buffer capacity depending on the concentration of the corresponding bases and acids (Switzenbaum *et al.* 1990, Moosbrugger *et al.* 1993). The hydrogen carbonate/ carbonate buffer system is also the main buffer system in different types of manure and minimizes pH changes caused by VFA and NH_4^+ ion formation in the process of anaerobic degradation.

Maintaining the pH in a biogas plant is crucial for an effective microbial degradation process. The pH optimum of fermentative bacteria involved in the first three steps of anaerobic degradation in a biogas reactor is 6.7 – 7.4 (Bryant 1979). The methanogenic archaea, responsible for last the step of CH_4 formation, grow at neutral pH values. Thus, the pH value in the anaerobic system of a biogas reactor has to be 6.5 – 8.5 for optimal growth of the microorganisms involved in the degradation process.

The proton binding effect of the buffer depends on the pH-dependent equilibrium reactions between carbonate (CO_3^{2-}) and hydrogen carbonate (HCO_3^-), and between hydrogen carbonate (HCO_3^-) and carbonic acid (H_2CO_3) (Fig. 7). The pKs values of the two reactions are 10.45 (CO_3^{2-}/HCO_3^-) and 6.52 (HCO_3^-/ H_2CO_3), respectively. As the pH value is typically 6.5 – 8.5 in the anaerobic degradation system of a biogas reactor, CO_3^{2-} concentrations are insignificant (Rozzi *et al.* 1994, Fig. 7). This can be explained by the buffer index diagram. At pH 6.5 – 8.5 mainly HCO_3^- is present in a biogas plant (Fig. 7). The equilibrium shifts to H_2CO_3 at increasing VFA concentrations in a biogas reactor (Fig. 7). In aqueous solution H_2CO_3 dissociates to CO_2 and H_2O. According to the HCO_3^-/CO_3^{2-} buffer system rising amounts of NH_4^+ lead to higher HCO_3^- concentrations (Moosbrugger *et al.* 1993, Fig. 7). Thus, changes in pH values can be balanced by the HCO_3^-/CO_3^{2-} buffer system to a certain extent.

The HCO_3^- buffer capacity is measured by titration and is defined as total alkalinity of carbonate (TAC). Various titration methods exist to determine TAC (Moosbrugger *et al.* 1993, Lahav and Morgan 2004). Correlation of TAC and VFA allows calculation of the amount of VFA and NH_4^+, which can be tolerated by the system.

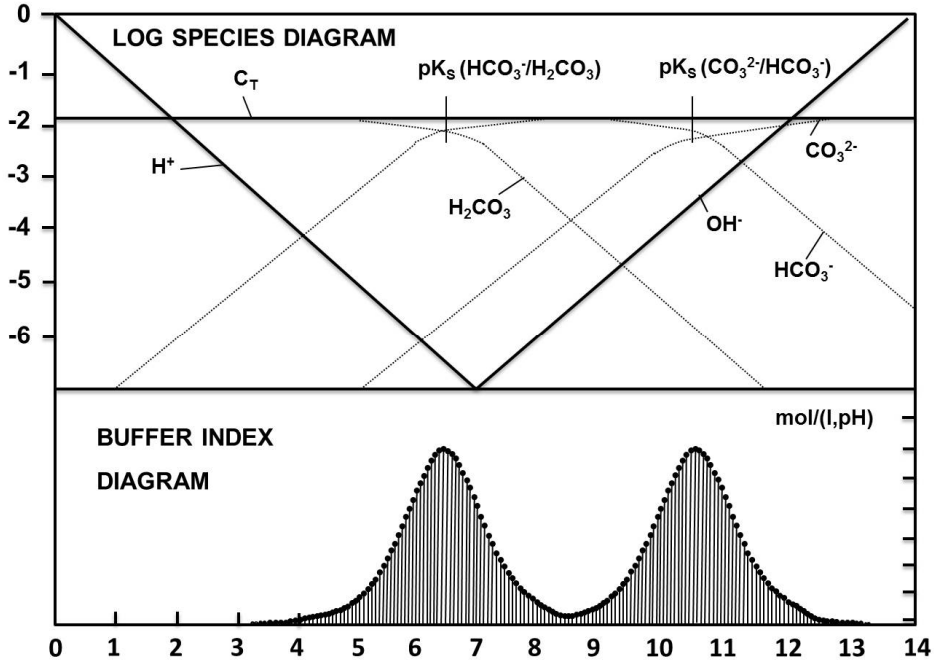

Figure 7: **Hydrogen carbonate/ carbonate buffer system.** Double logarithmic description of the concentration ratios of the carbonate/ hydrogen carbonate buffer system as a function of pH values. Modified from Moosbrugger *et al.* 1993.

Volatile fatty acid concentration

The determination of the VFA concentrations is a useful monitoring parameter for the biogas production process (Hill and Holmberg 1988, Hickey and Switzenbaum 1991, Anderson and Yang 1992, Moosbrugger *et al.* 1993; Ahring *et al.* 1995, Björnsson *et al.* 2000, Feitkenhauer *et al.* 2002, Mechichi and Sayadi 2005, Boe 2006, Boe *et al.* 2008). Additionally, it can be used as a typical warning system for process imbalances such as overfeeding, inhibition by mycotoxins, or micronutrient deficiency. Increasing VFA concentrations in a biogas reactor directly reflect a kinetic uncoupling between microbial acid producers and consumers (Switzenbaum *et al.* 1990). The concentration of different VFA like butyric acid, propionic acid, or acetic acid is measured via gaschromatography analysis. This analysis allows the determination of each acid concentration in the biogas sludge. Another common method to determine the amount of VFA in the biogas sludge is titration. This is a quantification method to determine the concentration of total VFA which is defined as FOS value and corresponds to the acid concentration in the sludge, expressed in acetic acid equivalent.

VFA concentration, pH value, and HCO_3^- buffer capacity directly influence each other. Consequently, all these parameters must be considered for monitoring a production process. In a low buffered system, pH, TAC, and FOS measurements are useful for process monitoring (Murto *et al.* 2004). In highly buffered systems only VFA is reliable for indicating process imbalance because the high TAC concentration stabilizes pH. Several studies have pointed out that different VFA concentrations can act as early warning systems for process incidents (Cobb and Hill 1991, Ahring *et al.* 1995). Often

propionic acid concentration itself can function as indicator for process imbalances. Besides propionic acid, iso-butyric, and iso-valeric acid as well as n-butyric and iso-butyric had been suggested as indicators of process imbalance (Cobb and Hill 1991, Ahring *et al.* 1995). So, VFA are excellent indicators of organic overload and of inhibitions caused by mycotoxins when acid oxidizers and methanogens are inhibited.

H_2 concentration

Another parameter useful to monitor the biogas formation process is the H_2 concentration or the H_2 partial pressure in a biogas plant. H_2 is an important intermediate and is used as an electron carrier in microbial metabolism. The H_2 partial pressure directly affects the anaerobic degradation process in the biogas sludge. Slightly increasing H_2 concentrations can be sufficient to impede degradation of VFA by syntrophic bacteria (Speece 1983). Thus, H_2 accumulation has been suggested as early indicator for process imbalances (Hickey and Switzenbaum 1991). However, in practice, accurate measurement of H_2 concentration in biogas sludge is challenging, hence, in standard agricultural biogas plants a technical implementation is not possible.

Besides all parameters already described, CO concentration, redox potential as well as the organic dry mass are useful parameters monitoring the biogas formation process. Furthermore, the analysis of the composition and quantification of the microbial community in a biogas plant can be used as stability marker for the process. Usually, the microbial community is analyzed by genomic approaches as described above. Although the microbial community structure could be theoretically used as stability marker, the determination is time consuming and expensive in practice. Therefore, this analytical method is rarely used and will not be discussed any further.

Microbial degradation potential

Besides characterization of microbial community structures based on sequence analysis, another analytic approach to characterize the microorganisms involved in the anaerobic breakdown of biomass is the quantification of the microbial degradation potential or the CH_4 production potential. The microbial degradation potential describes the metabolic ability of substrate degradation by microorganisms in a biogas reactor. The biochemical CH_4 potential is defined as the amount of CH_4 produced per 1 g of solid organic substrate (Labatut *et al.* 2011). In different studies, the microbial conversion of substrates with specific characteristics, e.g. protein-rich and lipid-rich, was investigated (Hatamoto *et al.* 2007, Palatsi *et al.* 2011, Wagner *et al.* 2013). Especially complex substrates were used for this kind of research. Labatut and coworkers (2011) published a study on biochemical analysis of the CH_4 production potential and the biodegradability of complex organic substrates. The authors measured the biodegradability of a mixture of dairy manure, renewable plant material, and food residues. The highest biochemical CH_4 potential could be detected with a lipid-rich, easily-degradable carbohydrate substrate while the lowest CH_4 potential was measured with lignocellulose-rich substrate (Labatut *et al.* 2011). Consequently, a limitation and a potential bottleneck might be present in the hydrolytic step of anaerobic degradation of organic material. Another study was done by Wagner *et al.* (2013), who investigate complex organic materials (classified as protein-rich, lipid-rich,

or cellulose-rich) as substrates for biogas production, to evaluate their microbial degradation potential. High CH_4 production and therefore a high microbial degradation potential was observed for protein-rich substrates; the degradation of lipid-containing and cellulose-rich substrates was problematic (Wagner *et al.* 2013). Thus, the metabolization of different substrates enables the analysis of metabolic processes in a biogas reactor.

Monitoring methods under lab-scale conditions

The biochemical analysis of the microbial metabolization of organic material fed into a biogas reactor is most frequently done in laboratory semi-continuous fermenters (Cuetos *et al.* 2008, Sreekrishnan *et al.* 2004, Pobeheim *et al.* 2011, Mähnert and Linke 2009). This process can be very time-consuming because long start-up phases and measuring periods are required (Sreekrishnan *et al.* 2004). Therefore, another system, the batch system, was introduced because it is easy to handle in the laboratory (Abouelenien *et al.* 2009, Mittweg *et al.* 2012, Llabrés-Luengo and Mata-Alvarez 1988). However, frequently, these systems turn out to be less time-efficient as shown in a batch analysis done by Wagner *et al.* (2013) where dilute reactor material of a thermophilic biogas plant was incubated for 55 d. One of the first attempts of time-efficient analysis was done by Schnürer *et al.* (1999) who established a short-term batch system where results can be obtained in less than 1 d. The set-up allowed the addition of radiolabeled acetate as an intermediate of anaerobic degradation to retrace the pathway of acetate in the biogas sludge (Schnürer *et al.* 1999). This batch system was an initial approach for a short-term and time-efficient analytic system in the laboratory. In summary, effective monitoring of the anaerobic process requires operational simplicity as well as time-and cost-effectiveness. In addition, the determination of the metabolic potential of each degradation step would allow assigning inefficient substrate conversion to a specific microbial deficit.

Figure 8: **Batch incubation system.** 20 g biogas sludge incubated anaerobically in a 120 ml serum bottle.

In this study, a batch system was established which allow to differentiate the metabolic potential of the individual degradation steps in biogas sludge within 24 h (Refai *et al.* 2014b, **Chapter 2**). Only 20 g reactor material of full operating biogas plants was required to perform this approach. The starting material was incubated under anaerobic conditions in 120 ml serum bottles at a certain temperature, according to the conditions of the full-scale biogas plant (Fig. 8). The process stability of this lab-scale batch system was analyzed by CH_4 production, pH value, and acetate concentration (Refai *et al.*

2014b, **Chapter 2**). For the analysis of the metabolic potential of individual degradation steps, the biogas sludge was supplemented with intermediate substrates (butyrate, ethanol, acetate, propionate, or H_2 + CO_2) and CH_4 production was determined (Refai *et al.* 2014b, **Chapter 2**). The addition of the substrates acetate or H_2 + CO_2 directly addresses the level of methanogenesis. The amount of CH_4 produced represents the metabolic capacity of the methanogenic archaea, responsible for the last degradation step. The addition of butyrate, propionate, or ethanol gives crucial evidence on the metabolic capacity of acetogenic bacteria. This analytic system is simple, exhibits authentic conditions of the analyzed biogas plant and allows determination of the metabolic potential of the organisms in the anaerobic digestion process.

However, until now, methods have not been published for quantifying the metabolic potential of each degradation step itself and of the total degradation process. In the course of this study a system was developed, the BEAP profile, which directly evaluate the metabolic activity of acetogenesis and methanogenesis and lead to a conclusion of the metabolic activity of the entire biogas production process (Refai *et al.* 2016, submitted, **Chapter 4**). Thus, the BEAP profile is a monitoring tool for the microbial capacity in a biogas reactor (Refai *et al.* 2016, submitted, **Chapter 4**). The test system is based on the addition of butyrate (BCON), ethanol (ECON), acetate (ACON) or propionate (PCON) to biogas sludge samples and subsequent analysis of CH_4 formation in comparison to control samples without supplementation. The combination of the four values (BCON, ECON, ACON, and PCON) was referred to as BEAP profile (from the first letters of the supplemented BEAP substrates). The added substrates are intermediate products, as already described, and educts for the microorganisms involved in different digestion levels. For the analysis of the BEAP profiles all additives were adjusted to the pH values of the biogas plant. An increasing CH_4 formation based on the utilization of these external substrates represents the metabolic capacity of the microorganisms responsible for the degradation process. Unchanged CH_4 formation in the presence of additional substrates indicates that the organisms in the biogas sludge sample already reached their maximal metabolic activity. Hence, depending on the added BEAP substrate, the metabolic bottleneck in anaerobic biogas formation can be identified. In summary, the BEAP profile indicates which microbial degradation level causes the rate-limiting step in anaerobic degradation of organic matter in biogas plants (Refai *et al.* 2016, submitted, **Chapter 4**). Due to a large number of experiments, a standard BEAP profile could be determined, representing efficiently running biogas plants with stable process stages (Refai *et al.* 2016, submitted, **Chapter 4**).

Process incidents during biogas formation in biogas plants

For the analysis of the operational conditions of a biogas plant a number of various analytical methods can be employed to characterize the state of the biological process. However, due to financial as well as time reasons, many plant operators apply monitoring methods only if a decrease in biogas production or CH_4 content has already occurred. The reduction of CH_4 production indicates a possible inhibition within the anaerobic degradation process, negatively affecting the breakdown of organic matter (Switzenbaum *et al.* 1990, Moletta *et al.* 1994). However, a decrease in biogas production

cannot be correlated with a specific process incident and cannot be used to identify process imbalances in the biogas production in detail.

As described above, biogas and CH_4 yield, FOS, TAC, VFA concentrations as well as NH_3 concentrations and the BEAP profile can be used to identify process imbalances. One additional option is the analysis of macro and trace element as well as H_2S concentrations (Demirel and Scherer 2011, Gadre 1989, Schieder *et al.* 2003). In the following section common process disturbances are described. The aim is to clarify how to recognize process incidents to identify underlying causes and potential countermeasures. It is also explained how the BEAP profile allows identification of the specific microbial community responsible for process imbalances (Refai *et al.* 2016, submitted, **Chapter 4**). Moreover, known and newly developed early warning systems for process incidents will be discussed.

Ammonia inhibition

Atmospheric nitrogen is processed or fixed by many microorganisms in a usable form to be taken up by plants. Many terrestrial animals such as cows, pigs, and horses eat that plant material at some stage of the food chain. Hence, nitrogen-containing compounds reach the biogas reactor by feeding different types of plants as well as different types of animal excrements such as cattle, pig, horse, dairy, or poultry manure. The degradation of nitrogen-containing substrates in a biogas reactor is important since nitrogen is required for cell growth and represents an essential nutrient for microorganisms. Bacteria convert organic nitrogen into NH_3, in a process called ammonification. However, high NH_3 concentrations can lead to process imbalances in biogas plants. In aqueous solution, NH_3 is protonated and forms NH_4^+. The pH-dependent equilibrium between NH_4^+ and NH_3 (pK_a value 9.25 (NH_3/ NH_4^+)) shifts with decreasing pH to the side of NH_4^+. In contrast, an increase in pH gives rise to unprotonated NH_3 which is responsible for the inhibitory effect during the biogas production.

Especially methanogenic cells are adversely affected by high NH_3 concentrations, often leading to a decrease in CH_4 production (Karakashev *et al.* 2005; Angelidaki and Ahring 1993; Nettmann *et al.* 2010). This is caused by NH_3 intrusion into archaeal cells resulting in a dissipation of the transmembrane pH gradient in an alkaline medium (Sprott *et al.* 1984). This has a negative effect on the membrane potential of methanogenic cells. A breakdown of the electrochemical ion gradient, which is the driving force for ATP synthesis, inhibits energy conservation in the cells. Aceticlastic methanogens such as *Ms. barkeri* are more sensitive to high NH_3 concentrations than hydrogenotrophic species (Sprott and Patel 1986). In aceticlastic methanogens an electrochemical transmembrane Na^+/H^+ gradient is the driving force for ATP synthesis (Schlegel and Müller 2011, Schlegel *et al.* 2012). Changes in the membrane potential and in the redox driven proton translocation is sensitively affected by NH_3. The electrochemical Na^+ ion gradient is less influenced by the dissipation of the transmembrane pH gradient. In hydrogenotrophic methanogens the electrochemical ion gradient is formed in the course of Na^+ ion translocation and is responsible for energy conservation (Schlegel and Müller 2011). Thus, reduction of CO_2 + H_2 to CH_4 and energy conservation in hydrogenotrophic methanogens is less affected by high NH_3 concentrations. However,

hydrogenotrophic methanogenesis is inhibited if the NH_3 concentration exceeds a certain threshold (~ 400 mM) in the reactor (Sprott and Patel 1986). Therefore, H_2 can no longer be degraded and accumulates in the biogas plant. Rising H_2 partial pressures negatively affect acetogenesis leading to VFA accumulation in the biogas sludge.

A wide range of NH_4^+ concentrations have been determined in agricultural biogas plants (0.15 - 6 g NH_4^+ l^{-1}), thus tolerable NH_4^+ concentrations cannot be generally identified (Resch *et al.* 2006, Angelidaki and Ahring 1994, McCarty and McKinney 1961). The range of nitrogen concentrations found in different biogas plants indicate that prokaryotes can adapt to various NH_3 concentrations (Angelidaki and Ahring 1993). Furthermore, the inhibitory concentration of NH_3 is process specific. For the calculation of the NH_3 concentrations in the biogas sludge and for the estimation of the toxicity potential an analysis of the NH_4^+ content should always be accompanied by determination of the pH and reactor temperature. However, the identification of process conditions by measuring the NH_4^+ concentration is difficult because of various tolerable NH_4^+ concentrations in different biogas reactors. In contrast, the BEAP profile, described above, represent a method to determine inhibitory NH_4^+ concentrations for the microorganisms involved in anaerobic degradation in biogas sludge. In contrast to the standard BEAP profile which indicates an optimal anaerobic degradation process in biogas plants, the BEAP profile of beginning NH_3 inhibition shows a substantially decreasing metabolic potential of methanogens (Refai *et al.* 2016, submitted, **Chapter 4**). This finding clearly demonstrates the inhibition of the methanogenic metabolism by high NH_3 concentrations (Refai *et al.* 2016, submitted, **Chapter 4**).

Inefficient hydrolysis

Inefficient hydrolysis is another common process incident in the biogas production process (Busch 2013). The enzymatic degradation of complex organic substrates is catalyzed by hydrolases like glycosidases, lipases, and peptidases. Especially lignocellulose, the main constituent of many plant materials, is highly complex and enzymatic break down is extensively difficult (Glass *et al.* 2013). Due to the presence of lignocellulose in a variety of plant silages, inefficient hydrolysis could constitute the bottleneck in the overall process.

The hydrolytic step depends on many different parameters such as substrate particle size, production of enzymes, pH, and temperature (Weiland 2010). However, enzymatic pretreatment of the substrates can prevent the hydrolytic step being the bottleneck of the whole system (Zheng *et al.* 2014, Mendes *et al.* 2006, Taherzadeh and Karimi 2008, Climent *et al.* 2007, Bruni *et al.* 2010, Carrere *et al.* 2016). Due to the complexity and variability of biomass, the optimal pretreatment methods and conditions depend on the types of lignocellulose present. Several structural and compositional properties were found to have impacts on the biodegradability of lignocellulosic biomass, including cellulose crystallinity, accessible surface area, the degree of cellulose polymerization, the presence of lignin and hemicellulose, and the degree of hemicellulose acetylation (Kim and Holtzapple 2005, 2006). The goal of substrate pretreatment is to alter such properties to improve biomass amenity to enzymes and microbes.

However, currently it is difficult to precisely monitor the hydrolytic process with standard analytical parameters. In contrast to the methods mentioned above, the BEAP profile is well suited to demonstrate an inefficient hydrolysis (Refai *et al.* 2016, submitted, **Chapter 4**). A limitation in the first degradation step leads to low intermediate substrate availability in the following digestion levels. Thus, the addition of intermediate substances such as butyrate, ethanol, acetate, or propionate results in sharp increases in CH_4 production indicated by the corresponding enhanced BEAP values (Refai *et al.* 2016, submitted, **Chapter 4**). Consequently, the BEAP profile demonstrates the additional metabolic capacity of syntrophic bacteria and methanogens in case of insufficient hydrolysis.

Acidification

Decreasing pH values in a biogas reactor can sustainably affect biogas formation. This process incident, called acidification, is caused by high concentrations of VFA produced in the course of acidogenesis. An accumulation of VFA occurs if the following degradation step, the acetogenesis, is inhibited. Acidogenesis is associated with a high net production of ATP, resulting in fast growth of the microorganisms involved. Thus, acidogenesis is never rate limiting in the biogas formation process (Gottschalk 1978). In contrast, the subsequent step, the acetogenesis, is an endergonic process under standard conditions (P_{H2} 1 atm, substrate and product concentrations 1 M, temperature 298 K, pH 7.0 (Thauer *et al.* 1977)). Hence, this process can only take place in a biogas reactor because of the syntrophic interaction of acid oxidizing bacteria and hydrogenotrophic methanogens as described in the chapter "*Microbial degradation processes in a mesophilic agricultural biogas reactor".* However, a process imbalance can disrupt this relationship. Consequently, VFA produced in acidogenesis accumulate in the biogas reactor, depleting HCO_3^- buffering capacity and resulting in a drop in pH and a metabolic breakdown of the overall process.

In the beginning of an acidification in a biogas reactor the BEAP profile is characterized by highly increased BCON values as well as slightly increased PCON values (Refai *et al.* 2016, submitted, **Chapter 4**). Enhanced BCON values point to a large metabolic potential for the oxidation of butyrate to acetate and H_2. This indicates an accumulation of butyrate oxidizers in the biogas sludge. A hypothesis could be that the reactors already suffer from an increased flux of butyrate from increasing acidogenesis, resulting in an enrichment of butyrate oxidizers and an increased metabolic capacity for butyrate oxidation. The same could be true for propionate oxidizers combined with an increase of the PCON value. Once the flow of butyrate and propionate exceeds a certain threshold, the metabolic capacity of syntrophic acid oxidizers becomes rate-limiting and butyrate and propionate accumulates in the biogas sludge leading to an acidification of the overall process. Potential countermeasures can be the reduction of the organic loading rate or TAC replenishment by the addition of buffering compounds.

Inhibitors

Most process incidents during biogas formation require high technical and financial efforts to be resolved. These incidents include process inhibitions by retardants such as antibiotics, disinfectants, mycotoxins, or a variety of heavy metals (such as chromium, copper, nickel, and zinc). The inhibitors

are added to the reactor by poor quality substrates and can severely damage the biogas production process.

Antibiotics are toxic to bacterial cells and can inhibit microbial catalyzed degradation to the point of total process breakdown. The highest amounts of antibiotics are added by different types of dairy manure into the reactor. Mastitis, a common udder disease, is routinely treated with antibiotics. On excretion, antibiotics are mixed with manure and thus enter the biogas plant. The most common mastitis pathogens are *Streptococcus* species; the applied antibiotics affect primarily gram-positive bacteria (Raemy *et al.* 2013).

In addition, mycotoxins are very effective inhibitors of biogas production. These inhibitors often reach the reactor in mouldy silage, especially, by grain silages infected with *Fusarium* spp. (Goertz *et al.* 2010). Based on the BEAP profile it was possible to determine that CH_4 formation from acetate is limited in a biogas plant stressed with mycotoxins (Refai *et al.* 2016, submitted, **Chapter 4**). The accumulation of acetate in biogas reactors suffering from a mycotoxin inhibition has been found on several occasions (personal communication Dr. Melanie Hecht). Accordingly, this process incident can be caused by inhibition of acetate degradation by aceticlastic methanogens. In the course of this study metabolization of ethanol in a mycotoxin stressed reactor was analyzed and it was found that free acetate was not formed during ethanol degradation (Refai *et al.* 2016, submitted, **Chapter 4**).

So far, a number of mycotoxins have been identified (e.g. alfa toxins, fumonisins, kojic acid, and moniliformin) (Battilani *et al.* 2009). These mycotoxins may exert different effects on eukaryotic cells, for example, inhibition of membrane, protein, or sphingolipid biosynthesis, inhibition of pyruvate oxidation, or act as a chelating agent for binding of iron, copper, or zinc ions. So far, the precise mycotoxin effects on bacterial and archaeal cells have not yet been clarified.

Micronutrient deficiency

Another process failure often discussed is the lack of micronutrients. Micronutrients, also called trace elements, are catalytically effective transition metals bound to microbial cofactors and enzymes. Trace elements such as iron, nickel, and molybdenum ions are essential components of methanogenic enzymes such as the F_{420}-reducing hydrogenase, the Ech hydrogenase, and the formyl-methanofuran dehydrogenase (Deppenmeier 2002). Iron deficiency has a serious impact on a number of key enzymes involved in methanogenesis and the methanogenic respiratory chain, e.g. all hydrogenases which contain iron-sulfur centers, essential for electron transfer (Deppenmeier 2002, Deppenmeier *et al*. 2002). When iron is deficient, these enzymes cannot catalyze H_2 oxidation any longer.

Moreover, the deficiency of elements like nickel, iron, cobalt, molybdenum, copper, and selenium are assumed to have negative effects in biogas formation (Schattauer *et al.* 2011). However, it is very difficult to induce a deficiency of these elements under anaerobic conditions in a biogas reactor because bacteria have efficient systems to bind metal ions such as iron from their surrounding media and subsequently, to take the trace element up into the cells (Dumas *et al.* 2013, Krewulak *et al.* 2008).

Nevertheless, if iron is deficient in a biogas plant, a process inhibition can be the consequence. A decreasing iron concentration can originate from the presence of H_2S. H_2S is produced by sulfur-

reducing or sulfate-reducing bacteria in their dissimilatory metabolism, e.g. *Desulfuromonas*, *Desulfovibrio*, or *Desulfobacter* (Rabus *et al.* 2015, Barton *et al.* 2014). In addition, fermentative bacteria release H_2S from organic substances with sulfhydryl groups (-SH) such as methionine and cysteine present in protein-rich substrates. H_2S, produced by sulfur-reducing and sulfate-reducing bacteria as well as by fermentative bacteria, is released into the headspace and accumulates in the gas storage as part of the biogas. Depending on the pH value more or less H_2S is dissolved in the sludge (the lower the pH, the more H_2S dissolved). H_2S and iron ions form water-insoluble complexes (Preissler *et al.* 2010). Thus, a possible way of controlling this process inhibition is the addition of iron salts into the biogas reactor. Another frequently used option is blowing air or purified oxygen into the headspace of the biogas plant, the so-called air desulphurization. Thereby H_2S is oxidized to elemental sulfur or sulfate by sulfur-oxidizing bacteria in the presence of O_2 (Nishimura and Yoda 1997, Kantachote 2008). However, elemental sulfur which is deposited in the fermenter space and in the gas space can lead to corrosion problems and lasting damages of the technical system in a biogas plant.

Monitoring- and early warning systems for process incidents

In the previous sections different monitoring parameters were described, which are used to analyze the biogas production process. As already indicated single parameters are not suitable for monitoring the biogas production process because they do not detect upcoming process imbalances at an early stage. Parameter variability and fluctuation can be differing between biogas plants making a determination of threshold values difficult for each single parameter (Resch *et al.* 2006, Angelidaki and Ahring 1994, McCarty and McKinney 1961). Therefore, the recognition of a process incident is often delayed to the point of process damage. In addition, a definite identification of process imbalances is difficult because many disorders start with the same ‘symptoms’ (for example, drop in CH_4 content and increase of VFA concentration) (Preissler 2010). An early warning system is required not only to indicate but also to diagnose a specific type of process incident.

Since a reduction of CH_4 production and an increase of VFA concentration only occurs after a process is sustainably damaged, these parameters are unsuitable to indicate a process incident at an early stage. A more significant and rapid early warning system could be the ratio of FOS and TAC (Rieger and Weiland 2006). In agricultural biogas plants fed with renewable raw materials, stable process conditions are present at a FOS/TAC ratio of ≤ 0.6. Accordingly, a process incident can only be determined if the ratio is higher than 0.6 or if the FOS/TAC ratio is constantly monitored and changes occur (Rieger and Weiland 2006). This ratio can detect process incidents earlier than variation in pH. Besides this, the ratio of VFA and calcium ions or phosphate can also function as an early warning system of a process failure (Kleyböcker *et al.* 2012).

Thus, the determination of upcoming process incidents at an early stage is possible only by the use of at least two monitoring parameters. In contrast, the BEAP profile can be used solely to detect a process incident without long-term observation and to specifically identify rate limiting steps during biomethanisation (Refai *et al.* 2016, submitted, **Chapter 4**). In addition, the BEAP profile indicates

ammonia inhibition at an early stage and thus, it can act as an effective early warning system (Refai *et al.* 2016, submitted, **Chapter 4**).

Improvement of process efficiency in biogas production

For a biogas plant's economic success mechanical as well as biological optimization and high efficiency is crucial. Therefore, it is necessary to develop strategies for efficient maximization of gas yields, CH_4 content, and full-load operational time of the power units. A first option is to ensure optimal feedstock balances in terms of energy and protein content, costs and handling. A second option is the optimization of microbial processes within the digester, avoiding substrate overload, or process disturbances.

Several strategies have been developed to improve process efficiency in biogas production such as increasing substrate availability and application of mechanical, thermal, chemical, or biological pretreatment of substrates.

Mechanical pretreatment

Reduced particle sizes and multiplying surface areas for enhanced substrate utilization can be achieved by various pretreatment methods such as mechanical pretreatment. This method does not cause odor generation, is easy to implement, and results in better dewaterability of the final anaerobic residue with only moderate energy consumption (Toreci *et al.* 2009a, 2009b, Pérez-Elvira *et al.* 2006). Izumi *et al.* (2010) studied the effect of particle size on biomethanisation. Increase of the surface using a beads mill resulted in 28 % higher biogas production yield. Sonication is another mechanical pretreatment method to crush the substrates and to increase their availability for microbial degradation. Sonication pretreatment, generated by a vibrating probe, mechanically disrupts the cell structure and floc matrix of organic material, thus increasing substrate availability for microbial cells (Elliott and Mahmood 2007). Hansen *et al.* (2007) found that screw press pretreatment results in a smaller substrate particle size, while a shredder with magnetic separation yield a higher CH_4 production (5.6 - 13.8 %) compared to the other methods (Jain *et al.* 2015). In contrast, Bernstad *et al.* (2013) reported that screw press pretreatment resulted in a loss of biodegradable material and nutrients, even though it enhanced biogas production in general. In summary, crushing of substrates increases the surface area and consequently ensures enhanced bioavailability.

Thermal pretreatment

Thermal pretreatment of substrates used in a biogas plant is one of the most studied pretreatment methods and has been successfully established in industrial scale (Carrère *et al.* 2010, Carlsson *et al.* 2012). The main effect of thermal pretreatment is the disruption of cell membranes, resulting in the release of organic compounds (Ferrer *et al.* 2008). Additionally, thermal pretreatment results in solubilization of proteins and increased biodegradability of lignocellulosic biomass. Thermal pretreatment methods lead to the breakup of cellulose crystallinity, increasing accessible surface area, reduction of the degree of cellulose polymerization or of the degree of hemicellulose acetylation (Neyens and Baeyens 2003, Kim and Holtzapple 2005, 2006). Moreover, thermal pretreatment of

sludge even at lower temperature (70 °C) has a decisive positive effect on pathogen removal (Skiadas *et al.* 2005).

The effects of thermal pretreatment depend on the substrate type and temperature range. Rafique *et al.* (2010) achieved a maximal biogas production of 178 % with 60 % CH_4 content by pretreatment of lignocellulose at 70 °C. The authors also studied pretreatment of pig manure at temperatures higher than 110 °C (Rafique *et al.* 2010). They observed hardening and darkening of manure, which resulted in a low biogas yield. Nevertheless, pig manure thermal pretreatment was investigated in different studies to maximize CH_4 production. Ma *et al.* (2011) determined an increase in CH_4 production of 24 % by pretreatment of the substrate at a temperature of 120 °C. The CH_4 potential of pig manure increased with temperature of thermal treatments higher than 135 °C (Carrerè *et al.* 2010). However, high temperatures were necessary to improve the CH_4 potential of the total fraction and the best results were obtained with the highest temperature (190 °C) (Carrerè *et al.* 2010). Obviously, different temperatures have to be used for pretreating different substrates to increase the biogas yield significantly.

Chemical pretreatment

Chemical pretreatments were implemented successfully to improve biogas production of lignocellulosic biomass. In a biogas plant enzymatic hydrolysis of lignocellulose is usually ineffective because of the stability of the polymers. Hence, extensive research has been done to develop effective pretreatment techniques for different types of lignocellulosic biomass feedstocks, but none have been commercialized due to high cost. However, alkaline and acidic pretreatment of lignocellulosic feedstocks showed positive effects on biogas formation. For alkaline pretreatment 1 - 10 % of NaOH, $Ca(OH)_2$, CaO, KOH or $NH_3 \times H_2O$ are added to the substrate. The mixture is incubated at –15 - 170 °C for 1 h to 10 d. In most cases these pretreatments of agricultural and forest residuals as well as grass plants lead to a 2-3 fold increase of CH_4 yield (Liew *et al.* 2011, Mirahmadi *et al.* 2010, Chandra *et al.* 2012a, 2012b). Besides alkaline pretreatment, acids can be used to make the substrates more accessible for microbial utilization. Chemicals like H_2SO_4, HCl, HNO_3, H_3PO_4, acetic acid, and maleic acid are used to render lignocellulosic biomass more degradable to microorganisms. This type of pretreatment is performed at temperatures between 24 - 170 °C and in a time frame of a few minutes to hours. The positive effect of pretreatment with acids results in an increase of CH_4 formation by 20 - 200 % (Xiao and Clarkson 1997, Monlau *et al.* 2013).

Furthermore, treatment of the sludge with diluted NaOH (e.g. 1.6 g l^{-1}) at room temperature (25 °C) is able to improve the volatile solid removal by 40 - 90 % (Lin *et al.* 1999, Heo *et al.* 2003). Thus, chemical pretreatment can be very effective and useful in enhancing biogas production, especially with indigestible plant materials.

Biological pretreatment

The aim of biological pretreatment is to increase the ability of raw material utilization and to cause an acceleration of the degradation process. Another goal is to increase in the gas yield of the individual substrates. The hydrolysis of organic material in the first degradation steps often represents the

bottleneck of the overall biogas production process. Therefore, the biological pretreatment by increasing the efficiency of hydrolysis is useful in order to raise effectiveness in full-operating biogas plants and to enable metabolization of persistent plant and animal materials. One option is the addition of preparations enriched with bacteria or fungi to biogas sludge to improve anaerobic degradation, especially with respect to the breakdown of lignocellulose, cellulose, and hemicellulose materials (Hendriks and Zeeman 2009, Taherzadeh and Karimi 2008, Sun and Chen 2002). Kurakake *et al.* (2007) studied the biological pretreatment of office paper with two bacterial strains, *Sphingomonas paucimobilis* and *Bacillus circulans*, for enzymatic hydrolysis. The authors could show that biological pretreatment with those strains improved the enzymatic hydrolysis of office paper from municipal wastes. Under optimum conditions, the sugar recovery for subsequent acidogenesis was enhanced up to 94 %. Other examples are the application of mushrooms, such as *Pleurotus sajorcaju,* and *Pleurotus florida* which are cultivated on a variety of agricultural residues. These fungi possess the capacity to degrade cellulose, hemicelluloses, and lignin components (Bisaria *et al.* 1983; Müller and Trösch 1986). After addition of the organisms biogas can be produced from the hydrolyzed agricultural wastes. In fact, the influence of enriched microorganisms on biogas production is widely discussed, but has very rarely been proven to be effective in practice.

The addition of hydrolytic enzymes is the most common strategy to increase the ability of polysaccharide degradation in biogas plants (Parawira 2012). Hydrolytic enzymes can be added to a one-step or to a multi-step fermentation process in a biogas plant. In addition, enzymes can be used in a separate pretreatment process of plant material. The enzymatic hydrolysis of lignocelluloses without chemical or physical pretreatment is often ineffective because of high resilience of the materials to enzymatic attacks, due to the tight association between lignin, cellulose, and hemicelluloses. The crystallinity of cellulose, its accessible surface area, protection by lignin and hemicelluloses, degree of cellulose polymerization, and acetylation of hemicelluloses are the main factors affecting the rate of enzymatic pretreatment of lignocelluloses (Parawira 2012). In this respect an enzymatic pretreatment study was done by Sonakya *et al.* (2001). Wheat grains were treated with trizyme, a mixture of different hydrolytic enzymes such as cellulase, α-amylase, and protease. The substrate pretreatment resulted in a 7 – 14 % increase in CH_4 production. Thus, enzymatic pretreatment methods are available which can enhance biogas formation.

Besides polysaccharides such as lignocellulose, animal excrements as well as waste from food industry are widely used for biogas production in agricultural biogas plants. Therefore, pretreatment of lipid-rich substrate such as waste from food industry with hydrolytic enzymes is of great importance. Mendes *et al.* (2006) used a lipase preparation from an animal source to perform an enzymatic hydrolysis of lipid-rich wastewater from dairy industry which resulted in increased levels of biogas production. The main advantages of biological pretreatment are the low energy demand and the nontoxic effect of the preparation for the microbial community in the biogas reactor. However, the increase in CH_4 production is very low in most biological pretreatment processes (Sun and Cheng 2002).

Application of ethanol to increase biogas formation

As shown in this work the addition of ethanol to biogas sludge is a different approach to increase biogas yield and CH_4 content in biogas production (Refai *et al.* 2014c, **Chapter 3**). Ethanol is an intermediate in the anaerobic digestion process. In the course of this study it was found that the addition of ethanol circumvents the butyrate/propionate-degradation bottleneck and allows getting around the rate-limiting step in many biogas plants. Ethanol is converted to acetate and H_2, which function as methanogenic substrates to produce CH_4. Hence, the great advantage of supplementation with ethanol is the fact that the compound is directly channeled into methanogenesis so that volatile fatty acids cannot be formed. The total biogas yield as well as the CH_4 content can be increased by the addition of ethanol because 1.5 mol CH_4 are formed per 1 mol ethanol. This advantage is crucial compared to other process improvements because currently, the CH_4 content in biogas has to be concentrated before it is fed into the natural gas grid (Hagen *et al.* 2001, Persson *et al.* 2006). However, this treatment would not be necessary when ethanol is applied. In the course of this study it was found that the addition of ethanol resulted in an increasing biogas formation on a short-term and over a longer period (Refai *et al.* 2014c, **Chapter 3**). Thus, the addition of ethanol can be integrated to improve long-term performance of biogas plants. Since biogas formation increases directly after the addition of ethanol, it is also possible to adjust CH_4 production to ensure power supply in times of daily or seasonal peak loads. However, due to high costs, addition of pure ethanol would not be economical, but a pre-fermenter with an alcoholic fermentation process could be used instead. Fermentation of maize silage by yeasts results in ethanol production and the pre-fermenter content can be fed stepwise into the main fermenter as needed to increase biogas production (Refai *et al.* 2014c, **Chapter 3**). Furthermore, it was shown that the addition of ethanol does not cause any lasting damage to the microbial degradation process in biogas plants. Thus, ethanol appears to be the ideal processing additive to increase the biogas production.

II. Aims of the thesis

The production of biogas is based on the fermentation of organic matter in an oxygen-free environment and is a central element for renewable energy production. It is crucial to understand the production process in detail in order to obtain the maximum CH_4 yield. Complex microbial consortia are involved in biogas production in a temperature-controlled, gas-tight reactor. The microbial formation of biogas proceeds in four interdependent steps referred to as hydrolysis, acidogenesis, acetogenesis and methanogenesis. A complete identification and quantification of the microorganisms involved in biomethanisation is required to improve the understanding of the biogas production process. Currently, efforts for the analysis of the bacterial community in biogas plants are mainly based on 16S rRNA-analysis and metagenome sequencing. However, these methods do not allow a quantification of metabolic activities or capacities of the microorganisms involved in the different steps of the anaerobic degradation of organic matter. In fact, the analysis of metabolic activities could serve to monitor microbial processes, to detect process imbalances, and to locate the rate-limiting step during methanogenesis from organic raw materials.
The methods usually used for monitoring are based on engineering parameters which only allow a statement about the entire process of biogas production. Differential analysis of the individual steps of anaerobic degradation is not yet possible. However, detailed monitoring based on microbial performance parameters could contribute to the early detection of biochemical bottlenecks in the production of biogas and thus, prevent the biogas formation from process incidents. The important and controversial discussed issue which reaction or which degradation level represents the bottleneck or the rate-limiting step in the production of biogas has not been fully elucidated. However, the prevention or circumvention of metabolic bottlenecks could provide potential to increase the biochemical conversion of the organic substrates. This strategy could lead to an improvement of the quantity and the quality of biogas.

Therefore, the aims of this thesis are:

- the development of a rapid and simple test system for the quantification of microorganisms involved in anaerobic biogas formation by activity measurements of key enzymes. The activity tests should focus on a key enzyme of methanogenic archaea responsible for the CH_4 formation as most important step in anaerobic degradation of organic matter.

- the establishment of time- and cost-effective methods for the analysis of biogas sludge in the laboratory that mirror operating conditions of a full-scale biogas plant and enable to analyze influences of substrates or inhibitors on biogas formation within 24 h.

- the design of a semi-continuous small-scale test system for long-term analysis (maximal 14 d) of biogas sludge. This test system would allow the investigation of the development of process incidents and other long-term influences on the microbial community.

- the development of a detection system for the quantification of the metabolic potential in each individual microbial step of biogas production. This system could then be used for the improvement of strategies to identify bottlenecks in metabolic processes in biogas plants and to establish an early warning system for process incidents.

- the generation of a new, economic approach to increase the efficiency of the microbial degradation process and to obtain a significant rise in biogas yield.

III. Publications included in this thesis

- **Refai S.**, Berger S., Wassmann K., Deppenmeier U. (**2014a**) Quantification of methanogenic heterodisulfide reductase activity in biogas sludge. *J Biotechnol* **180**: 66-69.

- **Refai S.**, Wassmann K., Deppenmeier U. (**2014b**) Short-term effect of acetate and ethanol on methane formation in biogas sludge. *Appl Microbiol Biotechnol* **98**: 7271-7280.

- **Refai S.**, Wassmann K., van Helmont S., Berger S., Deppenmeier U. (**2014c**) Increase of methane formation by ethanol addition during continuous fermentation of biogas sludge. *J Ind Microbiol Biotechnol* **41**: 1763-1772.

- **Refai S.**, Berger S., Wassmann K., Hecht M., Dickhaus T., Deppenmeier U. (**2017**) BEAP profiles as rapid test system for status analysis and early detection of process incidents in biogas plants. *J Ind Microbiol Biotechnol,* accepted.

Chapter 1 - Quantification of methanogenic heterodisulfide reductase activity in biogas sludge

The fermentation of organic matter during biogas production is performed by a variety of microorganisms. The identification and quantification of prokaryotes involved in the decomposition of organic material is crucial to understand the entire degradation process in detail. So far, quantification of microorganisms is mainly conducted by sequence analysis of 16S rDNA genes or other marker genes, or is performed by metagenomic sequencing. In contrast, the quantification of the activity of metabolic pathways in complex microbial consortia is extremely challenging. So, a detailed biochemical and enzymatic characterization of the biopolymer conversion by measuring activities of key enzymes is important for a complete understanding of the complexity of the microbial community.

The present study describes a new approach to quantify methanogenic archaea by analyzing a specific enzymatic reaction. The enzyme targeted in this approach is the heterodisulfide reductase (Hdr). The Hdr is a key enzyme of the methanogenic metabolism and catalyzes the reduction of the heterodisulfide CoM-S-S-CoB to HS-CoM and HS-CoB (Thauer *et al.* 2008, Heiden *et al.* 1993, Heiden *et al.* 1994, Smith and Ingram-Smith 2007). The reduction of the heterodisulfide is the key reaction for the process of energy conservation in all methanogenic archaea. Since the enzyme has high specificity for CoM-S-S-CoB as substrate no side activities can influence the measurement (Hedderich and Thauer 1988). Thus, the Hdr activity can be used for the quantification of methanogenic archaea.

Different groups of methanogens in a biogas plant possess Hdr enzymes, which are located at different sites within the cells. In hydrogenotrophic methanogens the Hdr forms a complex with a hydrogenase and is localized in the cytoplasm (Thauer *et al.* 2008). In aceticlastic methanogens the Hdr is tightly bound to the cytoplasmic membrane via a membrane-integral subunit and functions as the terminal reductase of an energy conserving respiratory chain (Heiden *et al.* 1993, Heiden *et al.* 1994, Smith and Ingram-Smith 2007). These different localizations of the Hdr enable to test the metabolic activity of hydrogenotrophic and aceticlastic methanogens separately by the analysis of the Hdr activity in the cytoplasm or the cytoplasmic membrane fraction.
A rapid test system for the quantification of the Hdr activity in biogas sludge was established. The assay is based on the preparation of cell-free extract from biogas sludge followed by the separation of cytoplasmic- and membrane fractions. In the assay reduced viologen derivatives were used as electron donor and the heterodisulfide as specific electron acceptor, respectively. 26 % of the total Hdr activity was found in the membrane fraction representing aceticlastic methanogens whereas the cytoplasmic fraction contained 74 % of the total Hdr activity that derived from hydrogenotrophic methanogens.

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Short communication

Quantification of methanogenic heterodisulfide reductase activity in biogas sludge

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ABSTRACT

Methanogenic archaea are essential for the production of methane in biogas plants. Here we present enzymatic test systems for the analysis of the metabolic activity of methanogens based on the heterodisulfide reductase reaction. The first rapid test shows that heterodisulfide reductase can be detected in 1 g of biogas sludge after sonication and centrifugation. The resulting cell lysate used reduced methylviologen for heterodisulfide reduction, a reaction that is specifically catalyzed by methanogenic heterodisulfide reductase. In the second test cell lysate from 60 g of biogas sludge was separated by ultracentrifugation. Both, cytoplasmic membrane and cytoplasmic fractions revealed heterodisulfide reductase activity, indicating the presence of hydrogenotrophic and aceticlastic methanogens, respectively.

1. Introduction

Biogas is a clean and renewable form of energy and is composed mainly of methane (CH_4; 48–65%) and carbon dioxide (CO_2; 36–41%) (Rasi et al., 2007). The production of biogas is based on the fermentation of organic matter in an oxygen-free environment and is performed in biogas plants that generate electric power and heat. Complex microbial consortia are involved in the decomposition of organic material and the formation of biogas. Microbial activity includes cleavage of complex organic polymers and the degradation of the monomers to organic acids (mainly butyric acid, propionic acid and acetic acid) and to a lesser extent to alcohols (ethanol, propanol). Subsequently, syntrophic bacteria convert these compounds to acetic acid, hydrogen and carbon dioxide, which are used to form CH_4 via the CO_2-reducing (hydrogenotrophic) or the acetate-utilizing (aceticlastic) pathway of methanogenesis. Methane forming microorganisms belong to the domain *Archaea* and are one of the key players for biogas production. They produce methane as their major metabolic end product and combine the pathway of methanogenesis with a unique energy conserving system for ATP synthesis and growth (Welte and Deppenmeier, 2013).

Methanogenesis finally results in the oxidation of two thiol-containing cofactors (HS-CoM and HS-CoB), leading to the formation of the heterodisulfide (CoM-S-S-CoB) that contains an intermolecular disulfide bridge. The enzyme that reduces this compound is referred to as heterodisulfide reductase (HDR) (Hedderich et al., 1989) and is one of the key enzymes in all methanogenic pathways. In aceticlastic methanogens that use acetate as substrate HDR is tightly bound to the cytoplasmic membrane via a membrane integral cytochrome *b* subunit and functions as the terminal reductase of an energy conserving respiratory chain. In hydrogenotrophic methanogens that use $H_2 + CO_2$ as substrates the enzyme is found in the cytoplasm and forms a complex with a hydrogenase. In the course of H_2-dependent CoM-S-S-CoB reduction reduced ferredoxin is formed in hydrogenotrophic methanogens as a powerful reducing agent for CO_2 fixation (Thauer et al., 2008). Hence, the HDR is a central enzyme in all methanogens and is essential for the energy metabolism of methanogenic archaea. The enzyme is very specific for CoM-S-S-CoB as substrate; no other disulfides (cystin, oxidized glutathione etc.) are reduced by the enzyme (Hedderich and Thauer, 1988). HDR-like proteins are also found in many *Archaea* and *Bacteria*. However, these enzymes do not react with CoM-S-S-CoB (Grein et al., 2013; Venceslau et al., 2014). Hence, the methanogenic HDR in combination with its substrate CoM-S-S-CoB is an ideal candidate for quantification of the metabolic activity of methanogens in complex microbial communities.

Abbreviations: BV, benzylviologen; BV_{red}, reduced BV; CoM-S-S-CoB, disulfide from HS-CoM and HS-CoB; HDR, heterodisulfide reductase; HS-CoB, N-7-mercaptoheptanoyl-L-threonine phosphate; HS-CoM, 2-mercaptoethanesulfonate; MV, methylviologen; MV_{red}, reduced MV.

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To our knowledge HDR activity has not been studied in biogas sludge. In general, the development of novel sensors in biogas plants is desirable to quantify the activities of key enzymes by simple detection systems. Sludge used for this work was obtained from a full-scale biogas plant near Cologne (Germany). The plant operated at 40 °C and was fed primarily with maize silage, cattle manure and poultry dry manure producing a constant power of 540–580 kWh. Important physico-chemical parameters of the full scale biogas plant were analyzed every two weeks during the sampling period indicating average values for pH (8.0), total inorganic carbonate (22 g $CaCO_3$/l), volatile organic acids (4.5 g HAc_{eq}/l), and acetic acid (0.8 g/l). Samples were collected in sealed plastic bottles and stored at 4 °C until use. The method for the preparation of cell extract from biogas sludge is described in the legend of Fig. 1.

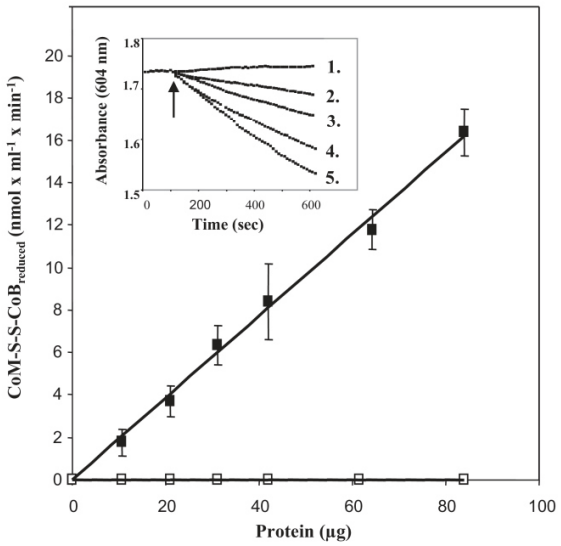

Fig. 1. Dependency of protein content and CoM-S-S-CoB reduction in cell free extract prepared from biogas sludge. Solid squares: assays containing CoM-S-S-CoB; open squares: assays containing H_2O instead of CoM-S-S-CoB. Inset: Time course of HDR activity in cell free extract: (1) 0 μg protein, (2) 10 μg protein, (3) 20 μg protein, (4) 30 μg protein, (5) 40 μg protein. All preparation steps were performed in an anaerobic chamber filled with N_2/H_2 (98/2%; Coy Laboratory Products, Michigan, USA). 1 g of biogas sludge was mixed with 1 ml anaerobic carbonate buffer (3 mM KH_2PO_4, 11.5 mM K_2HPO_4, 126 mM NH_4HCO_3, 1.3 mM $MgCl_2 \times 6\ H_2O$, 12 mM $NaHCO_3$, 0.1 mM $CaCO_3$, 40 mM K_2CO_3, 5 mM dithioerythritol, 1 ml/L trace element solution (SL6, Pfennig and Lippert, 1966), 1 ml/L vitamin solution (Wolin et al., 1964), 1 μg/ml resazurin, pH 8.0) and cells were disrupted on ice using an ultrasonic homogenizer (Sonoplus HD 2070; Bandelin, Germany) with an operating cycle of 5 min. The generator was running and resting in intervals of 0.5 s. Subsequently the sonicated extract was centrifuged at 14,500 × *g* for 5 min and the supernatant was transferred to a 3 ml glass vial and sealed with a rubber stopper. HDR activity was assayed under N_2 at 40 °C in 1 ml of 40 mM potassium-phosphate buffer (K-phosphate buffer) containing 5 mM dithioerythritol pH 7.0, in 1.6 ml glass cuvettes closed with rubber stoppers and preflushed with N_2. After addition of 20 μl 100 mM MV, 1 μl of 90 mM titanium(III) citrate (Hedderich and Thauer, 1988) was added to form MV_{red} (0.15 mM). Subsequently 2.5–20 μl of the extract was added and the cuvettes were incubated for additional 5 min until a stable baseline was reached. The reaction was started by the addition of CoM-S-S-CoB to a final concentration of 280 μM. Change of absorbance was monitored photometrically at 604 nm (ε_{MV} = 13.6 $mM^{-1} \times cm^{-1}$). According to Fry et al. (1982) the concentration of MV_{red} (0.15 mM) and MV (26.7 mM) in our test system resulted in a redox potential of −0.30 V. This calculation is in accordance with the finding that the production of H_2 from MV_{red} was not observed during CoM-S-S-CoB reduction (not shown). CoM-S-S-CoB was synthesized according to Welte and Deppenmeier (2011). Protein content was determined by the method of Bradford (1976) using the Bio-Rad microassay.1 U enzyme activity refers to the reduction of 1 μmol CoM-S-S-CoB per min. The values presented in Fig. 1 represent the average activities of preparations from five different samples of the full-scale biogas plant (samples taken between Nov. 2013 and Jan. 2014). Standard deviation is indicated by error bars.

2. Rapid test for quantification of HDR in biogas sludge

In aqueous solutions, methylviologen (MV) accepts electrons from titanium(III) citrate in a reversible reaction and turns to an intensive blue color. When CoM-S-S-CoB was added to the reaction mixture containing cell extract the blue colored reduced methylviologen (MV_{red}) was oxidized by HDR to the colorless form. In this test system MV_{red} replaces the natural electron donors of HDR, namely H_2 in hydrogenotrophic methanogens and reduced ferredoxin in aceticlastic methanogens. Electrons from MV_{red} are probably directly transferred to the active site of the HDR and are used to reductively cleave the disulfide bond in CoM-S-S-CoB. The time course of CoM-S-S-CoB reduction as catalyzed by the cell free extract obtained from sonicated biogas sludge is shown in Fig. 1 (inset). The HDR showed constant activity over 500 s as evident from the decrease of absorbance at 604 nm due to oxidation of MV_{red} and CoM-S-S-CoM reduction. The specific activity was 64 ± 17 mU × mg $protein^{-1}$ (1 U = 1 μmol CoM-S-S-CoB reduced per min). Total activity from 1 g of sludge was 0.40 ± 0.08 U. No reaction was observed when CoB-S-S-CoM was replaced by H_2O or when titanium(III) citrate, MV or extract were omitted from the assay. It was also evident that an increase of the amount of extract between 10 and 80 μg protein/assay led to a linear increase in HDR activity (Fig. 1) indicating a direct relationship between enzyme activity and protein concentration.

3. Detection of aceticlastic methanogens

As mentioned above the HDR of hydrogenotrophic and aceticlastic methanogens are located in the cytoplasm and the cytoplasmic membrane, respectively. The different localization within the cells allows to separate the proteins from the sludge into the two cellular fractions and to test whether metabolic activity of hydrogenotrophic and aceticlastic methanogens can be differentiated by the analysis of the enzymatic activity of HDR. For this purpose 60 g biogas sludge was used for anaerobic preparation of cytoplasmic membranes and cytoplasmic fractions as described in the legend of Fig. 2. Using MV_{red} as electron donor 84 ± 14 mU/g sludge or 26% of total HDR activity was found in the membrane preparation representing aceticlastic methanogenesis that depends on the activity of the membrane bound HDR. The cytoplasmic fraction contained 237 ± 40 mU/g sludge or 74% of total activity representing the soluble HDR of hydrogenotrophic methanogens. The specific activities were 0.37 ± 0.12 and 0.23 ± 0.11 U × mg $protein^{-1}$ in the membrane and cytoplasmic fraction, respectively. The total activities of the membrane and the cytoplasmic fraction sum up to 0.32 U/g sludge and were in the same range compared to the total HDR activity found in the cell free lysate (0.4 U/g sludge, see above). Hence, there was almost no loss or deactivation of HDR in the course of the steps for the preparation of membrane and cytoplasmic fractions. The relative amount of archaea (representing all methanogens) and aceticlastic methanogens (genera *Methanosarcina* and *Methanosaeta*) was also tested by the technique of qPCR using total DNA isolated from biogas sludge (Suppl. Table 1). It was found that 13–24% of all archaeal organisms belong to the genus *Methanosarcina*. *Methanosaeta* sp. were not detected. Hence, 76–87% of all archaea can be classified as hydrogenotrophic methanogens. The values are in agreement to the relative amount of HDR activities involved in aceticlastic methanogenesis (membrane-bound HDR) and hydrogenotrophic methanogenesis (cytoplasmic HDR) as described above.

Up to now the activity of purified HDR or HDR found in subcellular preparations was measured using reduced benzylviologen (BV_{red}) and CoM-S-S-CoB (Welte and Deppenmeier, 2011).

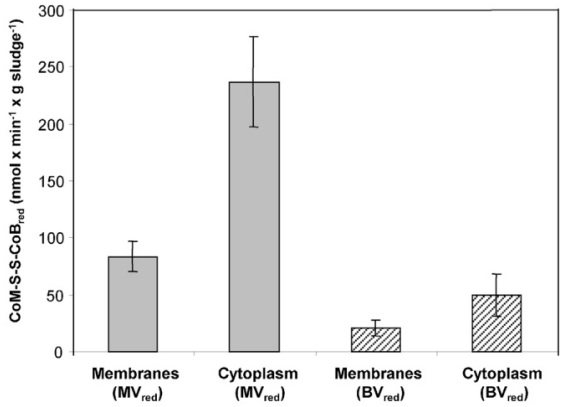

Fig. 2. Quantification of HDR activity in cytoplasmic membranes and soluble cytoplasmic fractions. Gray squares: HDR activity of cytoplasmic membranes; dashed squares: HDR activity of cytoplasmic fraction. The electron donor used for CoM-S-S-CoB reduction is indicated. The results represent the average HDR-activities of preparations from six different samples of the full-scale biogas plant (samples taken between Nov. 2013 and Jan. 2014). Standard deviation is indicated by error bars. Preparation of cytoplasm and washed membranes: All preparation steps were performed in an anaerobic chamber (98% N_2/2% H_2). 60 g of biogas sludge was intensively mixed with 50 ml anaerobic carbonate buffer. The liquid was pressed through a microstrainer with a pore diameter of 0.1 mm. The remaining solid material on top of the microstrainer was applied to a cotton cloth to press out remaining liquid, which was then also filtered through the microstrainer. The filtered preparation were combined and analyzed for methane production in comparison to untreated biogas sludge. It was found that about 85% of the total CH_4 forming activity remained in the filtered preparation indicating that the vast majority of methanogenic archaea was collected by this procedure. Subsequently, the filtered preparation was sonicated for 20 min as described in the legend of Fig. 1. The lysate was applied onto a 70% w/v sucrose cushion and ultracentrifuged (120,000 × *g*, 45 min, 4 °C). The cytoplasmic supernatant was separated and stored on ice. The membrane pellet on top of the sucrose cushion was carefully transferred to another centrifugation bottle and was washed with K-phosphate buffer by ultracentrifugation (120,000 × *g*, 45 min, 4 °C). The supernatants of both ultracentrifugation steps were combined and an aliquot was checked by GC–MS for the presence of phospholipids (Derichs et al., 2014) as indicator for remaining cytoplasmic membranes. The experiments showed that the concentration of phospholipids in the cytoplasm was below the detection limit. The resulting pellet of the second ultracentrifugation step was suspended in 1 ml K-phosphate buffer and referred to as cytoplasmic membranes. The preparations were transferred to serum bottles, sealed with a rubber stopper and stored on ice until use. Cytoplasmic and membrane fraction were analyzed with regard to the presence of soluble proteins using a NADH dependent test system for the detection of lactate dehydrogenase (LDH) that reduced pyruvate to lactate. It became evident that the membrane fraction was essentially free of LDH activity (>1 mU/g sludge) whereas the cytoplasmic fraction revealed a total LDH activity of 29 ± 9 mU/g sludge. MV_{red} dependent CoM-S-S-CoB reduction was assayed as described in the legend of Fig. 1. For the detection of BV_{red} dependent HDR activity 25 μl 25 mM BV was added to the reaction mixture. BV was reduced by 1 μl of 80 mM titanium(III) citrate leading to a final absorbance of about 2 (ε_{BV} = 8.6 $cm^{-1} \times mM^{-1}$) at 578 nm.

Therefore, the presence of HDR in our test system was also measured with BV_{red} as electron donor instead of MV_{red}. However, the sensitivity with BV_{red} was about 4 to 5-fold lower indicated by total HDR activities of 21 ± 6 mU/g sludge in the membrane fraction and 50 ± 18 mU/g sludge in the cytoplasmic preparation, respectively.

The importance of aceticlastic methanogens during biogas production is evident from the comparison of HDR activities in pure methanogenic cultures and biogas sludge. We found that the specific BV_{red} dependent CoM-S-S-CoB reduction was 0.14 ± 0.04 μmol × min^{-1} mg $protein^{-1}$ when membrane preparations from biogas sludge microbial communities were analyzed. Taking into account the specific activity of the purified HDR of 24–38 U/mg protein from the aceticlastic methanogen *Ms. barkeri* the relative amount of HDR can be estimated to be 0.4–0.6% of all membrane bound proteins in biogas sludge. (Heiden et al., 1993, 1994). Until now the quantification of aceticlastic methanogens was mainly conducted by sequence analysis of microbial 16S rRNA and other marker genes or by metagenomic approaches (Bergmann et al., 2010; Hanreich et al., 2013). Here we show that the measurement of enzymatic activity of HDR leads to direct evidence that aceticlastic methanogenesis plays an important role in acetate conversion in the selected biogas plant.

Currently the biogas production process is controlled by the detection of several physico-chemical parameters. These parameters (e.g. dry matter, volatile solids, pH, buffer capacity, volatile fatty acids, trace elements, NH_3/NH_4^+) have been the subject of numerous research projects and enable the operator of a biogas plant to analyze the fermentation process. However, a detailed biochemical and enzymatic characterization of the biopolymer conversion to CH_4 and CO_2 in biogas plants by measuring activities of key enzymes is still missing. Here we show that the direct analysis of HDR activity in biogas sludge is possible. The test system may be suitable to detect changes of metabolic activity of methanogens as a consequence of disorders of the biogas production process. Thus, it might be possible to take early action before it comes to serious process failures of the entire system.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.jbiotec.2014.04.001.

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Supplementary Tab. 1: Determination of the relative amount of archaeal organisms versus members of the genus *Methanosarcina* in biogas sludge

Sludge[a)]	Primer	Ct	Ratio (Archaea/*Ms.)*	*Ms.* (%)
Apr. 2013	*Methanosarcina*	31.5 ± 0.1	4.6 ± 0.6	21.9 ± 2.5
	Archaea	29.3 ± 0.2		
Oct. 2013	*Methanosarcina*	30.6 ± 0.8	7.1 ± 1.5	14.6 ± 3.0
	Archaea	27.8 ± 1.1		
Nov. 2013	*Methanosarcina*	21.8 ± 1.0	4.4 ± 1.2	24.0 ± 6.6
	Archaea	19.7 ± 0.6		
Jan. 2014	*Methanosarcina*	22.5 ± 0.6	8.2 ± 2.9	13.6 ± 2.9
	Archaea	19.6 ± 0.3		

a) Sludge was obtained from the model biogas plant as described in materials and methods

DNA was extracted from 0.3 g biogas sludge with the NucleoSpin® Soil DNA purification kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer´s instructions (with buffer SL1). The relative amount of methanogenic archaea and members of the genus *Methanosarcina* was quantified with specific primer pairs for the amplification of genes encoding the 16S rRNA (archaea_for: GYGCASCAGKCGMGAAW, archaea_rev: TTACCGCGGCKGCTG (Glöckner et al., 2012), ms_for CGTGCCCACTGTTACCAGC, ms_rev: CCCTTTTCAGGGGAGGGAC). *Methanosaeta* sp. were not detected. For relative quantification by qPCR, the FastStart Universal SYBR Green Master mix (Roche Diagnostics, Mannheim, Germany) was used according to the manufacturer´s instructions with reaction volumes of 25 µL and primer concentrations of 0.2 pmol/µL. Different dilutions of the sludge DNA preparations were used. qPCR was performed with the iCycler Thermal Cycler (BioRad, Munich, Germany) with an initial denaturation at 95 °C for 15 minutes (activation of FastStart Taq DNA Polymerase) and 40 cycles of DNA denaturation (95 °C, 15 s), Primer annealing (55 °C, 30 s) and DNA elongation (72 °C, 30 s).

Chapter 2 - Short-term effect of acetate and ethanol on methane formation in biogas sludge

The complex process of biogas production is catalyzed by an anaerobic microbial degradation chain. It has been shown that the degradation of organic material in many agricultural biogas plants does not run at its maximum capacity. This leads to an inefficient use of resources and to financial losses for plant operators. Consequently, it is important to perform biogas production more efficient. Many different factors for process optimization have been studied such as temperature, pH value, particle size of the substrate, the addition of trace elements as well as the recirculation of sludge material (Izumi *et al.* 2010, Elliott and Mahmood 2007, Carrère *et al.* 2010, Carlsson *et al.* 2012, Sharma *et al.* 1988, Jain and Mattiasson 1998, Umetsu *et al.* 1992, Lettinga et al. 1980, Wilkie and Colleran 1986, Sanders and Bloodgood 1965). However, all these factors did not significantly contribute to an increase in efficiency of biogas production. For a more precise and targeted optimization of biogas formation, it was crucial to understand the biological processes during anaerobic degradation of organic material.

For full understanding of the microbial degradation process in a biogas reactor, fast and efficient analytic methods are required. Moreover, the set-up of lab-scale reactors used for these methods had to mirror the conditions of a full-operating biogas plant. Therefore, biogas sludge was incubated in batch reactors and rapid analyzation methods were established. The reactors displayed stable biogas production rates, CH_4 concentrations, pH values, and acetate concentrations within 24 h and allowed the analysis of various factors influencing the biogas production process.

The addition of different intermediates of the anaerobic degradation chain enabled the identification of the metabolic capacity of each group of microorganisms involved in biogas formation. CH_4 formation was measured after supplementation with propionate, butyrate, or ethanol as substrates and was compared to control reactor without the addition of external substrates. Metabolic functionality of methanogens was determined by supplementation with acetate, as well as H_2 + CO_2. The results demonstrated significantly increased CH_4 formation when biogas plants were supplemented with acetate or ethanol. In contrast, all other analyzed fermentation products such as propionate, butyrate, or H_2 led only to slightly increased CH_4 formation rates.

These results indicate that aceticlastic methanogenic archaea and ethanol-oxidizing syntrophic bacteria do not constitute metabolic bottlenecks during biogas formation, respectively. In contrast, aceticlastic methanogenesis and syntrophic ethanol oxidation enable the optimization of biogas production because of their unused metabolic capacity during normal operation of a biogas plant.

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BIOENERGY AND BIOFUELS

Short-term effect of acetate and ethanol on methane formation in biogas sludge

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Abstract Biochemical processes in biogas plants are still not fully understood. Especially, the identification of possible bottlenecks in the complex fermentation processes during biogas production might provide potential to increase the performance of biogas plants. To shed light on the question which group of organism constitutes the limiting factor in the anaerobic breakdown of organic material, biogas sludge from different mesophilic biogas plants was examined under various conditions. Therefore, biogas sludge was incubated and analyzed in anaerobic serum flasks under an atmosphere of N_2/CO_2. The batch reactors mirrored the conditions and the performance of the full-scale biogas plants and were suitable test systems for a period of 24 h. Methane production rates were compared after supplementation with substrates for syntrophic bacteria, such as butyrate, propionate, or ethanol, as well as with acetate and H_2+CO_2 as substrates for methanogenic archaea. Methane formation rates increased significantly by 35 to 126 % when sludge from different biogas plants was supplemented with acetate or ethanol. The stability of important process parameters such as concentration of volatile fatty acids and pH indicate that ethanol and acetate increase biogas formation without affecting normally occurring fermentation processes. In contrast to ethanol or acetate, other fermentation products such as propionate, butyrate, or H_2 did not result in increased methane formation rates. These results provide evidence that aceticlastic methanogenesis and ethanol-oxidizing syntrophic bacteria are not the limiting factor during biogas formation, respectively, and that biogas plant optimization is possible with special focus on methanogenesis from acetate.

Sarah Refai and Kati Wassmann contributed equally to this work.

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Keywords Biogas plant · Aceticlastic methanogenesis · Anaerobic digestion · Biogas production · Syntrophic bacteria

Introduction

One of the most important renewable energy sources is biomass from energy crops and organic waste that can be used for the production of bioethanol or biogas. The production of biogas, which mainly consists of methane (48–65 %) and carbon dioxide (36–41 %) (Rasi et al. 2007), is based on anaerobic fermentation of organic matter and is performed in biogas plants that generate electric power and heat. Until 2012, more than 7,300 biogas plants were operating in Germany alone with an impressive output of approx. 3,200 MW (Fachverband Biogas e.V. 2012), which corresponds to the generation of energy of two to three nuclear power plants. This illustrates today's relevance of biogas as energy source and, in this regard, the need for research aiming at maximum performance of biogas plants. For their economic success, technical optimization together with an increased efficiency is crucial. The yield of every biogas plant depends on the composition and quantities of the respective substrates, which are fed to the reactor. Apart from plant silage, e.g., from maize, which is the major substrate for most biogas plants, cattle manure, dry chicken feces or other animal feces, and stillage from alcoholic fermentation of maize or other crops are often used as substrate for biogas production. Not only substrate composition but also system-dependent parameters such as volume load and the respective hydraulic retention time, pH, and temperature constitute the resulting substrate-specific methane yields.

During the last years, several attempts were made to find suitable methods for the improvement of biogas production. The optimization of process parameters such as temperature (Umetsu et al. 1992; Maurya et al. 1994; Takizawa et al.

1994), pH (Jain and Mattiasson 1998), or particle size (Sharma et al. 1988) of the substrate was a successful approach that contributed to an increase in the efficiency of biogas plants. Improvement of the nutritional supply of prokaryotes (Lettinga et al. 1980; Wilkie and Colleran 1986) by using different additives and manipulating the feed proportions (Sanders and Bloodgood 1965; Nyns 1986) as well as recirculating digested slurry back into the reactor and modification in the design of existing biogas plants are other possibilities to enhance gas production in biogas plants. Although a multiplicity of suggestions for the increase of biomethanation has been made in literature, there is still room for improvement of methane production. For a more precise and targeted optimization, it is crucial to understand the biological processes during biogas production. Emphasis should therefore be placed on the understanding of metabolic processes of the organisms involved in biomethanation and, in this respect, in the identification of metabolic bottlenecks.

Many groups of microorganisms are involved in the formation of biogas, which proceeds in four interdependent substeps. In the first step, complex organic polymers are cleaved into monomeric compounds (hydrolysis). These products (sugars, amino acids, purines, pyrimidines, fatty acids, and glycerol) are degraded in the subsequent fermentation (acidogenesis) to organic acids (mainly butyric acid, propionic acid, and acetic acid) and, to a lesser extent, to alcohols (ethanol and propanol). Byproducts are hydrogen and carbon dioxide. In the acetic acid phase (acetogenesis), the remaining compounds are converted to acetic acid, hydrogen, and carbon dioxide. These products are finally used by methanogenic archaea to form methane and carbon dioxide (methanogenesis). Methanogenesis is conducted by two groups of archaea: Aceticlastic methanogens conserve energy by the degradation of acetate, and hydrogenotrophic methanogens convert H_2+CO_2 to methane.

The aim of this study was to see whether methanogens constitute the bottleneck in the process of biogas production. To answer this question, biogas sludge was supplemented with substrates for syntrophic bacteria, such as butyrate, propionate, or ethanol, as well as with substrates used by methanogenic archaea, and methane production rates were compared. Results indicated that there is capacity to further increase methane yields in biogas plants by adding substrates such as ethanol or acetate.

Materials and methods

If not otherwise indicated, biogas sludge used in this work was obtained from a full-scale biogas plant, which was used as a model plant to analyze the effect of ethanol and acetate on methane formation. The plant is located near Cologne (Germany) and was operated with a temperature of 40 °C. The main feeding substrates were maize silage, cattle manure, and poultry dry manure producing a constant power of 540–580 kWh. Samples were collected in sealed plastic bottles and stored at 4 °C up to 2 weeks until use. For batch experiments, 20 g of original biogas plant sludge was transferred into 120-mL serum flasks in an anaerobic chamber with an atmosphere of N_2/H_2 (98/2 %) and sealed with a rubber stopper. The cultures were subsequently gassed for 10 min with N_2/CO_2 (50/50 %, 1 atm). Subsequently, batch fermenters were supplemented with different substrates: sodium acetate/acetic acid (25–100 mM, pH 7.9), ethanol (25–100 mM), butyrate (50 mM), propionate (50 mM), or H_2/CO_2 (50/50 %, 1 atm). Biogas sludge and substrates were mixed by shaking. Equal amounts of H_2O were added to control flasks. Incubation took place in a shaking incubator at 40 °C and 200 rpm.

Additionally, beer was tested as supplement. To determine whether it shows the same effect on methane production as pure ethanol, batch cultures were mixed with beer (Hefe-Weißbier, alcohol content of 5.5 vol%) to a final ethanol concentration of 20 mM. To reveal a possible effect of beer ingredients other than alcohol on biogas production, alcohol was eliminated from the beer by lyophilization. The lyophilizate was suspended in water and added to control fermenters. Fermenters supplemented with pure ethanol served as further controls.

Methane production was determined by correlation of overpressure and concentration of CH_4 in the headspace of the serum flasks. Overpressure was analyzed with a gastight glass syringe, and the respective methane concentration was determined by analyzing 20 μL samples from the headspace by gas chromatography (GC, PerkinElmer Clarus® 480, Rascon FFAP column 25 m 0.25 micron, PerkinElmer, Waltham, USA) with a flame ionization detector (FID). Measurements were performed with a column temperature of 120 °C, an injector temperature of 150 °C, and a detector temperature of 250 °C with N_2 as carrier gas. For methane yield calculations, a 10 % methane standard (90 % argon) was analyzed before and after every series of measurements. The values were normalized to standard conditions and were used to calculate the methane formation rates which are specified as micromole of CH_4 per gram sludge (wet weight) per hour ($\mu mol\ g^{-1}\ h^{-1}$).

For determination of acetate concentrations by GC, samples were prepared as follows: 1 g biogas sludge was centrifuged for 2 min at 12,840*g*. Two hundred fifty microliters of the supernatant was mixed with 50 μL Carrez solution I (300 g/L zinc sulfate heptahydrate), 50 μL Carrez solution II (150 g/L potassium hexacyanoferrate(II) trihydrate) (Carrez 1908), 150 μL H_2O, and 50 μL 2N HCl. The mixture was incubated at room temperature for 10 min and subsequently centrifuged for 2 min at 12,840*g*. The supernatant was filtered through a 0.45 μm nitrocellulose filter (Carl Roth, Karlsruhe, Germany), and 1 μL was injected to a GC (Shimadzu GC-

14A, Shimadzu, Duisburg, Germany with Agilent Chromosorb 101 column, Agilent Technologies, Santa Clara, USA) with N_2 as carrier gas and a constant column temperature of 170 °C. Injector and FID detector temperatures were set to 220 °C, respectively. Calibration curves were generated with sodium acetate standard solutions, which were processed according to the sludge samples. Determination of organic dry weight (oDM), volatile fatty acids (FOS), and total inorganic carbon (TAC) was performed as described in German Standard DIN 12879 and according to Nordmann (1977), respectively.

Results

Analysis of biogas formation under laboratory conditions does not necessarily reflect conditions of full-scale reactors. To minimize this problem, we established a test system with process conditions similar to those in the full-scale biogas plant. Twenty grams of biogas sludge, which was obtained from the model full-scale biogas plant with a process temperature of 40 °C and fed by maize silage, cattle manure, and dried chicken feces as substrates, was incubated at 40 °C and analyzed over a period of 26 h in anaerobic serum flasks without adding any supplements. Methane production rates, acetate concentrations, and pH values were chosen as indicator parameters for the determination of the performance of the test system. As shown in Fig. 1, methane production rates of 1.7 μmol g^{-1} h^{-1} were observed during the first 2 h of incubation. Within the following hours, methane production rates increased to 2.25±0.04 μmol g^{-1} h^{-1} and stayed constant for 24 h. After 26 h, the methane formation rate slowly decreased to 2.0 μmol g^{-1} h^{-1} probably because easily fermentable substrates became limiting. The full-scale biogas plant, from which the sludge was obtained, comprised a fermenter volume of 2,800 m^3 and produced approx. 140 m^3 methane per hour, which corresponded to a methane production rate of 2.23 μmol g^{-1} h^{-1}. The methane production rates of the small-scale batch fermenters during 24 h were therefore equal to those of the full-scale biogas plant, and lower methane yields during the first hours of incubation could be assigned to a slow warm-up of the cooled biogas sludge that was stored at 4 °C. However, not only methane production but also physicochemical parameters such as pH and the concentration of small chain organic acids are of great importance for the performance of a biogas plant. Therefore, acetate concentrations and pH values were also measured in our small-scale batch reactors (Fig. 1). While acetate concentrations varied between 2 and 20 mM in the full-scale plant, acetate concentrations of approx. 12 mM were determined in the small-scale batch fermenters in the beginning of incubation. After 15 h, acetate concentrations decreased by ~50 % to 6 mM, indicating the metabolic activity of methane-producing organisms in the sludge. The pH values of the full-scale plant between 7.8 and 8.0 also matched with the pH values measured in the small-scale batch fermenters (7.7–7.9). These results revealed that the batch reactors mirrored the conditions and the performance of the full-scale biogas plant and were suitable test systems for a period of 24 h. Further advantages of the small-scale batch fermenters were the capability to run dozens of reactors with different conditions in comparison with controls at a time. Statistical relevance of data was therefore assured by a range of technical and biological replicates.

To investigate whether it is possible to increase methane production under the given process conditions and to get an

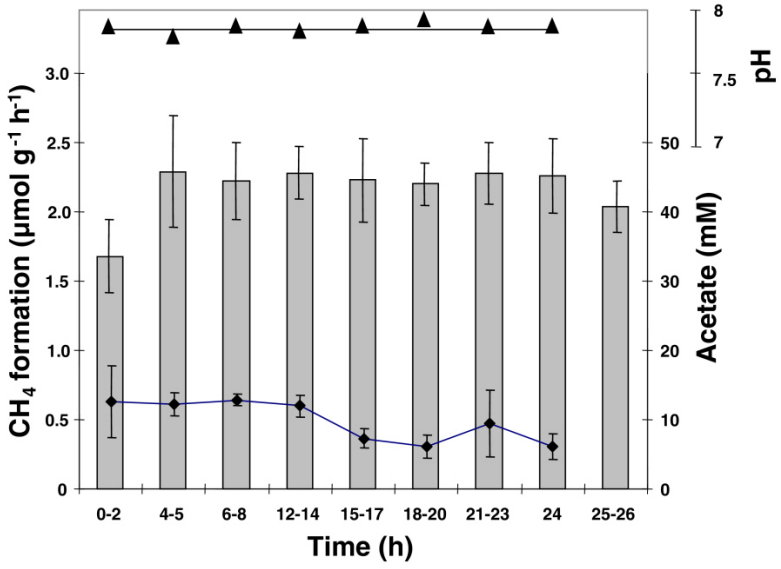

Fig. 1 Performance of batch reactors filled with biogas sludge from a full-scale operating biogas plant. Methane production rates (*bars*) and acetate concentrations (*squares*) of 20 g biogas sludge were determined during an incubation period of 26 h by GC analyses. The experiments were conducted in triplicate and repeated at least five times using different sludge samples. The respective standard deviations are indicated by *error bars*. *Black triangles* indicate pH value

idea about the potential bottleneck during biogas production, batch reactors containing 20 g biogas sludge were supplemented with metabolic intermediates of various groups of prokaryotes involved in anaerobic degradation of organic matter. Methane formation rates were determined after 24 h of incubation at 40 °C in serum bottles. Control cultures revealed a methane production rate of 2.2 μmol g^{-1} h^{-1}, which was in the range of methane production rates reached in the full-scale biogas plant (Fig. 2). Addition of 50 mM propionate or butyrate, which were chosen as substrates for fatty acid-oxidizing, fermenting bacteria (syntrophic bacteria), did not lead to significantly increased methane production rates compared to control fermenters. Hence, it is obvious that the metabolism of the fatty acid-oxidizing bacteria was already operating at full capacity so that biogas production could not be enhanced by the addition of propionate or butyrate. Similar results were obtained when H_2+CO_2 as substrates for hydrogenotrophic methanogens were added (1 atm; 80 % H_2/20 % CO_2). The resulting methane production rates of 2.2 μmol g^{-1} h^{-1} were similar to those of the control reactors (Fig. 2). In contrast, an almost twofold increase of methane production rate from 2.2 to 4.2 μmol g^{-1} h^{-1} was achieved after addition of 100 mM acetate as a substrate of aceticlastic methanogens. The simultaneous addition of acetate and H_2+CO_2 resulted in no further increase in the methane formation rate compared to the increase with only acetate. Thus, in contrast to fatty acid-oxidizing bacteria and hydrogenotrophic methanogenic archaea, it is tempting to speculate that the metabolic activity of aceticlastic methanogens was not working to full capacity with the substrates naturally present in biogas sludge, and biomethanation could be enhanced with augmented feeding of acetate. A similar effect was observed by supplementation with ethanol, which is converted to acetate by ethanol-oxidizing bacteria. Addition of 100 mM ethanol enhanced methane production by nearly 120 % compared to control reactors, resulting in a methane production rate of 4.8 μmol g^{-1} h^{-1} (Fig. 2).

These findings implicated the necessity to further investigate the biomethanation-enhancing effects of acetate and ethanol. Therefore, the effect of different concentrations of acetate and ethanol on methane production was monitored every 2 h and compared to control reactors, which were not supplemented with any substrate. As evident from Fig. 3, elevated acetate concentrations led to increased methane yields. Differences in the amount of produced methane after addition of acetate were already evident after a short lag phase of 2 h. The formation rate of methane of control fermenters was 0.96 mL methane per hour until 18 mL methane were produced after 19 h in the test system, which corresponds to a methane production rate of 2.14 μmol g^{-1} h^{-1}. This value again is in accordance with methane production rates of full-scale biogas plants. In the presence of 25, 50, and 100 mM acetate, 1.56, 1.81, and 2.08 mL methane were produced per hour, respectively. These production rates resulted in final methane volumes of 29.6, 34.4, and 39.5 mL after 19 h of incubation and correspond to methane formation rates of 3.27, 4.04, and 4.64 μmol g^{-1} h^{-1}, respectively. Concentrations above 100 mM did not enhance biomethanation any further (data not shown).

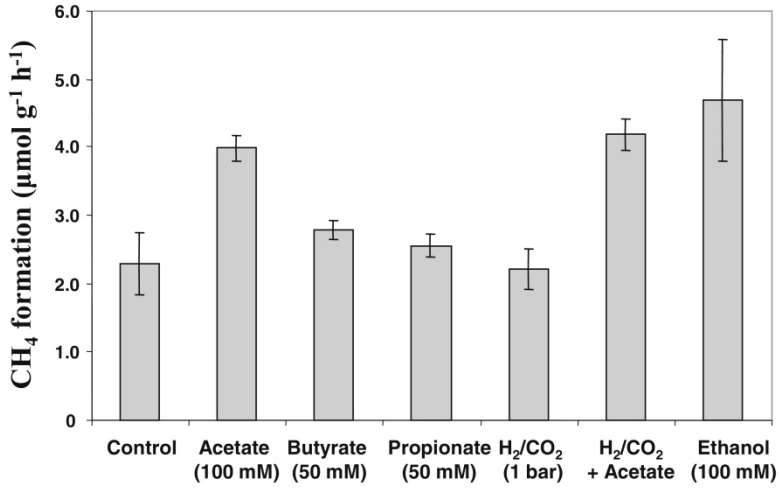

Fig. 2 Influence of addition of different fermentation end products on methane formation in biogas sludge. Biogas sludge (20 g) was analyzed after 24 h incubation in batch reactors supplemented with fermentation products as indicated. *Bars* represent the average methane production rates from four independent experiments performed in triplicate. Standard deviations are indicated by *error bars*. The *numbers in parentheses* indicated the final concentrations of the substrates. Before addition, acetate, propionate, and butyrate solutions were adjusted to pH 8. Biogas sludge samples contained between 200 and 300 mM of hydrogen carbonate buffer, and a change of pH was not observed after 24 h. The addition of 100 mM NaCl (final concentration) had no effect on methanogenesis (not shown)

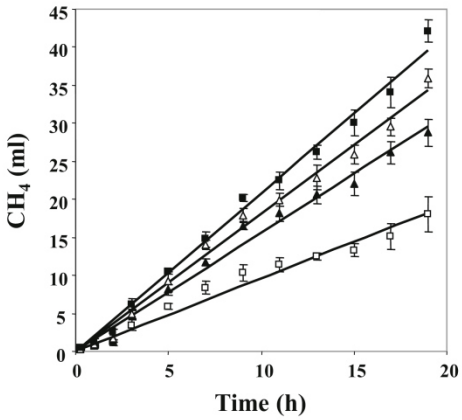

Fig. 3 Effect of acetate addition on methane formation of biogas sludge in batch reactors. The amount of methane was measured every 2 h as described in "Materials and methods" section: control without additions (*white squares*), 25 mM acetate (*black triangles*), 50 mM acetate (*white triangles*), and 100 mM acetate (*black squares*). Data of one representative experiment out of three independent experiments are shown. Average methane production and standard deviation were calculated from three repeats for each condition

Similar kinetics of methane formation as shown for acetate supplementation were observed when ethanol was added to the biogas sludge in different concentrations (data not shown). This observation prompted us to investigate the effect of a broader spectrum of ethanol concentrations on methanogenesis. Biogas sludge was supplemented with 0–250 mM ethanol, and the resulting methane production rates were calculated. As shown in Fig. 4, methane production increased by 20±6 % (10 mM ethanol), 38±15 % (25 mM ethanol), 60±13 % (50 mM ethanol), 76±16 % (75 mM ethanol), and 91±38 % (100 mM ethanol), respectively. Above a concentration of 100 mM ethanol, the increase of the methane formation rate slowly dropped. However, the addition of 250 mM ethanol still resulted in 30 % higher methane production rates compared to non-supplemented biogas sludge. GC analysis after 24 h of incubation showed that 10–20 mM ethanol was completely converted to CH_4 and CO_2. The addition of higher concentration of ethanol led not only to increased CH_4 formation rates as shown above but also to an accumulation of acetate, and ethanol was not completely consumed. Hence, the overall capacity of the biogas sludge to degrade ethanol completely to methane and CO_2 was reached at a concentration of 20 mM ethanol. In summary, the gas chromatographical analysis of the key compounds ethanol, acetate, and CH_4 clearly indicated that ethanol had a strong positive effect on methanogenesis and that methane formation resulted from the degradation of natural substrates present in the biogas sludge and from syntrophic oxidation of ethanol forming the methanogenic substrates acetate and H_2+CO_2. All the described findings raised the question of whether the effects of acetate and ethanol were exclusive for our model full-scale biogas plant or whether the effects can be generalized to biogas plants with similar substrate composition and process conditions. Therefore, biogas sludge of three other biogas plants was analyzed, and the impact of ethanol and acetate additions on methane production was determined. As evident from Table 1, methane formation increased by 35 to 126 % in serum bottles filled with sludge from all biogas plants tested when 100 mM of acetate or ethanol was added. The experiments clearly indicated that the effect of supplementation with acetate or ethanol was similar for sludge from biogas plants operated at 35–40 °C and fed with plant silage and animal manure.

With the results shown above, it became evident that acetate and ethanol addition to biogas sludge in small-scale reactors resulted in a dramatic increase of biomethanation. To check whether supplementation with acetate and ethanol led to changes of other process parameters or whether an

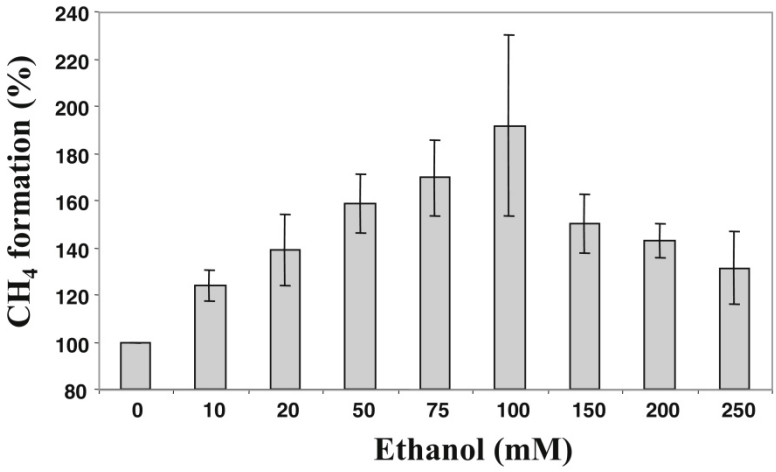

Fig. 4 Effect of different ethanol concentrations on methane formation of biogas sludge. *Gray bars* represent the average activities of sludge from at least four different samples with three replicates of the full-scale biogas plant. *Error bars* indicate standard deviations. The methane formation rate of the control assays was 2.2±0.6 μmol g^{-1} h^{-1} and was set to 100 %

Table 1 Increase of methane formation by addition of 100 mM ethanol or acetate in biogas sludge of different biogas plants

Biogas plant[a]	Feeding	CH_4 formation (ethanol)[b] (%)[e]	CH_4 formation (acetate)[c] (%)[e]
1[d]	MS/CM/PDM	195	174
2	MS/PDM/CS	224	135
3	MS/CM	226	161
4	MS/GS/HM/CS	163	156

MS maize silage, *CM* cattle manure, *CS* cattle slurry, *PDM* poultry dry manure, *HM* horse manure, *GS* grass silage

[a] Each experiment was performed in triplicate

[b] Percentage of CH_4 formation in cultures containing 100 mM ethanol in comparison to control cultures

[c] Percentage of CH_4 formation in cultures containing 100 mM acetate in comparison to control cultures

[d] Model biogas plant (please see "Materials and methods")

[e] Methane formation rates of control assays without addition of acetate or ethanol were set to 100 %

upscale of the experimental equipment had an influence on methane formation under the given test conditions, larger fermenters were filled with biogas sludge from a mesophilic biogas plant fed with maize silage and cattle manure. After 24 h incubation of 8 L biogas sludge in 9 L fermenters, methane production rates of control fermenters were 2.05 μmol g^{-1} h^{-1} (Table 2). In contrast, the addition of 50 mM acetate increased methane production rates to 2.9 μmol g^{-1} h^{-1}, and 50 mM ethanol led to a methane production rate of 3.55 μmol g^{-1} h^{-1}, which corresponds to a 73 % increase compared to control fermenters. These findings were in line with results obtained from small-scale reactors as shown above. Evidence that supplementation with acetate or ethanol does not cause any changes in the physicochemical conditions of the biogas sludge, which would otherwise lead to problems in long-term application, was provided by the analysis of different characteristic fermentation parameters (Table 2). It became evident that pH values, TAC (total inorganic carbon) FOS, (volatile fatty acids) and oDM (organic dry weight) were comparable in all fermenters after 24 h incubation, no matter if supplemented with acetate or ethanol or not. Organic acids such as acetic acid and butyric acid represent intermediates of anaerobic degradation of organic matter in a biogas plant. Their accumulation indicates a disturbance of the fermentation process. However, concentrations of butyric acid were stable in all reactors and the acetic acid concentration increased only slightly in the reactors with acetate and ethanol compared to the control fermenters.

To observe whether complex alcoholic solutions reveal similar effects on biomethanation as pure ethanol, biogas sludge was mixed with beer (Paulaner, unfiltered "Hefe-Weißbier," alcohol content of 5.5 % *v*/*v*) to a final ethanol concentration of 20 mM. The addition led to an increase of methane production rate by 40 % after 24 h compared to a control (Fig. 5), which is in accordance with the increase observed with the same concentration of pure ethanol. To reveal a possible effect of beer ingredients other than alcohol on biogas production, alcohol was eliminated from the beer by lyophilization. The lyophilizate was subsequently suspended in 425 μL H_2O and mixed with biogas sludge. Resulting methane production rates were slightly increased by ~10 % compared to control fermenters, which can be assigned to an increased input of fermentable organic substrate (oDM of beer was 39 mg g^{-1}). In summary, the results led to the conclusion that ethanol and acetate increase the metabolic activity of aceticlastic methanogens in biogas sludge without disturbing the steps of substrate digestion, which normally occur. As a consequence, formation of methane increased significantly.

Table 2 Increased methane production of biogas sludge by the addition of ethanol or acetate in 8 L reactors

Condition[a]	CH_4 (μmol g^{-1} h^{-1})	pH	FOS (g HAc_{eq}/L)	TAC (g $CaCO_3$/L)	oDM[d] (g/kg)	Acetate (mM)	Butyrate (mM)
Control	2.05±0.21	7.9±0.1	4.2±1.3	14.5±1.0	58.2±3.2	5.2±1.5	<1
Acetate[b]	2.90±0.07	8.0±0.2	4.7±1.6	13.9±1.2	55.5±5.6	9.7±3.8	<1
Ethanol[c]	3.55±1.18	8.0±0.1	4.2±0.8	14.1±0.9	57.5±3.2	8.7±2.0	<1

[a] Data were collected after incubation of 24 h at 37 °C

[b] 50 mM final concentration

[c] 50 mM final concentration

[d] According to DIN EN 12879

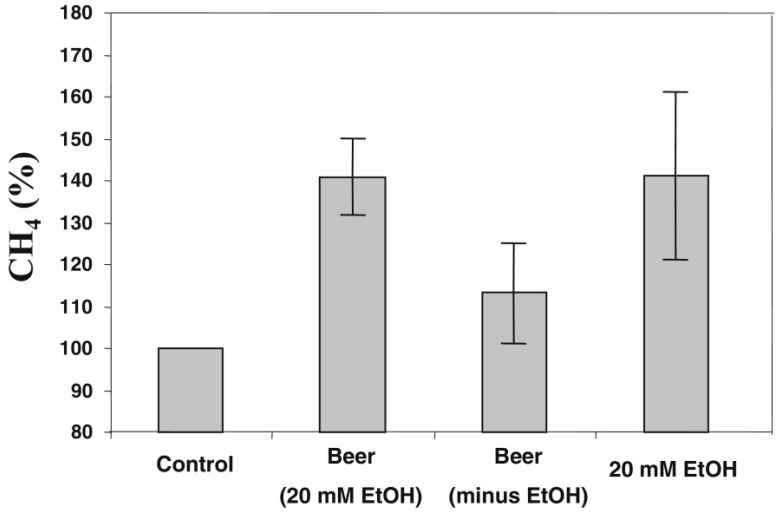

Fig. 5 Effect of the addition of beer to biogas sludge. Batch cultures were mixed with beer (alcohol content of 5.5 % *v/v*) to a final ethanol concentration of 20 mM (425 μL/20 g sludge). *Beer minus ethanol* indicates that alcohol was eliminated from the beer by lyophilization. The lyophilizate was suspended in 425 μL water and was added to the fermenters. Control assays were supplemented with 425 μL water instead of beer and revealed a methane formation rate of 1.9 μmol g^{-1} h^{-1}. This rate was set to 100 %. Batch reactors supplemented with pure ethanol served as further controls. At least three independent experiments were conducted in triplicate for each condition

Discussion

For a successfully operating biogas plant optimal process conditions such as temperature, substrate composition, NH_4^+ concentration, buffer capacity, and the resulting pH of the biogas sludge are of particular importance (Chen et al. 2008). Small changes in one of those factors might suffice to lead to a costly disturbance of the well-balanced and coordinated microbial processes. The accumulation of organic acids such as propionate, butyrate, or acetate often correlates with decreasing biogas production rates and indicates a potential imminent collapse of biogas production (Nielsen et al. 2007). In consideration of this fact, it is a fundamental task for research on biogas production to establish test systems, which simulate a full-scale biogas plant.

Here, we demonstrate that test series of small serum bottles with biogas sludge obtained from a full-operating biogas plant revealed constant pH values, methane production rates, and constant acid concentrations, shown on the basis of acetate concentrations, over a period of 24 h. All of these parameters were in the range of our model full-scale biogas plant, and the easy handling of the small batch fermenters enabled us to investigate large numbers of different culture parameters and biological replicates at the same time. With this batch system on hand, it was possible to address the question of which metabolic pathway constitutes the bottleneck during biogas production. For this purpose, the effect of different substrates on methane production was examined. Those substrates were educts for metabolic pathways of organisms involved in different digestion steps in the biogas plant.

Supplementation with butyrate and propionate, which are metabolized by fatty acid oxidizing, fermenting bacteria (acetogenic or syntrophic bacteria) (Schink 1997; Müller et al. 2010), did not lead to significantly increased methane production (Fig. 2). Pindt et al. (2003) found that the addition of 20–35 mM of the volatile fatty acids (VFA) propionate, butyrate, and acetate led to increased proportions of methane in the biogas, but since only gas quality but not quantity was considered, it cannot be concluded that methane production rates increased as well. They further showed that the period of time between addition of those VFA and an effect on methane content was extended when propionate or butyrate was applied compared to supplementation with acetate, which can directly be converted to methane by aceticlastic methanogens. Therefore, we have to consider two possible explanations for the missing effect of butyrate and propionate on biomethanation in our experiments. First of all, it is possible that the time frame of 24 h of incubation was too short to reveal a positive effect of those fatty acids on methane production, and second, it is worth considering that the metabolic activity of syntrophic bacteria was already limited within a time period of 24 h with the acids naturally produced in the tested biogas plant.

However, to uncover the "weakest link of the chain" in the process of biogas formation, thermodynamic considerations can provide further evidence and help to approach the question from a different angle: Conversion of organic matter to methane and carbon dioxide releases only 15 % of the energy that would be available in aerobic degradation (Schink 1997). As a consequence of this small energy gain, the reaction

product, methane, stores a major part of the energy available in aerobic biomass conversion. As a further consequence, the small amount of energy available in methanogenic conversion forces the involved microorganisms into a very efficient co-operation. The mutual dependence of bacteria and archaea with respect to energy limitation can go so far as neither partner can operate without the other and that together they exhibit a metabolic activity that neither one could accomplish on its own. The most important example of such a syntrophic interaction is the cooperation between fatty acid-oxidizing, fermenting bacteria with hydrogen-oxidizing methanogens (hydrogenotrophic methanogens) (Stams 1994; Schink 1997; McInerney et al. 2009; Sieber et al. 2012). Both partners cooperate in the conversion of fatty acids longer than two carbon atoms (mainly butyrate and propionate) or alcohols longer than one carbon atom (mainly ethanol) to acetate and methane by interspecies hydrogen transfer. Overall, breakdown reactions for fatty acids and H_2 production by syntrophic bacteria are energetically unfavorable, meaning that under standard conditions, these reactions are endergonic. Coculture systems provide favorable conditions for the decomposition only in combination with H_2-consuming methanogens. An extremely low partial pressure of H_2 is necessary for propionate and butyrate degradation under anaerobic conditions (Ahring and Westermann 1988). Such a low partial pressure can be reached with the help of hydrogenotrophic methanogens that form methane from H_2+CO_2 (Stams and Plugge 2009). Although we could not find a positive effect of increased H_2 concentration on methane formation under the chosen experimental conditions (80 % H_2/20 % CO_2, 1 bar), it may be possible that a higher gas pressure facilitates the distribution of H_2 in the sludge and therefore ensures accessibility of the substrate for hydrogenotrophic methanogenesis, which, in turn, produces more methane from H_2+CO_2 than under normal conditions (Prof. M. Rother, Technical University of Dresden, Germany, personal communication). Taking all this together, we assume that degradation of fatty acids by syntrophic bacteria, rather than hydrogenotrophic methanogenesis, is the limiting factor here and that anaerobic fatty acid oxidation constitutes one bottleneck in the process of biogas formation.

However, to answer the question of whether aceticlastic methanogenesis is also involved in the limitation of methane production, acetate that is metabolized to CH_4 and CO_2 by aceticlastic methanogens was added to the reactors. As a consequence, methane production rates rose significantly. Reactors supplemented with 100 mM acetate produced 2.0 to 2.2 mmol CH_4 per day in contrast to the control reactors that formed 0.9 to 1.1 mmol CH_4 per day. In this test system, a total amount of 2 mmol of acetate was added to 20 g of sludge. Taking into account the formation of one molecule of methane from one molecule of acetate, about 50 % of the supplemented acetate was consumed in a period of 1 day.

Accumulation of fatty acids is considered as one main disruptive factor when the feeding rate in biogas plants is increased (Nielsen et al. 2007). In contrast, the addition of acetate leads to an artificial increase of oDM without affecting the overall performance. Since acetate is directly converted by aceticlastic methanogens, the risk of accumulation of propionate or butyrate by additional feeding of substrates with high sugar or fat content is prevented. Results of our experiments are in line with indirect observations that an increase in acetate concentration, resulting from changed substrate feeding procedures, resulted in a temporary increased biogas production (Lv et al. 2013). In addition, Pindt et al. (2003) observed an increase in the methane content in biogas immediately after the addition of acetate.

As clearly shown in our experiments, the addition of ethanol to biogas sludge led to an even stronger increase of methane production compared to the addition of acetate. The observation is reasonable for the fact that one molecule of ethanol is converted to 1.5 mol CH_4 and 0.5 mol CO_2. When 2 mmol of ethanol was added per reactor, an additional amount of 1.3 mmol CH_4 was produced from 20 g biogas sludge per day. Methane production was therefore increased by ~130 % compared to the control. Since one molecule of ethanol can theoretically be converted to 1.5 molecules methane, one can conclude that about 40 % of the supplemented ethanol was consumed per day. From the economical point of view, pure ethanol as a supplement for biogas plants would be too expensive. However, a cost-efficient possibility to increase biomethanation by addition of ethanol would be the setup of a prefermenter, which would allow an alcoholic fermentation of renewable vegetable raw materials. Indications that this procedure might be successful are exemplified by the addition of beer to biogas sludge.

The phenomenon of syntrophic ethanol oxidation is long known and has originally been investigated on the basis of the *Methanobacillus omelianskii* culture (Barker 1940), which was later shown to be composed of two partner organisms, strain S (an ethanol-oxidizing bacterium) and strain M.o.H. (a methanogenic archaeon) (Bryant et al. 1967). Only in mixed culture the two strains are able to convert ethanol to acetate and methane, facilitated by interspecies hydrogen transfer.

$$CH_3CH_2OH + H_2O \rightarrow CH_3COOH + 2\ H_2 \qquad \Delta G^{0\prime} = +9.6\ \text{kJ/mol} \tag{1}$$

$$CH_3CH_2CH_2COOH + 2\ H_2O \rightarrow 2CH_3COOH + 2\ H_2 \qquad \Delta G^{0\prime} = +48\ \text{kJ/mol} \tag{2}$$

$$CH_3CH_2COOH + 2\ H_2O \rightarrow CH_3COOH + CO_2 + 3\ H_2 \quad \Delta G^{0\prime} = +76\ kJ/mol \quad (3)$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad \Delta G^{0\prime} = -36\ kJ/mol \quad (4)$$

$$CO_2 + 4\ H_2 \rightarrow CH_4 \quad \Delta G^{0\prime} = -130\ kJ/mol \quad (5)$$

Thermodynamics reveal that propionate and butyrate oxidation is much more endergonic than ethanol oxidation under standard conditions (Eqs. 1, 2, and 3). All these oxidation processes are dependent on H_2 concentration, but the partial H_2 pressures for the oxidation of propionate and butyrate need to be in the range of 10^{-4} bar (Scholten and Conrad 2000), whereas the limited H_2 pressure for ethanol oxidation is in the range of 10^{-2} bar (Seitz et al. 1988). Hence, under the given thermodynamic constraints, the microbial oxidation of ethanol is much easier than that of propionate and butyrate. Ethanol-oxidizing bacteria convert the substrate to acetate and H_2+ CO_2, which is then utilized by hydrogenotrophic and aceticlastic methanogens (Eqs. 4 and 5) to form CH_4. This demonstrates again that aceticlastic methanogenesis is not the limiting factor during biogas production and also reveals that it is possible to add substrates that are easily oxidized by syntrophic bacteria to enhance methane formation. Degradation of ethanol entails the same advantage as that of acetate: The critical step of propionate and butyrate oxidation is bypassed. Thus, the ethanol data support our hypothesis that bacterial propionate and butyrate oxidation constitutes one of the bottlenecks in the whole process.

To our knowledge, the positive effect of ethanol on biogas formation has not been described yet. However, addition of ethanol has already been suggested to enhance the synthesis yield of products other than methane. Agler et al. (2014) suggest applying ethanol and simultaneously inhibiting methanogenesis for the production of *n*-caproic acid, and Guo et al. (2008) found that fermentative hydrogen production was highest under elevated ethanol concentration, although there are some publications and patents which suggest a feeding of the fermentation reactor with stillage. This product remains after alcoholic fermentation from organic matter and subsequent distillation (Moestedt et al. 2013). McEniry et al. (2014) examined the effect of different silage characteristics on methane production and correlated high contents of products from heterofermentative lactic acid fermentation such as ethanol with positive effects on biogas formation. However, the direct effect of ethanol on biogas production has never been tested.

Although we have shown that the increased biogas production rates did not only occur when ethanol or acetate are added to biogas sludge of our model biogas plant but also to material from other plants, further studies are essential. Of course, one cannot draw a conclusion about long-term effects in biogas plants from the results of our 24 h batch fermentations. It will therefore be important to reveal if the addition of ethanol to biogas sludge is also practicable over longer periods of time under continuous conditions reflecting operation conditions in full-scale biogas plants. However, the fact that the addition of suitable substrates leads to enhanced methane production rates demonstrates that biogas plants might not be operating with full capacity yet and that optimization is possible with special focus on aceticlastic methanogenesis.

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Chapter 3 - Increase of methane formation by ethanol addition during continuous fermentation of biogas sludge

The increase of biomethanisation plays an important role in the efficient use of renewable resources to generate electric and thermal energy. Plenty of approaches are described in literature to increase biogas formation, e.g. mechanical, thermal, chemical, or biological pretreatments of the substrates used for biogas production (Bordeleau and Droste 2011, Frąc and Ziemiński 2012, Hahn *et al.* 2014, Krishania *et al.* 2012, Krishania *et al.* 2013, Morita and Sasaki 2012, Parawira 2012, Rajagopal *et al.* 2013). Depending on the type of substrates only some pretreatment methods lead to a slight increase in biogas or CH_4 formation.

In a previous study however, a highly efficient method was described to increase the production of biogas by adding ethanol to biogas sludge (Refai *et al.* 2014b, **Chapter 2**). This ethanol addition directly intervenes in the microbial degradation process of organic material. Ethanol is oxidized to acetate and H_2 by syntrophic bacteria. The end-products of this oxidation are metabolized by methanogenic archaea resulting in an increased CH_4 formation.

The addition of ethanol increased biogas production efficiently within 24 h. However, for economical use of ethanol for process improvement, it was necessary to investigate the effect of ethanol over longer time periods. The long-term analysis of the biogas production process was performed in small-scale continuous reactors filled with 200 g biogas sludge. These reactors were fed with the same substrates as the full-scale reactor where the sludge derived from. Stability of the process was monitored by quantification of biogas, CH_4 content, pH, TS as well as oTS content and VFA concentrations. The effect of ethanol on biogas formation was analyzed in the continuous reactors during a period of 14 d. Both, the effect of pulsed addition of ethanol at certain time points and the continuous supplementation with ethanol over a longer period was investigated. A pulse of 50 - 100 mM ethanol efficiently increased biogas production by up to 50 – 150 %. Continuous addition of 10 - 20 mM ethanol led to complete metabolization of this additive within 24 h. Consequently, rapid ethanol-conversion in the biogas sludge offers the opportunity to adjust the electric output of the biogas plant to peaks in daily and seasonal energy demands.

Thus, an efficient increase in biogas formation can be obtained by the addition of ethanol. Moreover, it was possible to increase CH_4 formation significantly by the addition of ethanolic solutions with relatively low ethanol contents that derived from alcoholic fermentations, e.g. beer. This finding opens up the potential for the setup of a pre-fermenter, which allows an alcoholic fermentation of renewable raw material and a stepwise addition of this alcoholic fermented digestate into the main reactor to increase biogas formation.

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Increase of methane formation by ethanol addition during continuous fermentation of biogas sludge

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Abstract Very recently, it was shown that the addition of acetate or ethanol led to enhanced biogas formation rates during an observation period of 24 h. To determine if increased methane production rates due to ethanol addition can be maintained over longer time periods, continuous reactors filled with biogas sludge were developed which were fed with the same substrates as the full-scale reactor from which the sludge was derived. These reactors are well reflected conditions of a full-scale biogas plant during a period of 14 days. When the fermenters were pulsed with 50–100 mM ethanol, biomethanation increased by 50–150 %, depending on the composition of the biogas sludge. It was also possible to increase methane formation significantly when 10–20 mM pure ethanol or ethanolic solutions (e.g. beer) were added daily. In summary, the experiments revealed that "normal" methane production continued to take place, but ethanol led to production of additional methane.

Keywords Biogas plant · Aceticlastic methanogenesis · Syntrophic bacteria · Anaerobic digestion · Methane production

Introduction

Biogas, which is formed during the degradation of organic material, is one of the most important renewable energy sources and is used for the generation of electric power and heat. In general, agricultural biogas plants (referred to as NawaRo biogas plants) typically use energy crops and animal manure as a fermentation substrate. In addition, anaerobic degradation also represents a suitable method for waste and wastewater treatment. The market for biogas plants and biogas production is constantly increasing because of the need to facilitate a sustainable development of energy supply and to reduce greenhouse gas emissions. More and more countries create the necessary framework conditions for a fast growth of the biogas industry. It is expected that the worldwide installed capacity will increase between 2012 and 2016 from 4,700 MWel to about 7,400 MWel [33]. Currently, more than two-thirds of the world's 10,000 operational biogas plants are located in Germany [23]. In 2012, the total installed electric capacity of these power plants was 3,352 MW and 22.84 TWh with a market value of 7.3 billion Euros were produced [9]. Because of the enormous economical importance of biogas production numerous studies have been performed to find ways for process optimization. There are plenty of approaches described in the literature to increase the biomethanation processes in biogas plants [3, 10, 12, 15, 16, 20, 25, 27].

For future perspectives of biogas production, one of the most critical issues is to understand the biological processes and to identify metabolic bottlenecks during the fermentation process. Biogas is formed in the course of anaerobic fermentation and consists mainly of methane (45–75 %) and carbon dioxide (25–55 %) [8, 26]. The decomposition of organic material during the fermentation process in a biogas plant is conducted by many groups of microorganisms. Renewable organic polymers are first degraded by enzymatic hydrolysis generating monomers such as sugars, amino acids, purines, pyrimidines, fatty acids and glycerol. In the second step, referred to as acidogenesis,

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these intermediates are converted to the short organic acids butyric acid, propionic acid and acetic acid and to a lesser extent to ethanol and propanol. Byproducts are hydrogen and carbon dioxide. Subsequently, organic acids and alcohols are converted by syntrophic bacteria to acetic acid, carbon dioxide and hydrogen (syntrophic acetogenesis). The last step comprises the process of methane formation (methanogenesis). Two different metabolic groups of archaea are responsible for methane production. The hydrogenotrophic methanogens generate methane from hydrogen and carbon dioxide, whereas aceticlastic methanogens cleave acetate and form CH_4 and CO_2.

As described above, the anaerobic digestion process is a sequential, complex biochemical process, in which organic compounds are mineralized to biogas. The slowest reaction of the overall degradation acts as the rate-limiting step and determines the overall performance of biogas plants. It has been suggested that the rate-limiting factor of biomethanation is either the activity of exoenzymes that hydrolyze large polymeric substrates [6, 35] or the process of methanogenesis [22]. A third possibility is that the conversion of propionic acid and butyric acid during syntrophic acetogenesis is the bottleneck because these volatile acids are the most important intermediates in an anaerobic digestion, and their degradation is extremely complicated because of thermodynamic restrictions [2].

Very recently, Refai et al. [28] demonstrated that aceticlastic methanogenesis is not the limiting factor during biogas production when biogas sludge is supplemented with acetate or ethanol. Since these results are only based on 24 h batch fermentations, it was not possible to draw conclusions on the long-term effects of ethanol and its effect on process stability in biogas plants. Here, we present data on the effect of ethanol on methanogenesis during continuous incubation of biogas sludge under real process conditions using a small-scale (200 g biogas sludge) and a laboratory-scale (8 L biogas sludge) system which copy the conditions of a full-operating biogas plant.

Materials and methods

Unless otherwise noted, the biogas sludge used for experiments in this work was obtained from a commercially operating full-scale biogas plant (in the following referred to as full-scale reactor), which is located near Cologne (Germany). The operation temperature of the full-scale reactor was 40 °C and maize silage, cattle manure and poultry dry manure served as substrates resulting in a constant power of 540–580 kWh. Samples were collected between September 2013 and February 2014 and were used to analyse the effects of ethanol on methane formation. Biogas sludge was stored at 4 °C up to 2 weeks in sealed plastic bottles until use.

Preparation of small-scale continuous reactors

Small-scale reactors were set up in an anaerobic chamber (98 % N_2/2 % H_2) using screw top transfusion glass bottles (1,000 mL, Müller-Krempel, Bülach, Switzerland) which were filled with 200 g of original biogas plant sludge, sealed with a butyl rubber stopper and locked with an aluminium screw cap that had a round opening to allow the insertion of needles. The cultures were subsequently gassed with N_2/CO_2 (50/50 %) for 10 min. Incubation took place in a shaking incubator at 40 °C. Biogas production was measured every day by correlation of overpressure and concentration of CH_4 in the head space of the reactors. Overpressure was determined by means of water replacement in an upside down measuring cylinder and the respective methane concentration was determined by taking 30 µL samples from the head space of the reactor which were then analysed by gas chromatography (GC, Perkin Elmer Clarus® 480, Rascon FFAP column 25 m 0.25 µm, Perkin Elmer, Waltham, USA) with an FID detector. Measurements were performed with a column temperature of 120 °C, an injector temperature of 150 °C and a detector temperature of 250 °C with N_2 as carrier gas. For methane yield calculations, a 10 % methane standard (90 % argon) was analysed before and after every series of measurements. The values were normalized to standard conditions and were used to calculate the methane formation rates which are specified as µmol CH_4 per g sludge per h (µmol g^{-1} h^{-1}).

Feeding and sampling procedures of small-scale reactors

Samples (2 g) were taken every day under N_2-aeration and served for the determination of organic dry weight (oDM), pH and fatty acid concentration. After sampling, the reactors were fed with 2.5 g premixed and shredded substrates (8.6 g/L maize silage, 2.7 g/L cattle manure and 1.4 g/L dry chicken faeces) and 0.4 mL recirculate (supernatant of centrifuged biogas plant sludge) which resulted in an organic loading rate of 4.1 g oDM d^{-1} L^{-1}. Feeding was the same as in the full-scale reactor from which the sludge was derived. Ethanol was added after feeding as indicated. Fermenters were kept in a preheated water bath (40 °C) during the daily feeding and measuring procedures. Finally, fermenters were gassed with N_2/CO_2 (50/50 %) for 10 min and then incubated in shaking incubators at 40 °C for 24 h before the feeding and sampling procedures were repeated. In addition, beer was tested as supplement. To determine whether beer shows the same effect on methane production as pure ethanol, small-scale reactors were fed as described above and were mixed with beer (Paulaner, unfiltered "Hefe-Weißbier", alcohol content of 5.5 % v/v) to a final ethanol concentration of 10 mM. To reveal a possible effect of beer ingredients other than alcohol on biogas production,

alcohol was eliminated from the beer by lyophilisation. The lyophilisate was suspended in water and added to control fermenters. Fermenters supplemented with pure ethanol or with lyophilisate complemented with pure ethanol served as further controls.

Analysis of volatile acids and ethanol concentrations

For determination of acetate and ethanol content in small-scale reactors, 1 g biogas sludge was centrifuged for 2 min at 12,840*g*. 250 µL of the supernatant was mixed with 50 µL Carrez solution I (300 g/L zinc sulphate heptahydrate), 50 µL Carrez solution II (150 g/L potassium hexacyanoferrate(II) trihydrate) [7], and 150 µL H_2O and 50 µL 2 N HCl. The mixture was incubated at room temperature for 10 min and subsequently centrifuged for 2 min at 12,840*g*. The supernatant was filtered through a 0.45 µm nitrocellulose filter (Carl Roth, Karlsruhe, Germany) and 1 µL was injected to a GC (Shimadzu GC-14A, Shimadzu, Duisburg, Germany with Agilent Chromosorb 101 column, Agilent Technologies, Santa Clara, USA) with N_2 as carrier gas and a constant column temperature of 170 °C. Injector and FID-detector temperature was set to 220 °C, respectively. Calibration curves were generated with sodium acetate or ethanol standard solutions, which were processed according to the sludge samples. Determination of organic dry mass (oDM) was performed as described in German Standard DIN 12879.

Setup of lab-scale continuous reactors

To ensure the technical viability of enhanced biogas formation as a consequence of ethanol supplementation, upscale experiments were performed. Therefore, acryl glass vessels with a capacity of 9 L (ATB Potsdam) and stirring devices (Stirring devices: IKA RW 20, Heidolph RZR 2051, controlling device: Conrad Electronics) were used (from here on referred to as lab-scale reactors). The double walled reactors were connected to a 39 °C water bath. To eliminate loss of heat and to protect the fermenter content from light, reactors were isolated with foam plastics. The reactors were filled with 8 L of 100 % microbial active digestate from a NawaRo plant and fed with maize silage and cattle slurry every day. The amount of substrates that were fed every day was increased over 4 weeks until an organic loading rate of 3.5 g oDM d^{-1} L^{-1} was reached, which was then maintained during the remaining time of the experiment. Addition of substrate was carried out once a day and 200 mL of the fermenter content was removed. Generated biogas was collected in gas collection bags (Tecobag, Fa. Tesseraux Spezialverpackungen GmbH, Bürstadt, Germany). Methane content was measured as described above. Ethanol (95 %) was added up to a final concentration of 50 mM on incubation days 42–44, 53–54 and 63–65. For the determination of FOS/TAC values, which represent the proportion of volatile fatty acids (FOS or VFA) and carbonate buffer capacity in terms of total inorganic carbon (TAC), the method according to Nordmann [24] was used.

Results

Development of small-scale continuous reactors

For evaluation of the biological process within NawaRo biogas plants usually laboratory-scale continuous fermenters are used that are typically filled with several liters of effluent from existing anaerobic digester facilities. Usually, the reactors are technically complex and a start-up process is necessary to increase the organic loading rate until stable biogas production is observed. This procedure is time consuming, the number of fermenters is limited and there is the danger that the experimental conditions do not directly reflect the conditions in biogas plants. In contrast, batch fermenters are technically more easy to handle and dozens of reactors can be analysed at a time. However, batch cultures do not allow the investigation of biogas production over a longer period and the long-term effects of additives on biogas production cannot be analysed. Therefore, there is a need for simple procedures to mimic the process of methane production in full-scale biogas plants using simple and space saving continuous reactors that can be run with authentic biogas sludge over days without changing the physico-chemical parameters. Therefore, we developed a small-scale continuous reactor system that was filled with 200 g biogas sludge from the model biogas plant. The fermenters were incubated at 40 °C for 14 days, and feeding and analysis of different process parameters were conducted daily as described in materials and methods. Methane production rates, oDM and volatile acid concentration were chosen as parameters to determine the stability of the continuous test system. All three parameters stayed constant in the daily fed fermenters (Fig. 1a, b) and were in line with parameters measured in the full-scale biogas plant from which the sludge was obtained. The full-scale biogas plant comprised a fermenter volume of 2,800 m^3 and produced approximately 140 m^3 methane per hour. This corresponded to a methane production rate of 2.2 µmol g^{-1} h^{-1} and was similar to the rate of 2.16 ± 0.07 µmol g^{-1} h^{-1} observed in the small-scale continuous reactors over a period of 14 days (Fig. 1a). Interestingly, the methane formation rate stayed constant even over a period of 30 days with daily feeding of the small-scale reactors (not shown). oDM was approximately 90–100 g per kg biogas sludge in the small-scale reactors from day 1–14 and varied between 83 and 105 g kg^{-1} in

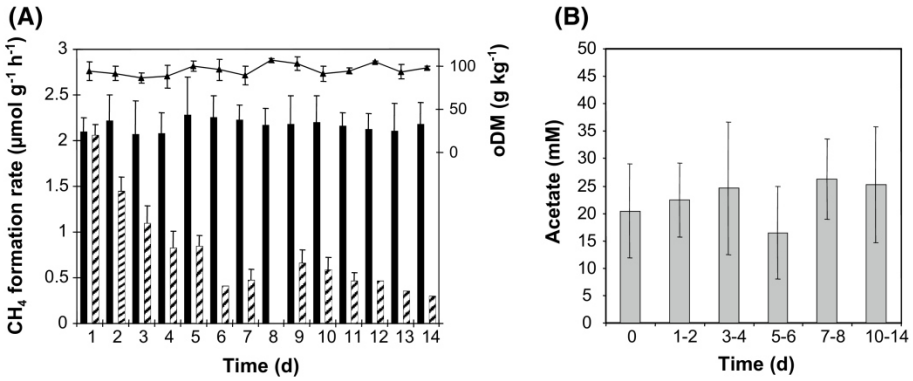

Fig. 1 Performance and stability of small-scale reactors. All reactors were daily fed with maize silage, cattle manure and dry chicken faeces as indicated in materials and methods. **a** *Black bars*, methane formation rate of reactors with daily feeding. *Dashed bars*, methane formation rate of reactors without feeding. (*filled triangle*) oDM in daily fed fermenters. **b** Acetate concentration in small-scale reactors with daily feeding. The experiments were conducted in duplicate and repeated at least five times using different sludge samples. The respective standard deviations are indicated by *error bars*

the full-scale plant. Acetate concentrations of 14–30 mM were determined in the full-scale biogas plant and were 16–26 mM in the small-scale reactors (Fig. 1b). Also, propionate concentrations were similar with 1.7–2.8 mM in the full-scale plant and 2–4 mM in the small-scale reactors (not shown). Butyrate concentrations were always below detection limit. Fermenters, which were not fed, served as control and results clearly showed that feeding was necessary to maintain constant biogas formation. Without the addition of substrate, methane production rates decreased from 2.1 to 0.3 µmol g^{-1} h^{-1} within 14 days (Fig. 1a). From these results, it became evident that the continuous fermentation in small-scale reactors is a suitable system to observe the effect of different conditions or additives on biogas formation within a period of 14 days.

Effect of ethanol supplementation on methane formation in small-scale continuous reactors

Experiments in small batch cultures pointed to potential bottlenecks during biogas formation from organic matter [28] and it was shown that neither ethanol oxidizing bacteria nor aceticlastic methanogenic archaea are involved in the limitation of methane production. Furthermore, methane production increased as a consequence of ethanol addition without interfering with the normal digestion processes during a period of 24 h [28]. However, investigation of long-term effects of ethanol additions is indispensable before possible biotechnological applications can be taken into consideration. Therefore, the effect of ethanol supplementation in the above-mentioned small-scale continuous reactors was studied with normal feeding over a time period of 14 days. In Fig. 2, the relative change of methane production following the addition of 100 mM

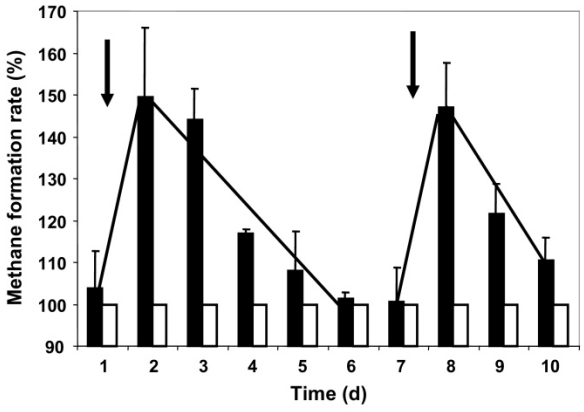

Fig. 2 Increase of methane formation rate after ethanol pulses in small-scale reactors. *Bars* represent the average methane production rates from three independent experiments performed in triplicates. Standard deviations are indicated by *error bars*. *Black bars*, fermenters with ethanol pulses (100 mM final concentration). *White bars*, control fermenters. *Black arrows* indicate addition of ethanol. All reactors were daily fed as indicated in materials and methods

ethanol in the small-scale reactors after 1 day and 7 days is shown, respectively. Already 24 h after the first ethanol pulse, the methane production rate reached its maximum and was enhanced by approximately 50 % in comparison to the control. During the following days, methane production slowly decreased until the rate of the control fermenter was reached again. When a second 100 mM ethanol pulse was applied after 168 h, methane formation increased again by about 50 % and slowly aligned to that of control fermenters after a few days. Furthermore, the basic methane formation rate of 100 % in the control fermenters was determined to an average of 1.93 ± 0.22 µmol g^{-1} h^{-1} over

14 days and was similar to the full-scale operating biogas plant productivity with an average of 2.2 µmol g^{-1} h^{-1}. The maximum methane production rate in ethanol fermenters was 2.83 ± 0.27 µmol g^{-1} h^{-1} and pH, acetate concentration and oDM were constant and comparable to control fermenters during the time of fermentation (not shown).

Effect of ethanol supplementation on methane formation in 9-L continuous lab-scale fermenters

To investigate whether the ethanol effect was also prominent in lab-scale reactors for a time period which is similar to the hydraulic retention time of full-scale biogas plants, 9 L acryl glass reactors were filled with 8 L of microbial active digestate from a NawaRo biogas plant and were fed with increasing amounts of maize silage and cattle manure every day. After 4 weeks, an organic loading rate of 3.5 g oDM d^{-1} L^{-1} was reached and maintained. Ethanol was added up to a final concentration of 50 mM on incubation days 42–44, 53–54 and 63–65. The impact on methane formation is depicted in Fig. 3a. Every ethanol pulse resulted in an immediate increase in methane production by nearly 150 % compared to control fermenters. In each case, maximum values were reached 2–3 days after supplementation. Subsequently, methane production of ethanol fermenters progressively aligned with that of control fermenters until they almost matched after 8–10 days. The additional production of methane after ethanol supplementation was directly linked to the activity of ethanol oxidizing bacteria, which is well reflected in slightly enhanced acetate concentrations after each ethanol pulse. Acetate concentrations increased from initially 5 to 10–20 mM within 2 days after the addition of ethanol and decreased to the initial concentration during the following 4 days (Fig. 3b). However, determination of pH values revealed that the buffer capacity of the biogas sludge was high enough to countervail acidification as a consequence of slightly increased acetate concentrations. The pH values of control and ethanol fermenters equalled each other during the whole time of incubation and averaged between 7.7 and 8.0 (Fig. 3c). Moreover, the oDM values of control and ethanol fermenters were similar and ranged from 55 to 68 g kg^{-1}. The FOS/TAC value represents the proportion of volatile fatty acids (FOS or VFA) and carbonate buffer capacity in terms of total inorganic carbon (TAC). This proportion is an important stability parameter during the anaerobic digestion in biogas plants. FOS/TAC is mainly depending on substrate composition and values between 0.2 and 0.4 have proven to be optimal [14]. As shown in Fig. 3e, FOS/TAC varied between 0.21 and 0.4 in the large-scale continuous fermenters (with a VFA of 3–5 HAceq L^{-1} and a TAC of 13–16 g $CaCO_3$ L^{-1}) and was therefore in accordance with the recommended value for full-scale plants. With these results, it became evident that ethanol does not only enhance biogas formation in small scale during a short observation period in batch fermenters but also when realistic conditions of full-scale biogas plants are imitated. Since this effect could be observed in both, the small-scale and the lab-scale continuous fermentation, these test systems can be regarded as comparably suitable to study long-term effects of ethanol on biogas formation. Hence, in terms of saving time, space and costs, further investigations were conducted in the more convenient small-scale reactors.

Influence of daily addition of ethanol on methanogenesis in biogas sludge

Besides the addition of pure ethanol every 7 days (pulsed supplementation) as shown above, the effect of a continuous supplementation with diluted ethanolic solutions was of great interest with regard to possible future biotechnological applications. Therefore, small-scale continuous reactors were fed as described in materials and methods and supplemented with 10 and 20 mM ethanol every 24 h, respectively. Methane formation rates of control fermenters without addition of ethanol were set to 100 % corresponding to methane formation rates of 2.0 ± 0.3 µmol g^{-1} h^{-1}. When the ethanol concentration was adjusted to 20 mM once a day, methane production increased in average by 30 % after 1 day and 60 % after 6 days, respectively, and revealed a constant rate of 3.2 ± 0.3 µmol CH_4 g^{-1} h^{-1} till the end of the experiment. The addition of 10 mM ethanol per day enhanced methane production by 30 % compared to control reactors and reached a methane formation rate of 2.6 ± 0.3 µmol g^{-1} h^{-1}. The values are in agreement with the theoretically expected increase of methane formation. The final concentration of 10 mM ethanol for example corresponded to 2 mmol of ethanol that was consumed every 24 h in the fermenters with a sludge content of 200 g (equals about 200 mL). Keeping in mind that 3 mmol of methane is formed from 2 mmol of ethanol, the amount of methane produced from ethanol should have been in the range of 0.6 µmol g^{-1} h^{-1}. The rate of the ethanol-supplemented fermenter was 2.6 µmol CH_4 g^{-1} h^{-1} indicating that 2.0 µmol CH_4 g^{-1} h^{-1} was generated from normal feeding (as in the control fermenters) and 0.6 µmol CH_4 g^{-1} h^{-1} from ethanol. Important physico-chemical parameters such as pH values, oDM and acetate concentration stayed constant over the whole period of incubation and no ethanol remained when 10 or 20 mM was added daily. Hence, ethanol was completely metabolized to methane in a range of 10–20 mM within 24 h. Therefore, the continuous addition of ethanol did not disturb processes, which normally occur in the biogas plant. The "normal" methane formation from digestion of the daily fed substrates continued to take place, indicating the possibility to constantly increase biogas formation by ethanol supplementation.

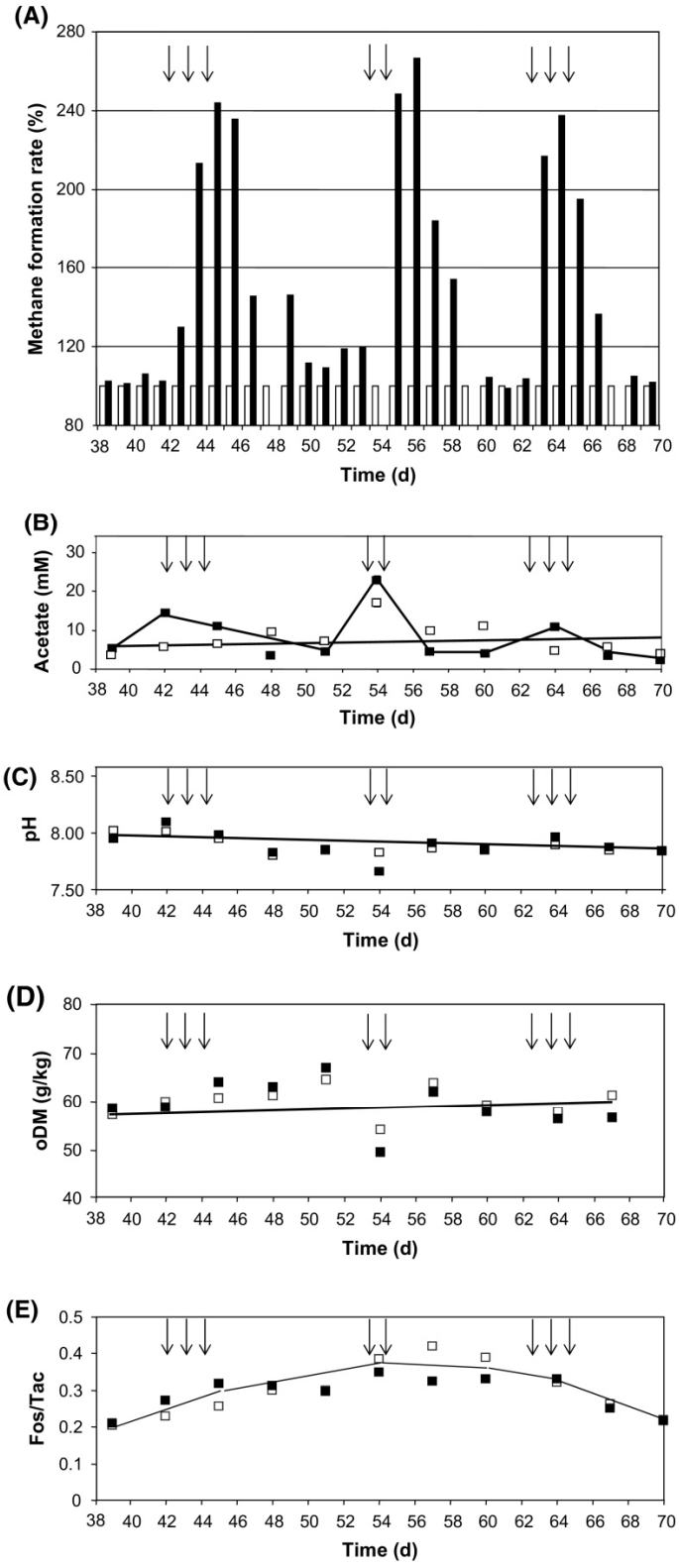

Fig. 3 Effect of ethanol on CH_4-formation in 8 L lab-scale reactors. Feeding of the reactors was performed daily as described in materials and methods. *Arrows* indicate ethanol (95 %) addition to a final concentration of 50 mM each on incubation days 42–44, 53–54 and 63–65. **a** Methane formation rate. *Black bars*, fermenters with ethanol pulses. *White bars*, control fermenters. **b** Acetate concentration (*open square*) control fermenters, (*filled square*) fermenters with ethanol pulses. **c** pH values, (*open square*) control fermenters, (*filled square*) fermenters with ethanol pulses. **d** oDM, (*open square*) control fermenters, (*filled square*) fermenters with ethanol pulses. **e** FOS/TAC value, (*open square*) control fermenters, (*filled square*) fermenters with ethanol pulses

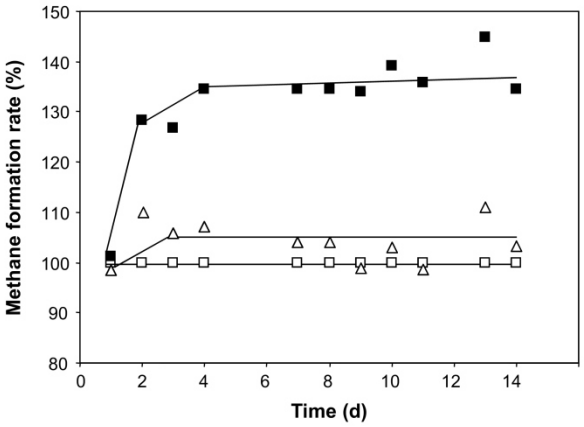

Fig. 4 Effect of beer on methane formation in continuous small-scale reactors. All reactors were daily fed with maize silage, cattle slurry and dry chicken faeces as indicated in materials and methods. The methane formation rate of control fermenters was 1.8 ± 0.3 µmol g^{-1} h^{-1} (*open square*) and was set to 100 %, (*filled square*) daily addition of beer to a final ethanol concentration of 10 mM ethanol, (*open triangle*) daily addition of ethanol-free beer. Data of one representative experiment out of three independent experiments are shown. Average methane production was calculated from two repeats for each condition

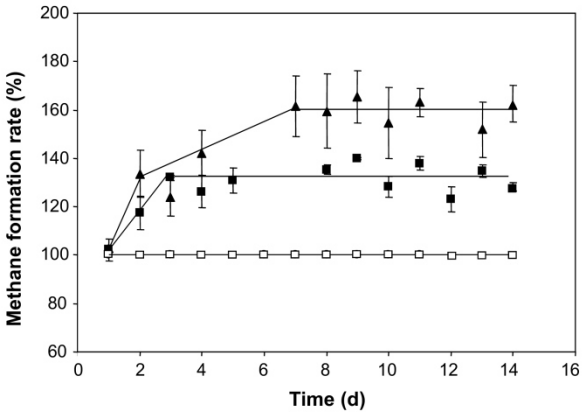

Fig. 5 Effect of daily addition of ethanol on methane formation. A substrate mixture of maize silage, cattle slurry and dry chicken faeces was fed to all reactors daily as indicated in materials and methods. The methane formation rate of control fermenters was 2.0 ± 0.3 µmol g^{-1} h^{-1} (*open square*) and was set to 100 %, (*filled square*) daily addition of 10 mM ethanol (final concentration), (*filled triangle*) daily addition of 20 mM ethanol (final concentration). Data represent the average activities of sludge from three different samples with two replicates of the full-scale biogas plant. *Error bars* indicate standard deviations

However, with respect to economic issues, the use of ethanolic solution produced by alcoholic fermentation of organic material instead of pure ethanol is preferable. In this work, beer served as an example for such alcoholic fermented substrates. With an ethanol content of 5.5 %, beer reasonably represents a diluted complex ethanol containing solution. To investigate the potential of beer to increase methane formation, small-scale continuous reactors were supplemented with 2 mL beer (Paulaner, unfiltered "Hefe-Weißbier", alcohol content of 5.5 % v/v) per day to a final ethanol concentration of 10 mM in addition to the normal feeding. This resulted in a constant increase in methane formation by approx. 35 % (Fig. 4). Similar to fermenters, which were supplemented with pure ethanol, no ethanol could be determined after 24 h of incubation. With an oDM of beer of ~40 g kg^{-1}, the additional input of fermentable organic substrates was slightly increased by ~0.4 g kg^{-1} per day in fermenters supplemented with beer, which corresponds to an increase of total oDM in the fermenter by ~5 %. Therefore, methane production rates resulting from supplementation with beer were naturally slightly higher than those observed with pure ethanol (Fig. 5). This was also in accordance with methane production of control fermenters, which were supplemented with beer without ethanol. Methane production rates were approximately 5 % higher than those of non-supplemented control fermenters. From these results, it can be calculated that the use of beer with a lower percentage of ethanol would lead to a lower increase in methane production. But even a beer with an oDM of ~40 g kg^{-1} and an ethanol content of 1 % (corresponds to 1.8 mM final concentration of ethanol in the 200 mL fermenters) would theoretically lead to an enhanced methane production of about 11 % in our small-scale continuous fermenters when 2 mL of beer is fed every day. In summary, not only pure ethanol but also reasonably priced complex alcoholic fermented substrates with low alcohol content are suitable to raise methane production. This finding supported the biotechnological relevance and practicability of the addition of ethanol to enhance biogas formation.

Discussion

Recently, a test system for the analysis of the performance of biogas sludge using small-scale batch fermenters was established which facilitated the investigation of effects of ethanol and acetate supplementation on methane formation during 24 h incubation. The convenience to receive statistically firm data within a short period of time due to the potential to run a multitude of small-scale batch fermenters simultaneously was one advantage of the batch experiments. However, batch cultures did not necessarily reflect conditions of full-scale reactors. To determine if increased methane production rates due to ethanol addition can be maintained over a longer time period, it was necessary to establish a reliable and convenient test system that allowed

a continuous and stable biogas production. We demonstrated that conditions of the full-scale biogas plant were well reflected during 14 days of observation in our small-scale continuous reactors. Hence, it can be concluded that the experimental setup allowed the simulation of realistic operation conditions of biogas plants. It is worth to mention that the application of small-scale continuous fermenters for analysis of full-scale biogas plants has a number of advantages. There is no need for a time-consuming start-up phase which is characterized by a gradually increase of oDM before steady state conditions are reached [4]. Our system allows a direct use of active biogas sludge from a running system and steady state conditions are already reached after a few hours [28]. The small size of the reactors enables handling in an anaerobic hood preventing possible inhibitory effects of oxygen during inoculation or feeding.

When small-scale or lab-scale continuous fermenters were pulsed with 50–100 mM ethanol, biomethanation increased by 50–150 %, depending on the consistency and composition of the biogas sludge. It was also possible to increase methane formation by 30–60 % in the small-scale reactors when pure ethanol or ethanolic solution (e.g. beer) was added daily to a final ethanol concentration of 10–20 mM. Furthermore, different important process parameters such as FOS/TAC, organic dry mass (oDM), acetate concentration and pH in the control and ethanol-supplemented fermenters were in the range of the model biogas plant. In summary, the experiments revealed that methane production, which normally proceeds in a biogas plant, is not inhibited by the addition of ethanol. This means that basic methane production continues to take place (100 % efficiency), but ethanol leads to the production of additional methane in the biogas plant. In principal, an increase in organic loading rate was achieved without influencing normal fermentation processes.

Multiple renewable organic materials can be applied to 'NawaRo'-biogas plants; however, most often maize is the dominant substrate, which is usually combined with grass silage and cattle or pig manure [8]. Depending on the type of substrate, a wide range of values can be obtained for the substrate-specific methane yield. In addition, system-dependent parameters such as volume load or hydraulic retention time play a role for the resulting substrate-specific methane yields [13]. It was observed that increased loading rates often led to acidification and to a breakdown of the methanation process [17]. Therefore, an improvement of efficiency of biogas plants by increased feeding is obviously very difficult or even impossible. The synthesis of methane depends on a variety of microorganisms and includes a huge number of biochemical reactions that form a reaction chain for the conversion of biopolymers into CH_4. However, the "weakest link of the chain" determines the performance and the speed of the overall system. Previous experiments and thermodynamic considerations already gave a clue about this "weakest link of the chain": it was assumed that anaerobic fatty acid oxidation constitutes one bottleneck in the process of biogas formation [28]. This hypothesis is based on the fact that many bacteria grow in obligate syntrophy with methanogens on substrates that are not fermentable under standard conditions. In these cases, methanogens are essential to reduce the concentrations of hydrogen to make the reaction sufficiently exergonic to support energy conservation, cell maintenance and growth. In fact, hydrogen partial pressures below ca. 10^{-4} and 10^{-3} atm are necessary for degradation of propionate and butyrate (Eqs. 1, 2), respectively [1, 5, 31]. Such low hydrogen partial pressures in methanogenic systems are achieved by interspecies transfer of molecular hydrogen or formate from syntrophic bacteria to hydrogen-oxidizing methanogens [19, 21, 30, 32].

$$\text{Propionic acid} + 2\,H_2O \rightarrow \text{Acetic acid} + CO_2 + 3\,H_2 \quad \Delta G^{o\prime} = +76\ \text{kJ/mol} \tag{1}$$

$$\text{Butyric acid} + 2\,H_2O \rightarrow 2\,\text{Acetic acid} + 2\,H_2 \quad \Delta G^{o\prime} = +48\ \text{kJ/mol} \tag{2}$$

$$\text{Ethanol} + H_2O \rightarrow \text{Acetic acid} + 2\,H_2 \quad \Delta G^{o\prime} = +9.6\ \text{kJ/mol} \tag{3}$$

$$CO_2 + 4\,H_2 \rightarrow CH_4 \quad \Delta G^{o\prime} = -130\ \text{kJ/mol} \tag{4}$$

In this respect it is to note that biogas sludge is not a homogenous material and consists of particles, granules, cell aggregates and biofilms with different composition and diameters, which can be defined as micro-scale habitats or microenvironments. It is tempting to speculate that the actual H_2 pressure is not always the same in these microenvironments because of mass transfer imbalances and different physico-chemical conditions (e.g. pH, substrate and product concentration). Hence, only a part of the microenvironments may possess the proper thermodynamic conditions to allow fatty acid oxidation by syntrophic bacteria. The consequence would be a fluctuation of active and inactive microenvironments depending on the mass transfer and activity of hydrogenotrophic methanogens. Therefore, the overall performance of a biogas plant might depend on the number of active microenvironments that are able to perform butyrate and propionate oxidation to form acetate. The average of active microenvironments in turn depends on the overall H_2 pressure, which varies between 10 and 1,000 ppm in a normally operating biogas plant [18]. The lower the overall hydrogen concentration the more microenvironments can degrade butyrate and propionate and the higher the H_2 concentration the less microenvironments are active in short fatty acids oxidation to acetate. However, ethanol oxidation (Eq. 3) already turns to an exergonic reaction at a

H_2 pressure of about 10^{-2} atm. That means the H_2 concentration in ethanol oxidation can be much higher compared to butyrate/propionate oxidation. Taking together these facts, the oxidation of ethanol to acetate can be performed by the majority of microenvironments found in the biogas plant. It can also take place in those microenvironments that are temporally inactive with respect to propionate and butyrate oxidation. Thus, our hypothesis is that the addition of ethanol circumvents the butyrate/propionate bottleneck and leads to an increase in the velocity of methane production. Furthermore, it allows getting around the rate-limiting step in biogas production leading to an optimized methane formation and an increase of the overall throughput and the electricity yield per time. In this way, the entire capacity and the full potential of the biogas plant can be exploited. Therefore, the great advantage of supplementation with ethanol is the fact that ethanol added to the fermenter is directly channelled into methanogenesis so that volatile fatty acids such as propionate or butyrate cannot be formed from ethanol. Thus, the risk to head toward acidification after ethanol addition in biogas plants is eliminated.

With this knowledge, ethanol seems to be suitable to be applied to full-scale reactors to enhance biogas formation without disturbing normally occurring fermentation processes. The following factors are important for possible technical applications of ethanol with respect to increase in biogas formation:

(i) Cost-efficient production of ethanolic solutions: despite the fact that the addition of pure ethanol to a full-scale biogas plant will presumably enhance methane formation similarly to the effects we observed in our laboratory-scale fermenters, economic relevance is not given due to high ethanol costs. A cost-efficient alternative to increase biomethanation by the addition of ethanol is the application of diluted ethanol, whereas its origin can be diverse. To test whether complex diluted ethanolic substrates generally affect biomethanation positively, biogas sludge was supplemented with beer to a final ethanol concentration of 10 mM in addition to the usual daily feeding and methane formation was determined over 14 days. Methane production rates were increased by approximately 35 % which is in line with results of continuous fermentations with 10 mM pure ethanol and theoretical values. To reach an ethanol concentration of 10 mM in the fermenters, ~2 mL beer was added daily. With an oDM of beer of ~40 g kg^{-1}, the input of additional oDM per day was very low so that a continuation of normal feeding was possible. From these results, one can conclude that complex substrates with relatively low ethanol contents deriving from alcoholic fermentations are just as well applicable to improve biogas formation as pure ethanol. This opens up potential for biotechnological application. The addition of not saleable alcoholic drinks, e.g. because of exceeding expiration dates, is one possibility to increase cost effectiveness. A further industrially practicable opportunity is the setup of a pre fermenter, which allows an alcoholic fermentation of renewable vegetable raw material. The alcoholic fermented digestate of the pre fermenter could be added stepwise to the main fermentation vessel. Considering a final ethanol content of 10 % v/v in the pre fermenter, its volume could amount to only 10 % of the main fermentation vessel, but production of methane would be enhanced to a considerable degree. However, the principle idea of setting up a pre fermenter for alcoholic fermentation of organic material is not new [34]. But in contrast to other inventions, ethanol is not meant to be removed before the digestate is transferred to the main fermenter.

(ii) Adjustment of methane production to fluctuant power demands: biogas formation increases directly after the addition of ethanol. As shown in previous experiments in small-scale batch fermenters, a significant increase in methane production already occurs within 2 h after supplementation with ethanol [28]. In case of a future industrial application, this outstanding feature facilitates the adjustment of methane production to fluctuant demands and to secure power supply at any time. Addition of ethanol to the biogas plant can be used to ensure power supply in times of peak loads or temporary occurring maximum demands in the electric supply network arising from time of the day or season.

(iii) Increased methane concentration: Though the increase in biogas formation is not the only advantage, ethanol addition entails. Before biogas is fed into the natural gas grid, it has to be upgraded to concentrate methane in the gas mixture [11, 26, 29]. With the conversion of ethanol to methane, the methane content in the biogas is increased compared to "normal" biogas formation. A higher methane content involves a higher quality of the biogas and alleviates the gas reprocessing to natural gas quality.

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Chapter 4 - BEAP profiles as rapid test system for status analysis and early detection of process incidents in biogas plants

The decomposition of organic material in a biogas plant is performed by a complex microbial community. There are four key metabolic processes (hydrolysis, acidogenesis, acetogenesis and methanogenesis) degrading the substrates mainly to CO_2 and CH_4.

To monitor this degradation process and the biogas production it is crucial to ensure optimal performances in a biogas reactor. The biogas quantity and quality as well as the efficiency of the microbial conversion of organic material depend on the composition and the amount of substrates fed into a biogas reactor. Additionally, system-dependent physico-chemical parameters play a crucial role on biogas formation. The most common physico-chemical parameters for monitoring the process are the production of biogas, CH_4 content, pH, temperature, the NH_4^+-N concentration, FOS and TAC values, and the concentration of individual VFA. These parameters are routinely used to check the anaerobic degradation of organic matter. However, the metabolic capacities of the individual microorganisms catalyzing the anaerobic conversion of biopolymers into CH_4 represent the most important factor of the overall performance of the entire system. A test system for the quantification of the metabolic capacity and thus, the performance of the microorganisms involved in the different digestion levels in biogas plants was not available. Consequently, it was difficult to determine the metabolic pathway constituting the bottleneck during biogas production and to identify specific reasons for imbalances in the microbial degradation process.

In the present study, a system is described allowing for the first time to quantify the performance of microorganisms involved in different digestion levels in biogas plants. The test system (BEAP profile) was based on the addition of intermediates of the anaerobic degradation process, butyrate (BCON), ethanol (ECON), acetate (ACON), or propionate (PCON), to biogas sludge samples and subsequent analysis of CH_4 formation in comparison to control samples without supplementation within 24 h. The study showed that the BEAP profile enables to monitor the metabolic capacity or metabolic potential of each of the four main microbial degradation levels. Thus, with the help of the BEAP profile it is possible to identify the rate-limiting step in biogas formation. Furthermore, a targeted optimization of biogas production during process incidents is now possible.

The analysis of agricultural biogas plants showing different process incidents allowed to distinguish between specific BEAP profiles. The beginning of NH_4^+-N intoxication, the start of acidification, insufficient hydrolysis, and potential mycotoxin effects were determined by BEAP profiles. The BEAP profiles also function as a warning system to predict critical NH_4^+-N concentration thresholds leading to a drop of CH_4 formation and a sustainable process failure. Thus, the BEAP profiles enable to identify process limitations with respect to microbial degradation level and to avoid process incidents in biogas plants.

BEAP profiles as rapid test system for status analysis and early detection of process incidents in biogas plants

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Key Words

Biogas, methane, renewable energy, renewable raw material, process failure, early warning system

Abstract

A method was developed to quantify the performance of microorganisms involved in different digestion levels in biogas plants. The test system was based on the addition of butyrate (BCON), ethanol (ECON), acetate (ACON) or propionate (PCON) to biogas sludge samples and the subsequent analysis of CH_4 formation in comparison to control samples. The combination of the four values was referred to as BEAP profile. Determination of BEAP profiles enabled rapid testing of a biogas plant's metabolic state within 24 h and an accurate mapping of all degradation levels in a lab-scale experimental setup. Furthermore, it was possible to distinguish between specific BEAP profiles for standard biogas plants and for biogas reactors with process incidents (beginning of NH_4^+-N inhibition, start of acidification, insufficient hydrolysis and potential mycotoxin effects). Finally, BEAP profiles also functioned as a warning system for the early prediction of critical NH_4^+-N concentrations leading to a drop of CH_4 formation.

Introduction

Biogas is one of the most important renewable energy sources and is produced from biomass of energy crops and organic waste. Most biogas plants in Germany are operated on silaged material from renewable energy crops, such as maize, grass and cereal (whole-crop silage) as well as solid and liquid manure [21, 28, 41]. Additionally, waste from the food industry, paper production, as well as biologically degradable packing material and fat can be used as substrates in a biogas plant [6, 10, 23, 46, 55, 64].

Biogas from organic matter is generated during an anaerobic, fermentative degradation process and consists mainly of methane (CH_4) and carbon dioxide (CO_2). The anaerobic degradation taking place in a biogas reactor can be divided into a four step process. In the first step, referred to as hydrolysis, polysaccharides, lipids and proteins are converted into sugars, amino acids and fatty acids. In the following step, a process called acidogenesis, fermentative bacteria convert these substrates to volatile fatty acids (VFA; e.g. butyrate, propionate and acetate) and to a minor extent to alcohols (ethanol and propanol). Byproducts are hydrogen (H_2) and CO_2. Subsequently, acetate, H_2 and CO_2 are produced from VFA and alcohols during the acetogenic metabolism of syntrophic bacteria. In the last step methanogenic organisms produce CH_4 from H_2 and CO_2 (hydrogenotrophic methanogenesis) or CH_4 and CO_2 from acetate (aceticlastic methanogenesis).

Biogas quantity, quality and the efficiency of anaerobic conversion differ depending on the composition and the amount of substrates [4, 9, 21, 22, 64]. In addition, system-dependent physico-chemical parameters such as pH, carbonate buffer capacity (TAC; total alkalinity of carbonates) and nitrogen content as well as operating procedures such as organic loading rate or hydraulic retention time play key roles in the resulting substrate-specific CH_4 yields [62]. However, the most important factor for the biogas production process is the activity of the microorganisms catalyzing the biochemical reactions for the conversion of biopolymers into CH_4. It is evident that the weakest link in this biological chain determines the overall performance of the entire system. Unfortunately, methods do not exist to quantify the performance of microorganisms involved in the different digestion levels in biogas plants to address the question which metabolic pathway constitutes the bottleneck during biogas production. Moreover, it is difficult to determine a specific reason for incidents in the microbial degradation process. However, recently a new parameter referred to as Metabolic Quotient was introduced for the determination of methanogenic activity and as a warning system of process acidification, which is based on qPCR and RT-qPCR methods [43].

In general, three common incidents contribute to the reduction of biogas: the reduction of the hydrolytic potential, high ammonium/ ammonia concentrations or high VFA concentrations. Firstly, the efficiency of the hydrolytic breakdown depends on the availability of hydrolytic exo-enzymes (e.g. glycosyl hydrolases). These enzymes cleave large polysaccharides into oligo-, di- and monosaccharides that can then be transported into the cells for further degradation. Consequently, the presence of a large number of enzymatically active proteins is essential to facilitate the conversion of the original feedstocks into biogas [8, 16, 25, 50, 59]. Secondly, a high concentration of ammonium ions (referred to as ammonium nitrogen, NH_4^+-N) in equilibrium with ammonia (NH_3) can also lead to reduced biogas production [27, 47, 51, 52]. Finally, increasing concentrations of VFA can lead to process disruption [1, 3, 31, 48]. VFA accumulation can be caused by various factors: e.g. high organic loading rates, fat-rich substrates [26, 30], trace element deficiency and toxic substances. In summary, a successful degradation of complex organic

polymers depends on the substrates themselves and the fermentative potential of the corresponding microorganisms. This leads to the conclusion that any incident involving a reduced biogas production results from insufficient metabolic activities by one or more groups of microorganisms in biogas plants. The aim of this study was to develop a test system that allows the quantitation of the performance of biogas sludge samples. The system is based on the application of metabolic intermediates formed naturally during anaerobic degradation of organic matter. Additionally, these intermediates are educts for metabolic pathways involved in different digestion levels. The test system can be used to map the biogas formation process on each level of anaerobic degradation. Furthermore, it can be employed as a warning system to identify upcoming process failures in a biogas plant.

Material and methods

Setup of small-scale batch reactors to determine BEAP values

The biogas sludge samples used in this study were obtained from 53 mesophilic and two thermophilic biogas plants operated with temperatures of 38 - 47 °C and 50 – 55 °C, respectively (Table S1). The main input substrates of the biogas plants were maize silage, green rye silage, wheat grains, sugar beets, cattle/ horse/ swine manure, and poultry dry manure. Samples from full-scale reactors were collected in sealed plastic bottles and stored at 4 °C till use. Batch experiments were performed according to Refai *et al.* [53]. Briefly, 20 g of original biogas sludge were transferred into 120 mL serum flasks under anaerobic conditions, sealed with a rubber stopper and purged with N_2/CO_2 (50/50 %, 1 atm) for 10 min. For the analysis of the BEAP profiles, butyrate/ butyric acid, acetate/ acetic acid, ethanol or propionate/ propionic acid solutions were adjusted to pH 8 and added to a final concentration of 50 mM. Equal amounts of H_2O were added to control flasks. The small batch fermenters were incubated at 40 °C for 24 h and 200 rpm. After 24 h the amount of CH_4 was measured according to Refai *et al.* [53] and was indicated as µmol CH_4 formed per 1 g sludge per day (µmol CH_4 g^{-1} d^{-1}). The overpressure within the serum flasks was quantified with a gastight glass syringe. The CH_4 content was determined by injecting 20 µl from the gas phase into a gas chromatograph model Clarus® 480 (PerkinElmer, Waltham, USA) equipped with a flame ionisation detector and a Rascon FFAP column (25 m 0.25 micron, PerkinElmer, Waltham, USA. The temperatures of injector, detector, and column oven were set to 150 °C, 250 °C and 120 °C, respectively. A gas mixture of 10 % CH_4 and 90 % argon (Air Liquid, Düsseldorf, Germany) was used as standard.

CH_4 formation in semi-continuous lab-scale reactors

For the determination of the CH_4 production and the ECON- and ACON values, samples were taken from the reactors and incubated for 24 h at 40 °C as indicated above [53]. Biogas produced by semi-continuous lab-scale fermenters was collected in gas collection bags (Tecobag, Fa. Tesseraux Spezialverpackungen GmbH, Bürstadt, Germany) and the gas volume was determined by a drum gas meter (Fa. Ritter, Bochum, Germany). CH_4 and CO_2 concentrations were detected by a biogas analysis unit (VISIT 03, Messtechnik EHEIM GmbH, Germany). Calibration of the instrument was carried out daily (Messer, Industriegase GmbH, Germany).

Calculation of the theoretical CH_4 yield

Amon *et al.* published mean values for biogas and CH_4 yields from various energy crops and livestock manures [5]. These values are based on batch fermentation experiments according to the methods of VDLUFA [61]. Using these data, we calculated the theoretical CH_4 yield of all biogas plants analyzed in this study. The calculation was based on the size of the fermentation reactor and the composition and

quantities of the substrates fed to each biogas plant. E.g. a full-scale biogas plant with a fermentation reactor of 1,500 m^3 is fed with 2 t fresh mass (FM) of maize silage and 8 t FM of cattle manure every day. Maize silage yields 202 m^3 biogas t $[FM]^{-1}$ with a CH_4 content of 53 %. 48 m^3 biogas t $[FM]^{-1}$ is produced from cattle manure containing 55 % CH_4 [5]. Hence, under steady state conditions 425.3 m^3 CH_4 or 18975 mol CH_4 is produced per day. Taking into account the volume of the reactor and the density of the biogas sludge (1.05-g-L^{-1}) 12.1 µmol CH_4 d^{-1} mL^{-1} should be produced in the biogas plant. This value is then compared to the CH_4 yield obtained from small-scale batch reactors within 24 h.

Set up of semi-continuous lab-scale reactors

To analyze the effect of increasing NH_4^+-N concentrations on biogas formation acryl glass vessels with a capacity of 9 L (ATB Potsdam) and stirring devices (Stirring devices: IKA RW 20, Heidolph RZR 2051, controlling device: Conrad Electronics) were used [54]. The double walled and plastic foam isolated lab-scale reactors were connected to a 39 °C water bath. The reactors were filled with diluted biogas sludge from a mesophilic biogas plant and fed with maize silage. During the startup phase the amount of substrate was increased over 2.5 weeks until an organic loading rate of 3 g oDM d^{-1} L^{-1} was reached (day 1 of the experiment), which was then maintained until the end of the experiment. The OLR of 3 g oDM d^{-1} L^{-1} corresponded the biogas plant, where the inoculum came from. The stirring period of the lab-scale reactors was 15 min followed by a break of stirring for 60 min. Once a day 300 mL of the fermenter content was removed. Maize silage as substrate was diluted in 200 mL fermenter content and was added to the fermenters. The remaining 100 mL samples from the lab-scale reactors were collected in sealed plastic bottles and stored at 4 °C till analyzing the BEAP values in small scale batch fermenters as described above. After 5 days of steady state fermentation with constant NH_4^+ concentrations, BEAP values and CH_4 production (day 1-5), the NH_4^+-N concentration in the test fermenters was increased by adding 3.6 g urea L^{-1} biogas sludge. To further increase the NH_4^+-N concentration 0.23 g urea L^{-1} biogas sludge was added on days 7, 8, 9 and 13.

Determination of physico-chemical parameters

Acetic-, butyric-, and propionic acid concentrations were determined by gas chromatography as described by Refai *et al.* [54] with sample preparation following the Carrez method [19]. The volatile fatty acid content (VFA) and the carbonate buffer capacity (TAC) were analyzed according to Nordmann [49]. Briefly, a sample of biogas sludge is titrated with 0.1 N H_2SO_4 up to pH 5.0 to calculate the TAC value, expressed in mg L^{-1} of $CaCO_3$ (TAC= A* 250 [mg $CaCO_3$ L^{-1}] – with A = consumption of H_2SO_4 in mL). The FOS value is obtained after a second titration step between pH 5.0 and pH 4.4 and is expressed in mg L^{-1} of acetic acid (FOS = ((B *1.66) – 0.15)* 500 [mg L^{-1} HAc] – with B = consumption of H_2SO_4 in mL). The NH_4-N concentration was measured by a gas-sensitive NH_3-Elektrode [60]. The determination of organic dry mass (oDM) was performed as described in German Standard DIN 12879. Briefly 2 g of biogas sludge was incubated at 150 °C for 2 h to measure the dry weight (DM). The resulting material was then heated to 550 °C for 75 min to determine the oDM.

Results

Definition of BEAP profiles

The entire reaction chain from biopolymers to CH_4 can be described by four interdependent steps referred to as hydrolysis, acidogenesis, acetogenesis and methanogenesis. In general, the weakest link of this chain limits the performance of the overall process. However, there is no analytic method available to

quantify the performance of each degradation step in one experimental setup. Therefore, we developed a test system to analyze the efficiency and the catalytic capacity of various groups of prokaryotes involved in anaerobic degradation of organic matter. In the test system, authentic biogas sludge samples were supplemented with the metabolic intermediates butyrate and propionate as substrates of syntrophic bacteria, respectively, or with acetate as substrate for aceticlastic methanogens. The forth substrate was ethanol, which is converted to acetate and H_2 by ethanol-oxidizing bacteria and further on to CH_4 and CO_2 by aceticlastic methanogens. H_2, in turn, is used as reductant in the process of CH_4 formation from CO_2 catalyzed by hydrogenotrophic methanogens. To investigate the effect of these additives on CH_4 formation, batch reactors containing 20 g biogas sludge were used which reflect process conditions similar to those in the full-scale biogas plant [53]. From percental differences between CH_4 formation of supplemented and non-supplemented biogas sludge, so called BEAP profiles were generated. The respective experimental setup was referred to as BCON (addition of 50 mM **b**utyrate in comparison to the **con**trol), ECON (addition of 50 mM **e**thanol in comparison to the **con**trol), ACON (addition of 50 mM **a**cetate in comparison to the **con**trol) and PCON (addition of 50 mM **p**ropionate in comparison to the **con**trol). The corresponding values indicate CH_4 formation (%) in comparison to control reactors (set to 100%). The term "BEAP" derives from the first letters of the additives.

BEAP profiles of efficiently running biogas plants

BEAP profiles of more than 50 samples from different German agricultural biogas plants were determined in this study (Table S1). 16 of these biogas plant operated very well and did not reveal process failures or a decrease in performance in the three months before sampling, indicating a stabile microbial degradation of organic matter. Biogas sludge samples of these plants showed an extensive increase in CH_4 formation after addition of ethanol or acetate. In contrast, the addition of propionate or butyrate did not result in significantly increased CH_4 yields. These criteria were used to group these reactors in biogas plant cluster 1. The selected plants were operated with different amounts of renewable organic materials (maize silage, wheat grain, rye grain, grass silage and animal feces, e.g. cattle- or horse manure, chicken litter). CH_4 production, acetic-, butyric- and propionic acid concentrations, pH values, NH_4^+-N content and VFA- and TAC values were analyzed to monitor the status of the biogas plants and to determine correlations between these parameters and specific BEAP profiles (Table S1). The operating temperature of the plants was between 39 °C and 55 °C. The pH value was 7.9 ± 0.1. Each plant exhibited different NH_4^+-N-contents (1.5 - 4.5 g L^{-1}) and acid concentrations (e.g. 1.0 - 18.8 mM acetate and < 0.4 - 5.5 mM propionate) (Table S1). Biogas sludge of these plants was supplemented with the BEAP substrates and was incubated for 24 h in serum bottles. CH_4-formation was measured and calculated as indicated in materials and methods. The average CH_4 production of the small-scale batch fermenters without any supplementation was 57.4 ± 10.8 µmol g^{-1} d^{-1} and was slightly higher than the theoretically calculated CH_4 formation in the full-scale biogas plants. Strongly increased CH_4 production was observed after addition of acetate or ethanol, indicated by ECON and ACON values of 190 – 254 % and 163 – 209 %, respectively (CH_4 production of control fermenters was set to 100 %) (Table S1). In contrast, CH_4 formation was only 111 ± 8 % after butyrate addition and 102 ± 10 % after propionate supplementation (Fig. 1). From these data the BEAP profile for a cluster 1 biogas plant was defined as BCON ≤ 120 %, ECON 190 - 260 %, ACON 160 - 210 % and PCON ≤ 115 %.

The use of BEAP profiles for the identification of process incidents in biogas plants

As shown above cluster 1 biogas plants are characterized by a specific BEAP profile with high ECON and ACON values and low BCON and PCON values. So the question arose whether process failures can be detected by deviant BEAP profiles and whether BEAP values allow to distinguish between various process incidents (Table S1). Besides cluster 1 biogas plants, four other clusters could be identified each with a characteristic BEAP profile. Biogas plants of cluster 2 revealed increased NH_4^+-N concentrations (4.8 ± 0.8 g L^{-1}) in comparison to cluster 1 reactors (2.9 ± 0.7 g L^{-1}). Ammonium is a product of urea hydrolysis or anaerobic protein degradation in biogas sludge. It is essential for bacterial growth, but high concentrations may lead to a decrease or even a failure of CH_4 production due to toxicity caused by NH_3 formation [20]. Interestingly, increased NH_4^+-N contents in biogas plants resulted in a specific BEAP profile, characterized by the reduction of all BEAP values (BCON $\leq$ 120 %, ECON 140 - 160 %, ACON 105 - 150 % and PCON $\leq$ 100 %; CH_4 production of control fermenters was set to 100 %) (Fig. 2a). In contrast, CH_4 yields were still $\geq$ 100 % of the calculated CH_4 yield. pH and TAC values were slightly increased. Total VFA concentrations as well as acetate, butyrate and propionate concentrations were in the same range as in cluster 1 biogas plants (Table S1).

Cluster 3 comprises malfunctioning biogas plants with biogas production of < 100 % (average of 75 %) of the calculated CH_4 yield (Fig. 2b). NH_4^+-N concentrations, VFA/TAC ratios and acid concentrations were within normal range [32] and therefore, no evidence for process failures could be observed based on parameters (Table S1). But interestingly, all BEAP values were strongly increased. This process limitation could be detected in seven biogas sludge samples and a typical range of each BEAP value could be defined: BCON 180 - 300 %, ECON 240 - 350 %, ACON 190 - 260 %, PCON 140 - 270 % (CH_4 production of control fermenters was set to 100 %) (Fig. 2b).

Cluster 4 biogas plants were characterized by increased BCON- and PCON values (Fig. 2c). ACON- and ECON values did not differ from those of cluster 1 biogas plants. Additionally, the CH_4 yield was $\geq$ 100 % compared to the theoretical calculated values (Fig. 2c). The BEAP profile of this cluster of facilities could be defined as BCON 150 - 250 %, ECON 190 – 260 %, ACON 160 – 210 % and PCON 120 – 170 %. Besides strongly increased BCON values, slightly reduced pH (7.8 ± 0.3) and TAC (14.8 ± 6.6 g $CaCO_3$) values could be detected in comparison to those of cluster 1 (Table S1).

One biogas plant belongs to cluster 5, which was accidentally fed with mildew permeated maize silage and it was suggested that this reactor suffered from mycotoxin intoxication. The biogas plant had a reduced CH_4 yield of 52 % and a slightly increased VFA/TAC ratio (0.3 g HAceq g^{-1} $CaCO_3$) as well as an elevated concentration of acetate (43 mM). In contrast, pH value, NH_4^+-N content, and the concentrations of butyrate and propionate were within normal range [32]. However, the BEAP profile was uniquely characterized by a very low ACON of 112 % and a normal ECON value of 205 %. BCON (136 %) and PCON (116 %) were slightly increased. This was the only example for a biogas plant revealing divergent ECON and ACON values.

In summary, all four clusters with non-standard BEAP profiles could be mapped to dysfunctions in the digestion levels during the anaerobic breakdown of renewable organic material. This context is explained in detail in the discussion. As indicated above many biogas plants could be grouped into the five BEAP profiles. However, it is important to note that not all biogas plants fit into these clusters. This was especially true for biogas

plants with severe process incidences and CH_4 yields < 50% of the theoretical value (Table S1). Most of these reactors suffered from severe NH_4^+-N intoxication or acidification. A few biogas plants revealed normal physico-chemical parameters but showed reduced BEAP profiles and CH_4 yields. Possible reasons could be other process limitations (e.g. trace elements limitation, abrupt substrate change, access of air) [11] which were not analyzed in this project. A third group of biogas plants showed BEAP profiles that indicated a transient state between two clusters. These findings suggested that the microbiome in the biogas sludge is not static but is subject to change depending on the overall process management. The five clusters presented here represent snap shots of the metabolic status of biogas plants. It is also possible to analyze the time course of the metabolic capacity of the microbial community in biogas plants by regular probing and BEAP profile analysis as shown below.

BEAP profiles for the early detection of NH_4^+-N induced process failure

As mentioned above, NH_4^+ is essential for the growth of prokaryotes, but an excess of these ions can inhibit biogas formation [38]. However, the inhibitory threshold concentration of NH_4^+-N is different for each biogas plant and cannot be predicted by measuring standard parameters or by theoretical considerations. Therefore, we used semi-continuous laboratory-scale reactors to simulate a process failure due to high NH_4^+-N concentrations and to analyze the development of BEAP profiles in the course of NH_4^+-N intoxication. Six acryl glass reactors were filled with 8 L of diluted biogas sludge from a mesophilic biogas plant operated on renewable energy crops and manure. Feeding of the fermenters was slowly raised by adding increasing amounts of maize silage until an OLR of 3 g oDM d^{-1} L^{-1} was reached (Phase 1, Fig. 3). This OLR was identical to the OLR of the biogas plant which was used to inoculate the fermenters. The OLR was maintained throughout the entire experiment.

In phase 1 of the experiment the fermenters were kept under non-stress conditions for 5 days. All physico-chemical parameters and the BEAP values were stabile during that period (Fig. 3; Phase 1; Table S2). The NH_4^+-N concentration of the reactors at this time point was 2.6 ± 0.04 g L^{-1} and was set to 100 %. At the beginning of the second phase 28.6 g of urea was added to the stress fermenters followed by the addition of 1.8 g urea on days 7, 8 and 13. The additions led to a continuous increase of NH_4^+-N up to 183 % in the stress reactors compared to the control reactors (Fig. 3, Phase 2; Table S2). During that time period the CH_4 production in all stress reactors did not differ from the rate of biomethanization in the control reactors. In contrast to the CH_4 formation, the ECON- and the ACON values in the stress reactors already decreased significantly by 38 % and 50 %, respectively when the NH_4^+-N concentration reached 120 % at day 8 (Fig. 3, Phase 2). Also, the BCON value was reduced by more than 30 %, while the PCON value revealed ambiguous results (data not shown). In contrast to the BEAP values, VFA and TAC values increased by less than 10 %, which is within the range of the normal fluctuation in a biogas plant. Thus, ECON- and ACON values can function as an effective warning system that detects a NH_4^+-N induced process inhibition earlier than common performance parameters.

The inhibitory effect of elevated NH_4^+-N concentrations in Phase 2 reached its maximum on day 13 with a reduction of ECON and ACON values by 65 % and 60 %, respectively. VFA and TAC values increased dramatically at this point to 150 % and 130 % indicating an imminent malfunction in the stress reactors. Interestingly, CH_4 formation was constant in Phase 2 and started to drop not before day 15 (Fig. 3, Phase 3; Table S2). In parallel, ECON and ACON values further decreased by 65 % and 60 % compared to the ECON and ACON values of the control

fermenters, respectively. Obviously, the microbiome of the biogas sludge could cope with an increased NH_4^+-N concentration up to a certain threshold (Fig. 3, Phase 2, Table S2). Above this threshold the entire system was inhibited, indicated by a reduced CH_4 yield (Phase 3). Hence, it is evident that the ACON and ECON values are sensitive parameters to predict critical NH_4^+-N thresholds that lead to reduced biomethanization. After 35 days, CH_4 production in the stressed reactors was 89 ± 9 % compared to the control reactors (Fig. 3, Phase 4; Table S2). This recovery was due to the decrease of the NH_4^+-N concentration to 143 % compared to control fermenters. However, ACON and ECON values were still reduced by 56 % and 52 % compared to the control indicating that the original metabolic capacity for ethanol and acetate degradation had not been regained completely.

Monitoring an ammonia induced incident in a full-scale commercial biogas plant

ECON and ACON values as a warning system were also tested in a mesophilic full-scale biogas plant, which was fed with maize silage, cattle manure and poultry litter. The process of biogas production was observed for 13 months. Biogas sludge samples of the biogas plant were taken monthly and analyzed in lab-scale batch reactors to investigate CH_4 production, ECON and ACON values. Additionally, physico-chemical parameters such as pH, NH_4^+-N concentration, acid concentrations and the VFA and TAC values were determined. Daily substrate input records and standard gas yields were used to calculate the theoretical CH_4 formation (Material and methods) [5]. The experiments revealed that the CH_4 production of sludge samples remained at a constant level of 51.7 ± 0.4 µmol g^{-1} d^{-1} during the first five months of monitoring and was ≥ 100 % of the theoretical production rate (Fig. 4; Phase 1). In addition, the BEAP profile fit the criteria of a cluster 1 biogas plant (BCON 116 ± 7 %, ECON 219 ± 8 %, ACON 180 ± 15 % and PCON 107 ± 2 %). After five months, the composition of substrate input changed. In Phase 2 the amount of poultry litter was increased from 4.2 to 6.1 t fresh mass d^{-1}. Consequently, the NH_4^+-N concentration raised from 3 g L^{-1} to 4.4 g L^{-1} but no significant changes could be observed in terms of biomethanization (Fig. 4, Phase 2). In contrast, ECON and ACON values decreased by 52.8 % and 44.1 %, respectively. During the following months, the NH_4^+-N concentration further increased and the CH_4 formation slightly dropped by 14 % (Fig. 4, Phase 3). After 11 months, NH_4^+-N concentration peaked at 5.8 g L^{-1} and ECON and ACON values decreased by 111 % and 78 %, respectively, compared to the values at the end of Phase 1. In parallel, the VFA value increased by 125 % and the TAC value by 65 %. In Phase 4, poultry litter input was reduced with the NH_4^+-N concentration subsequently decreasing to 4.6 g L^{-1}. Consequently, the CH_4 yield recovered until reaching the plant's initial CH_4 formation (51.7 ± 0.4 µmol g^{-1} d^{-1}). However, ECON and ACON values were still low at 142.8 % and 132.4 %, respectively. The results indicate that methanogenic archaea and syntrophic ethanol-oxidizing bacteria had not completely recovered from the NH_4^+-N intoxication of the biogas reactor.

To shed light on the question whether BEAP values can be used as an effective warning system for NH_4^+-N intoxication, it is necessary to have a closer look at the transition from Phase 1 to Phase 2. As mentioned above, CH_4 formation was not influenced by the early increase in NH_4^+-N concentration in Phase 2. In contrast, ECON and the ACON values severely dropped from 227 % to 185 % and from 192 % to 155 %, respectively, at the transition from Phase 1 to Phase 2. Interestingly, the VFA and TAC values only marginally increased and were still in range of cluster 1 biogas plants (Table S1). For this reason, it is difficult to evaluate whether inhibition of biogas production is imminent or whether the parameters are still in a tolerable range. However, the ECON and ACON values

clearly indicated reduced metabolic capacity of the biogas plant at an early stage before the CH_4 formation dropped. Taking together all results, the BEAP values can clearly be used as a sensitive warning system to determine an upcoming process failure because of high NH_4^+-N concentrations.

Discussion

The breakdown of organic matter in commercial biogas plant involves many interacting groups of microorganisms, which degrade complex organic polymers into biogas [39]. Because of the complexity of the system, small changes in any process parameters can lead to increasing or decreasing CH_4 production and a shift of the delicate equilibrium within the microbial community. Usually the whole biological process is monitored by biogas formation, CH_4 content, pH value, temperature, NH_4^+-N concentration, TAC- and VFA values and the concentrations of acetic-, butyric- and propionic acid [1, 12, 14, 15, 33, 37]. Thus, each parameter reflects the total biogas formation process. In contrast, test systems to analyze individual steps of the whole process are scarce [39]. In general, the entire degradation process in a biogas plant can be described as a chain of reactions where the slowest reaction defines the overall rate of biomethanization. The BEAP profiles presented in this study allow for the first time to monitor the metabolic functionality of each of the four main microbial degradation levels and to identify the rate-limiting step of the process. Each BEAP value indicates the change of the CH_4 formation after feeding the corresponding BEAP substrate butyrate, ethanol, acetate or propionate (Fig. 5). In case of increasing CH_4 formation it is evident that the organisms responsible for the degradation possess additional capacity for the utilization of external substrates which are degraded in addition to the substrates normally formed in the biogas sludge. Hence, with this test system, it was possible to answer the question which degradation level causes the bottleneck during CH_4 formation.

The metabolic intermediates butyrate (BCON) and propionate (PCON) are substrates of syntrophic bacteria and the degradation of these compounds represent the most important pathways during acetogenesis [56]. The ACON value gives information on the conversion of acetate by aceticlastic methanogens [65]. Control experiments revealed that ACON values are not influenced by high H_2 concentrations in the headspace [53]. Moreover, it was shown that the addition of H_2 significantly increases the rate of CH_4 formation if mass transfer from the gas phase to the sludge was improved [36]. These results showed that the process of acetate conversion does not depend on syntrophic acetate oxidation, which is thermodynamically unfavorable under this condition [58]. Supplementation with ethanol results in the formation of acetate which is converted to CH_4 and CO_2 (aceticlastic methanogens) and H_2 which is metabolized to CH_4 by CO_2 reduction (hydrogenotrophic methanogens). Hence, the ECON value determines the metabolic potential of ethanol-oxidizing bacteria and methanogenic archaea. Furthermore, the comparison of the ACON and the ECON values allows an estimation of the performance of hydrogenotrophic methanogens (Fig. 5). In summary, the BEAP values directly reveal the potential capacity of acetogenesis and methanogenesis in biogas plants. Acidogenesis describes the primary fermentation of cleavage products from biopolymers (especially polysaccharides) and is not considered as rate-limiting step in biogas plants. This statement is proven by the fact that the addition of monosaccharides or disaccharides always led to an acidification of the biogas sludge samples used in this study. This pH drop resulted from a rapid degradation of sugars by fermentative bacteria and a strong increase of the VCA concentrations (data not shown). Hence, the formation rate of butyrate and propionate was faster than their degradation rate. In full scale

biogas plants it was also observed that an increased feeding with eupeptic polysaccharides can lead to acidification and to a breakdown of biomethanization [34]. The estimation of the performance of hydrolysis as the first digestive level is possible by comparing the BEAP values and the determination of the rate-limiting step. In case all BEAP values show a significant higher level than the values of cluster 1, the hydrolytic step is rate-limiting (see below).

As shown above it is possible to define different clusters of biogas plants differing with respect to their BEAP profiles. Cluster 1 comprises efficiently running biogas plants with stable process stages that were characterized by high ECON (190 – 260 %) and ACON (160 – 210 %) values. As evident from these values, the addition of ethanol to biogas sludge can cause a stronger increase of CH_4 formation compared to the addition of acetate. The observation is explained by the fact that 1 mol of ethanol is converted to 1.5 mol CH_4 and 0.5 mol CO_2 by the catalytic activity of ethanol-oxidizing bacteria, aceticlastic methanogens and hydrogenotrophic methanogens. In contrast, acetate is converted to 1 mol CH_4 and 1 mol CO_2. Hence, the expected ratio of ECON/ACON should be 1.5. Cluster 1 biogas plants also displayed a slightly increased biomethanization after supplementation with butyrate (BCON $\leq$ 120 %) and propionate (PCON $\leq$ 115 %). The data indicate that the biogas plants would produce about 20 % and 15 % more CH_4 if additional butyrate and propionate would be fed to the fermenter, respectively. However, with respect to economic and technical issues, the supplementation with VFA is not feasible. Furthermore, it is evident that the capacity of ethanol-oxidizing bacteria and methanogenic archaea is largely unused. It was already suggested that the addition of ethanol produced by alcoholic fermentation could lead to an increased CH_4 production in biogas plants without disturbing the normal flux of metabolites [54].

The second cluster of biogas plants is characterized by reduced ECON- and ACON values and increased NH_4^+-N concentrations indicating the beginning of NH_4^+-N inhibition. It is known that high NH_4^+-N concentrations can be responsible for reduced biogas production in biogas reactors [27, 47, 51, 52]. Furthermore, it has been suggested that especially methanogenic organisms are negatively affected by high NH_4^+-N concentrations, leading to a drop in CH_4 production [7, 35, 45]. These observations fit the decreased ACON- and ECON values of cluster 2 biogas plants.

In cluster 3 biogas plants all four BEAP values were strongly increased and the CH_4 yield was below 100 % of the theoretical calculated CH_4 values. The data indicate that the degradation levels of acidogenesis, acetogenesis and methanogenesis possessed an excess of metabolic capacity. Hence, it is tempting to speculate that the rate-limiting step was located at the level of hydrolysis. Many studies have been conducted to circumvent this potential bottleneck by increasing the hydrolytic potential e.g. by the addition of hydrolytic enzymes and pretreatment of biopolymers for improved biodegradability [18, 44]. In summary, cluster 3 comprises biogas plants suffering from insufficient hydrolysis.

Cluster 4 biogas plants were characterized by a CH_4 yield of $\geq$ 100 % and highly increased BCON values as well as slightly increased PCON values. ACON- and ECON values did not differ from those of a standard biogas plant. Enhanced BCON values point to a large potential of the microorganisms in the biogas sludge to oxidize butyrate to acetate and H_2, indicating an accumulation of butyrate oxidizers. Hence, it is tempting to speculate that cluster 4 biogas plants are in the beginning of acidification which is one of the most common incidences

in biogas formation [13]. The hypothesis is that the reactors already suffer from an increased flux of butyrate from acidogenesis. This scenario could result in an accelerated proliferation of butyrate oxidizers and an increased metabolic capacity for butyrate oxidation leading to an elevated BCON value. The same could be true for propionate oxidizers accompanied by an increase of the PCON value. Once the flow of butyrate and propionate exceeds a certain threshold, the metabolic capacity of syntrophic acid oxidizers becomes rate-limiting and butyrate and propionate concentrations begin to rise. In this respect it is to mention that acetogenesis is a delicate life style because fatty acid oxidation coupled to H_2 (or formate) production by syntrophic bacteria is a highly endergonic process under standard conditions [42, 56]. In fact, an extremely low partial pressure of H_2 is necessary to allow propionate and butyrate degradation under anaerobic conditions [2, 17, 57]. Consequently, syntrophic bacteria can only grow in close association with H_2-oxidizing methanogenic organisms that form CH_4 by the H_2-dependent reduction of CO_2 [41]. The depletion of acetate as product of butyrate oxidation by aceticlastic methanogens is also important. Fortunately, the methanogenic processes are not rate-limiting as ECON- and ACON values are high in Cluster 4 biogas plants.

The cluster 5 biogas plant, predicted to suffer from mycotoxin inhibition, revealed an unusual BEAP profile where the ECON value was much higher than the ACON value. This was the only biogas plant with divergent ECON- and ACON values that differed significantly from the expected ratio ECON/ACON of 1.5. Obviously, ethanol oxidation and methanogenesis from H_2 and CO_2 were highly effective but CH_4 formation from acetate was limited. Therefore, we suggest that acetate is not a free intermediate of ethanol conversion in this special biogas plant. Hence, acetyl-CoA formed from ethanol is not converted to acetate for substrate-level phosphorylation but is oxidized to H_2 and CO_2 in a process that resembles the metabolism of syntrophic acetate-oxidizing bacteria [28, 66]. Another possible explanation is that a special group of aceticlastic methanogens may be able to utilize ethanol as substrate thereby forming H_2 and acetyl-CoA which is converted to CH_4 and CO_2 intracellularly without acetate as intermediate. However, such a group of aceticlastic methanogens has not been discovered yet.

The use of the BEAP profile enables a rapid testing of the metabolic state of biogas plants within 24 h and an accurate mapping of all levels of anaerobic biopolymer degradation in a lab-scale experimental setup. In addition, it became evident that the BEAP profile can be used as a warning system to detect upcoming process failures during biogas formation e.g. caused by increased NH_4^+-N concentrations. Many reports about the NH_4^+-N content in anaerobic digesters are available and it is known that the main component causing NH_4^+-N intoxication is free NH_3 which is formed from NH_4^+ depending on temperature and pH value [20]. However, conflicting results about inhibiting concentrations were obtained because toxic NH_4^+ concentrations for anaerobic processes range from 2.8 to 8 g kg^{-1} biogas sludge [24]. Furthermore, the inhibitory concentration depends on long-term adaptation of the microbiome [7, 63]. Especially, methanogenic archaea, responsible for the last degradation step, are sensitively affected by high NH_4^+-N concentrations [7, 35, 45]. However, as shown in this publication the NH_4^+-N concentration in full operating biogas plants can rise to a certain threshold with no significant changes in biomethanization. Only after exceeding this critical concentration a drop in CH_4 formation was observed. Furthermore, it is evident that the inhibitory threshold NH_4^+-N concentration varies between different biogas plants and unfortunately cannot be predicted from current analytic parameters. However, ACON- and ECON values are suitable parameters to

determine the metabolic capacity of methanogenic archaea and to predict the critical NH_4^+-N threshold that leads to a drop in biogas formation.

The knowledge about degradation processes in biogas reactors is currently still limited. However, to understand CH_4 production in detail and to demonstrate opportunities to optimize the process or to avoid incidents, a test system monitoring single levels of the biogas formation process is essential. Besides analyzing physico-chemical parameters, a microbial monitoring of the production process is crucial. Therefore, we developed the BEAP profile presented in this study. The BEAP profile allows an analysis of the performance and metabolic limitations of each step in the anaerobic breakdown of organic matter. Additionally, a classification of biogas plants with respect to process incidences (e.g. NH_4^+-N intoxication, acidification or insufficient hydrolysis) is possible. Finally, when applied as a regular monitoring analysis, BEAP profiles can function as warning systems for NH_3-induced process disruption, thus preventing a commercial biogas plant from reduced biogas production and the ensuing financial losses.

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Compliance with Ethical Standards

The authors affirm that the said publication complies with obligations and norms of the ethical standards of research.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

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Figure legends

Figure 1: BEAP profile of standard biogas plants. CH_4 production of the control was 57.4 ± 10.8 µmol g^{-1} d^{-1}. BEAP values correspond to increase in CH_4 formation (in percentage) in comparison to the control. BCON, CH_4 production after adding butyrate and incubation for 24 h as indicated in materials and methods. ECON, CH_4 production after supplementation with ethanol. ACON, CH_4 production after addition of acetate. PCON, CH_4 production after addition of propionate. Each supplement was added at a final concentration of 50 mM. All experiments were conducted at least three times using 16 different biogas sludge samples. Standard deviations are indicated by *error bars*.

Figure 2: BEAP profiles of different biogas plant clusters. Butyrate (BCON), ethanol (ECON), acetate (ACON) and propionate (PCON) were added to small lab-scale batch reactors and CH_4 formation was measured after 24 h and compared to controls without supplementation. Each supplement was added at a final concentration of 50 mM. All experiments were conducted at least three times using different biogas sludge samples. The respective standard deviations are indicated by *error bars*. a) Cluster 2 (10 samples); b) Cluster 3 (seven samples) c) Cluster 4 (nine samples); d) Cluster 5 (one biogas plant). The CH_4 formation rate of the control was set at 100 % in each class. The increase [%] of the CH_4 formation rate in comparison to the control is indicated. *White boxes* in the background indicate BEAP values of standard biogas plants (Cluster 1).

Figure 3: Effect of increasing NH_4^+-N concentrations on the performance of lab-scale reactors. *Bars*, NH_4^+-N concentrations compared to the control reactors [%]. *Black diamonds*, CH_4 production compared to control reactors [%]. *White squares*, CH_4 production after addition of ethanol in stress reactors compared to ethanol supplemented control reactors [ECON %]. *Black squares*, CH_4 production after addition of acetate in stress fermenters compared to acetate supplemented control fermenters [ACON %]. *Black circles*, comparison of TAC values in control and stress fermenters [%]. *White circles*, comparison of VFA values in control and stress fermenters [%]. The values derive from three control and two stress reactors, respectively. CH_4 formation and ACON and ECON values were analyzed in triplicates as described in materials and methods. The respective standard deviations are indicated by *error bars*. Urea was added on day 5 (3.6 g L^{-1} biogas sludge) and on days 7, 8, 9 and 13 (0.23 g L^{-1} biogas sludge) to the reactors.

Figure 4: ECON and ACON value as warning system in a full-scale biogas plant. *Black diamonds*, CH_4 production [µmol g^{-1} d^{-1}]. *Gray bars*, NH_4^+-N concentration [g L^{-1}]. *White squares*, ECON value [%] (CH_4 production after addition of ethanol compared to non-supplemented biogas sludge). *Black squares*, ACON value [%] (CH_4 production after addition of acetate compared to non-supplemented biogas sludge). *White triangle*, TAC value [g $CaCO_3$ L^{-1}]. *Black triangle*, VFA value [g HAceq L^{-1}]. CH_4 production experiments were conducted in triplicates. The respective standard deviations are indicated by *error bars*.

Figure 5: Scheme of the anaerobic degradation process in a biogas reactor and relevance of BEAP values. *Continuous arrows*, direct correlation between the supplement and the metabolic potential of the digestion level. *Dashed arrows*, indirect correlation between the substrate and the microbial metabolism of the anaerobic degradation step.

Figure 1:

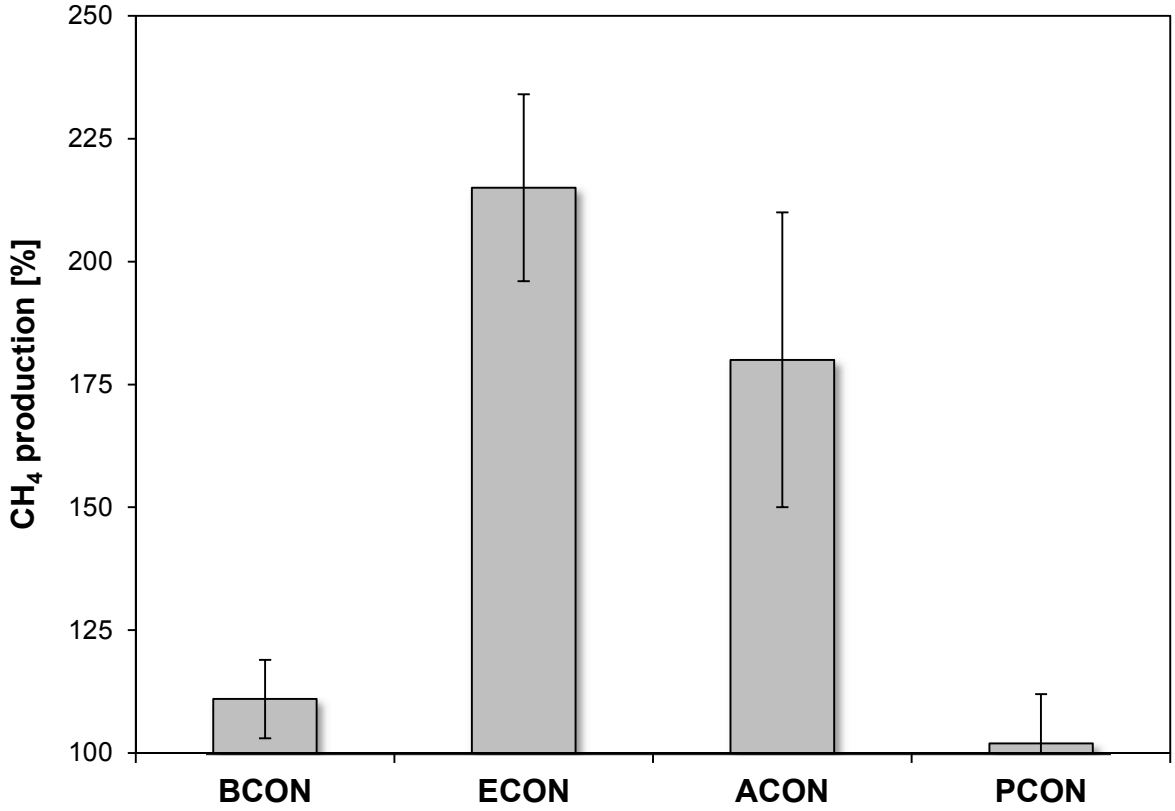
250
225
200
175
150
125
100
CH4 production [%]
BCON
ECON
ACON
PCON

Figure 2:

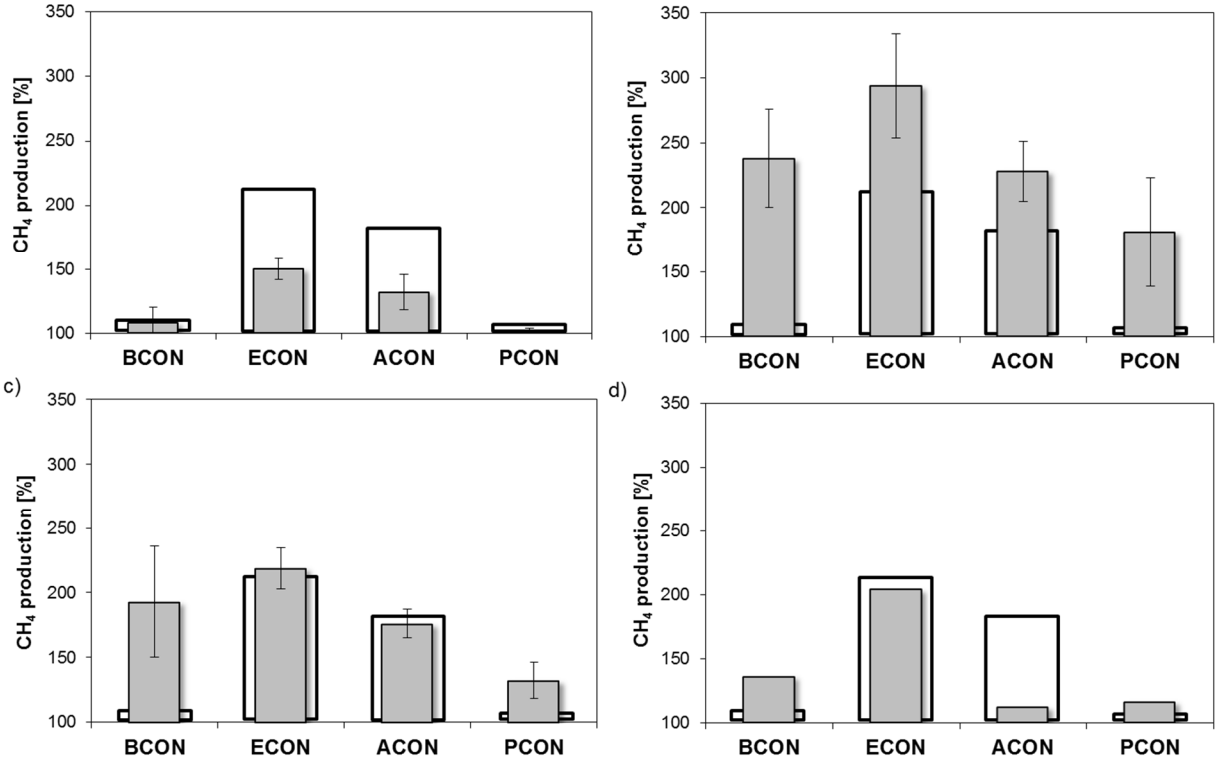

Figure 3:

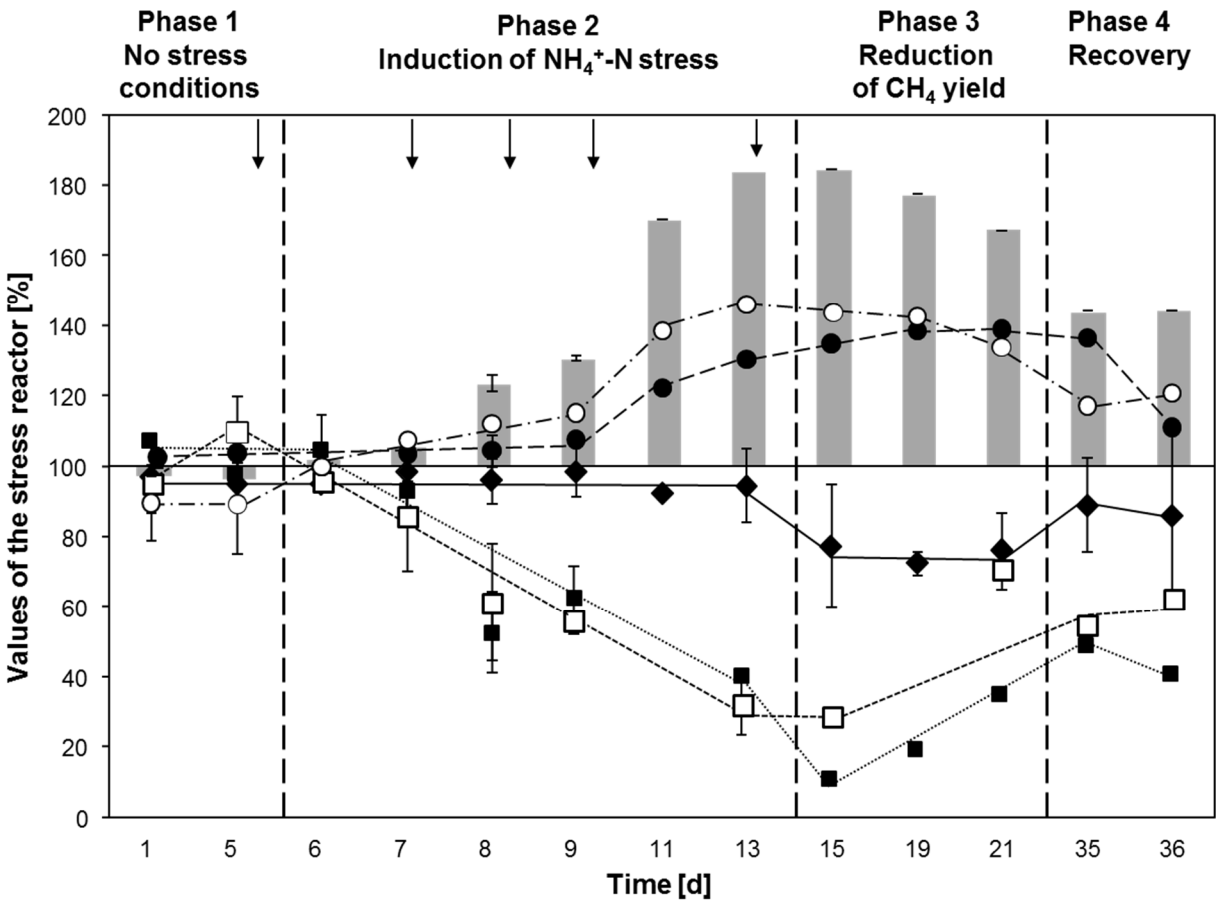
Phase 1
No stress conditions
Phase 2
Induction of NH4+-N stress
Phase 3
Reduction of CH4 yield
Phase 4
Recovery
Values of the stress reactor [%]
Time [d]
0
20
40
60
80
100
120
140
160
180
200
1
5
6
7
8
9
11
13
15
19
21
35
36

Figure 4:

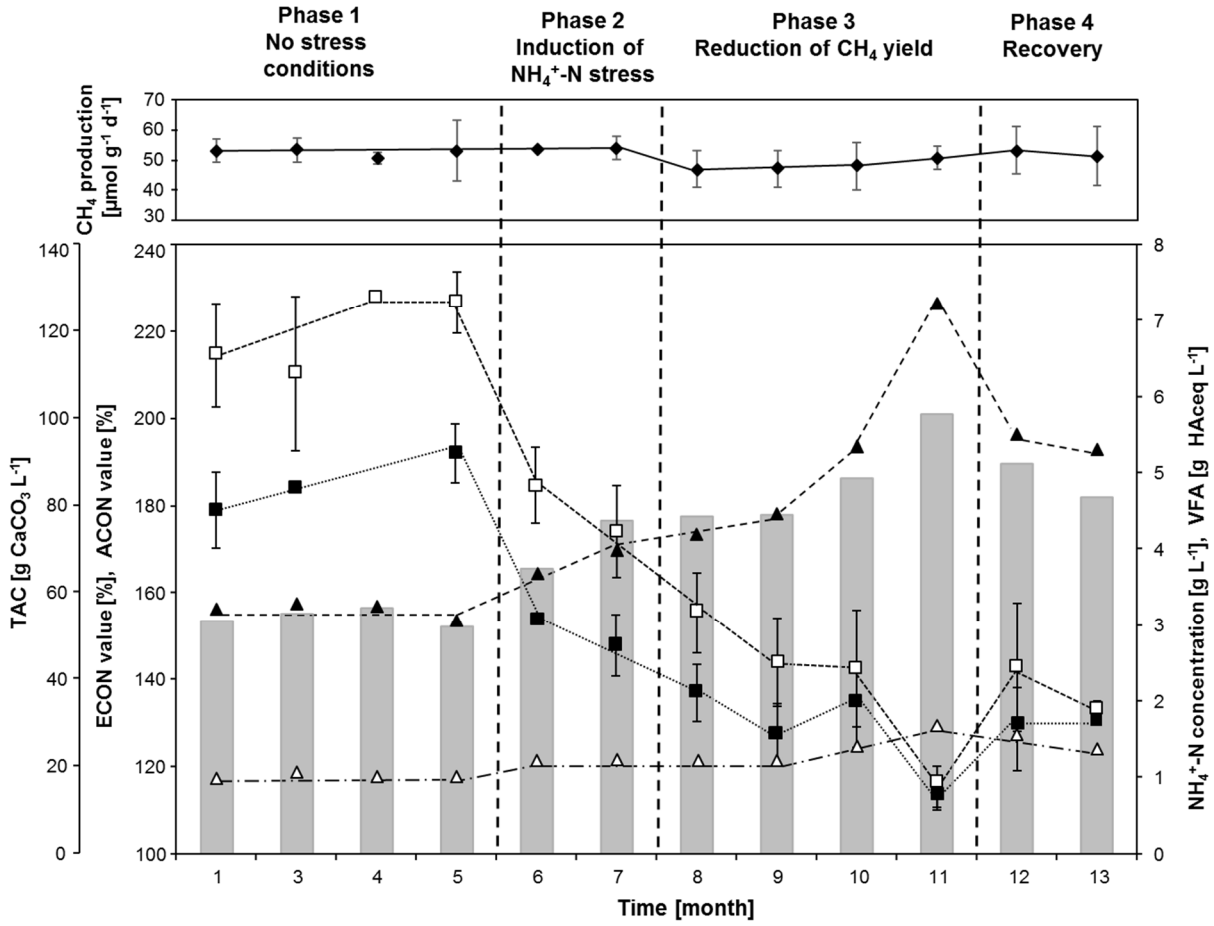
Phase 1
No stress conditions
Phase 2
Induction of NH_4^+-N stress
Phase 3
Reduction of CH_4 yield
Phase 4
Recovery
CH_4 production [µmol g^{-1} d^{-1}]
TAC [g $CaCO_3$ L^{-1}]
ECON value [%], ACON value [%]
NH_4^+-N concentration [g L^{-1}], VFA [g HAceq L^{-1}]
Time [month]

Figure 5:

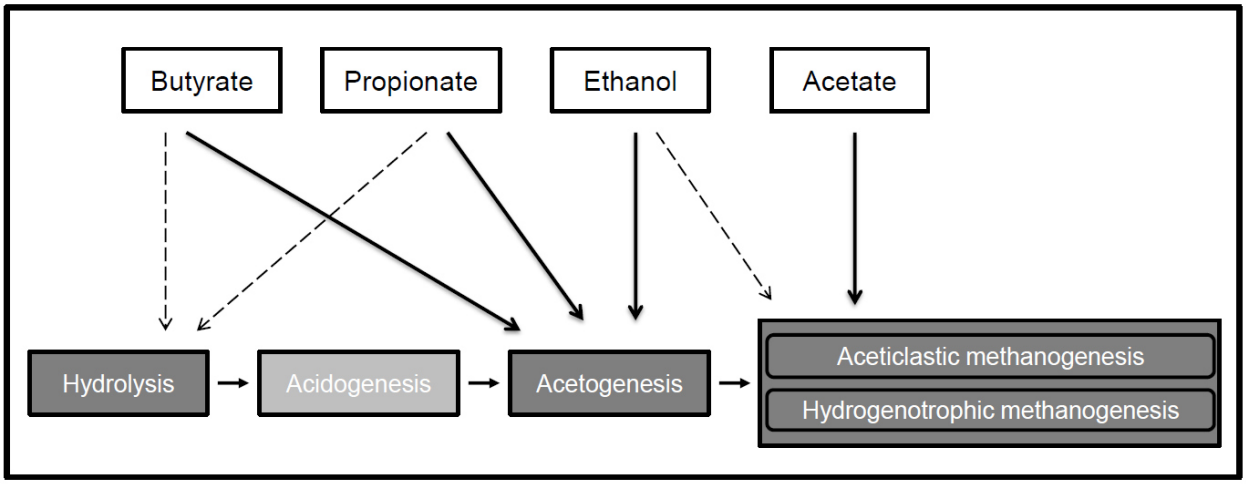
Butyrate
Propionate
Ethanol
Acetate
Hydrolysis
Acidogenesis
Acetogenesis
Aceticlastic methanogenesis
Hydrogenotrophic methanogenesis

Table S1: BEAP values and physico-chemical parameters of agricultural biogas plants

Cluster	Biogas Plant	temperature [°C]	pH-value	CH_4 production [µmol h^{-1} d^{-1}] measured	CH_4 production [µmol h^{-1} d^{-1}] calculated[a]	CH_4 production [%]	BCON[b] [%]	ECON[c] [%]	ACON[d] [%]	PCON[e] [%]	VFA/ TAC [g HAceq $g^{-1}CaCO_3$]	VFA[f] [g HAceq]	TAC[g] [g $CaCO_3$]	NH_4^+-N[h] [g L^{-1}]	acetic acid[i] [mM]	butyric acid[j] [mM]	propionic acid[k] [mM]
1	1	40	7.9	52.08 ± 4.8	47.04	110.7	116.8	210.3	183.8	111.1	0.18	3.28	18.7	3.14	4.50	< 0.33	< 0.41
1	2	40	8.0	48.72 ± 2.4	48.72	100.0	n.d.	227.8	171.2	n.d.	0.18	3.25	17.7	3.23	2.16	< 0.33	< 0.41
1	3	40	8.0	51.84 ± 11.8	45.84	113.1	n.d.	226.7	191.8	n.d.	0.17	3.06	17.7	2.98	4.83	< 0.33	< 0.41
1	4	40	8.0	48.24 ± 1.7	39.12	123.3	101.5	253.7	202.5	97.5	0.20	3.75	19.0	3.05	2.50	< 0.33	0.54
1	5	55	7.8	60.48 ± 3.1	49.44	122.3	111.7	190.5	182.2	102.0	0.28	2.83	10.2	1.54	11.82	< 0.33	1.22
1	6	55	7.8	49.68 ± 6.5	49.44	100.5	n.d.	218.2	173.3	n.d.	0.28	2.83	10.2	1.54	11.82	< 0.33	1.22
1	7	40	7.9	73.68 ± 6.2	54.00	136.4	106.6	234.1	165.6	75.5	0.20	3.60	17.9	3.05	2.50	< 0.33	< 0.41
1	8	40	8.1	48.96 ± 9.1	40.32	121.4	115.4	229.5	209.4	106.1	0.18	4.24	23.9	4.54	4.00	< 0.33	0.41
1	9	39	8.1	50.88 ± 15.4	46.08	110.4	119.5	245.0	187.2	111.7	0.20	3.31	16.7	2.98	1.00	< 0.33	0.81
1	10	39	7.8	80.40 ± 7.0	63.60	126.4	116.1	194.9	180.7	110.3	0.22	3.32	15.4	2.77	6.49	< 0.33	0.81
1	11	39	7.8	72.96 ± 16.3	63.60	114.7	101	196.8	184.9	98.2	0.22	3.28	15.2	2.79	6.16	< 0.33	0.68
1	12	45	8.0	51.60 ± 0.7	48.72	105.9	98.1	203.8	163.2	92.5	0.20	2.97	14.7	2.61	2.33	< 0.33	< 0.41
1	13	49	8.1	67.68 ± 0.7	67.92	100.0	104.1	202.5	184.7	93.5	0.25	4.14	16.3	3.07	7.00	< 0.33	1.76
1	14	41	7.9	49.44 ± 1.9	39.84	124.1	118.9	203.8	163.3	109.3	0.24	4.44	18.4	3.20	18.80	< 0.33	1.76
1	15	41	7.9	47.76 ± 1.0	40.08	119.2	114.0	198.8	173.0	109.7	0.20	2.98	15.1	2.53	3.80	< 0.33	0.54
1	16	43	8.0	61.92 ± 1.9	45.60	139.5	119.2	198.1	170.6	107.9	0.30	4.22	14.0	3.73	13.80	< 0.33	5.54
2	17	40	8.1	57.60 ± 7.9	50.16	114.8	n.d.	146.6	129.5	n.d.	0.20	4.61	22.8	4.28	6.49	< 0.33	0.68
2	18	39	8.2	55.44 ± 10.3	52.80	105.0	103.3	152.2	141.8	96.0	0.17	3.54	20.4	4.25	6.00	< 0.33	0.54
2	19	39	8.3	68.64 ± 13.9	62.40	110.0	81.2	137.3	105.3	91.3	0.19	4.44	23.6	5.38	18.15	< 0.33	0.81
2	20	39	8.2	49.20 ± 5.3	46.80	105.1	105.3	144.4	118.2	92.6	0.22	4.66	21.3	4.75	15.82	< 0.33	1.35
2	21	39	7.9	54.24 ± 5.0	51.60	105.1	113.9	138.9	123.1	98.6	0.24	4.64	19.4	3.94	7.66	< 0.33	1.08
2	22	39	7.9	35.52 ± 1.2	32.40	109.6	117.3	156.2	124.8	92.0	0.19	4.35	22.4	4.35	12.49	0.57	2.03
2	23	41	7.9	37.20 ± 1.4	27.84	133.6	112.1	149.9	139.3	94.3	0.19	4.40	23.0	3.82	6.33	0.45	0.54
2	24	43	8.1	60.96 ± 3.8	58.56	104.1	118.3	162	146.8	100.3	0.21	5.33	25.3	5.32	11.32	< 0.33	1.22
2	25	40	8.2	51.60 ± 2.2	39.36	131.1	119.5	156.7	138.9	87.0	0.22	6.00	27.4	5.58	9.66	< 0.33	1.49
2	26	45	8.3	37.68 ± 0.7	32.64	115.4	100.9	157.7	147.8	94.8	0.21	5.35	25.4	6.02	9.99	< 0.33	0.68

Cluster	Biogas Plant	temperature [°C]	pH-value	CH4 production [µmol h^{-1} d^{-1}] measured	CH4 production [µmol h^{-1} d^{-1}] calculated[a]	CH4 production [%]	BCON[b] [%]	ECON[c] [%]	ACON[d] [%]	PCON[e] [%]	VFA/ TAC [g HAceq $g^{-1}CaCO_3$]	VFA[f] [g HAceq]	TAC[g] [g $CaCO_3$]	NH_4^+-N[h] [g L^{-1}]	acetic acid[i] [mM]	butyric acid[j] [mM]	propionic acid[k] [mM]
3	27	40	8.1	29.52 ± 2.2	44.64	66.1	202	313.0	241.3	143.7	0.18	4.24	23.9	4.54	4.00	< 0.33	0.41
3	28	47	7.9	69.60 ± 5.3	86.40	80.6	224.5	247.6	203.5	161.4	0.24	2.48	10.3	1.49	7.66	< 0.33	0.95
3	29	40	7.7	51.82 ± 5.5	69.12	75.0	187.8	280.7	226.8	153.6	0.21	2.61	12.5	1.76	4.50	< 0.33	0.54
3	30	43	7.8	34.08 ± 5.5	37.68	90.4	260.2	341.3	257.6	167.8	0.20	2.65	13.5	2.79	3.83	< 0.33	0.41
3	31	40	7.8	41.04 ± 3.4	46.80	87.7	300.5	342.9	248.4	267.1	0.21	3.56	16.8	2.74	2.00	< 0.33	0.54
3	32	42	7.7	49.20 ± 1.0	94.56	52.0	245.8	278.3	221.5	172.7	0.21	2.49	11.6	1.89	2.83	< 0.33	0.54
3	33	40	7.9	61.44 ± 10.8	72.48	84.8	242.9	253.6	193.5	201.8	0.24	2.88	12.2	1.76	3.66	< 0.33	0.81
4	34	47	7.7	102.72 ± 6.0	80.16	128.1	217.9	228.8	202.6	142.0	0.25	2.50	10.0	1.93	7.83	< 0.33	0.95
4	35	40	7.8	64.56 ± 9.6	47.04	137.2	216.3	253.0	188.2	166.2	0.18	2.89	15.7	2.69	3.00	< 0.33	< 0.41
4	36	n.d.	n.d.	30.96 ± 2.2	n.d.	n.d.	186.8	193.9	168.0	120.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4	37	43	8.2	59.28 ± 7.4	58.80	100.8	158.8	207.1	164.1	124.8	0.20	5.12	25.4	4.99	14.65	< 0.33	1.22
4	38	43	7.3	74.40 ± 1.0	66.48	111.9	253	232.0	193.6	161.3	0.26	1.96	7.4	0.92	3.16	< 0.33	0.54
4	39	42	7.8	20.16 ± 15.4	19.92	101.2	154	240.8	169.7	132.1	0.25	2.84	11.5	1.95	5.50	< 0.33	0.41
4	40	38	8.0	52.56 ± 0.2	52.32	100.5	151.1	224.6	187.7	123.0	0.19	4.34	22.6	3.69	6.16	< 0.33	0.54
4	41	40	8.0	18.72 ± 2.2	n.d.	n.d.	245.6	219.0	177.4	136.7	0.23	3.85	16.9	2.76	3.50	< 0.33	0.54
4	42	42	7.4	82.80 ± 8.6	74.16	111.7	199.8	215.3	169.7	126.8	0.23	2.05	8.8	1.11	2.33	< 0.33	0.54
5	43	41	8.0	25.52 ± 2.4	45.36	51.9	136.3	204.4	111.6	115.7	0.29	3.71	12.9	2.27	42.80	< 0.33	0.53
unclassified	44	42	7.5	27.84 ± 1.2	43.92	63.4	109.9	144.6	110.4	96.5	0.28	3.72	13.1	2.01	23.31	< 0.33	1.89
unclassified	45	43	8.0	67.68 ± 1.7	91.68	73.8	108.9	159.2	150.1	101.2	0.29	4.52	15.6	2.36	13.32	0.45	2.16
unclassified	46	41	7.7	28.08 ± 4.3	39.84	70.5	99.1	118.7	120.4	94.0	0.24	2.92	12.1	2.09	11.32	< 0.33	0.81
unclassified	47	45	7.9	95.28 ± 23.5	118.80	80.2	122.3	150.3	121.9	97.0	0.30	4.90	16.1	2.58	14.20	< 0.33	0.81
unclassified	48	41	8.0	57.36 ± 3.1	103.68	55.3	135.8	192.6	150.7	104.7	0.21	4.63	21.8	5.13	13.49	< 0.33	1.35
unclassified	49	44	7.8	14.64 ± 0.7	48.96	29.9	115.5	298.0	222.4	106.3	0.70	10.40	14.8	3.36	5.33	< 0.33	125.73
unclassified	50	48	8.1	4.8 ± 4.6	47.76	8.5	89.4	813.1	453.5	257.9	0.54	9.19	17.0	3.38	13.66	< 0.33	83.86
unclassified	51	50	7.6	117.12 ± 1.4	234.72	49.9	120.7	128.1	130.9	89.3	0.84	8.52	10.2	2.32	20.15	< 0.33	41.05
unclassified	52	44	7.8	37.92 ± 1.7	38.64	98.1	106	149.2	110.1	105.5	0.73	8.45	11.6	2.73	70.77	1.59	19.99
unclassified	53	40	7.8	80.64 ± 9.4	53.04	152.0	108.7	138.3	97.5	113.9	0.23	3.45	14.8	2.29	12.49	< 0.33	0.95

a) The amount of the substrate fed into the biogas plants and specific data for gas- and CH_4 yields of the used substrates were used to calculate the maximum CH_4 yield [53].

b)-e) CH_4 production of biogas sludge supplemented with butyrate (BCON), ethanol (ECON), acetate (ACON), propionate (PCON) [50 mM].

f)-g) The volatile fatty acid content (VFA) and the carbonate buffer capacity (TAC) were analyzed according to Nordmann [49].

h) The NH_4-N concentration was measured by an ion-sensitive electrode [60].

i)-k) Acid concentrations were determined by gas chromatography as described by Refai et al. [54].

Table S2: Process parameters of lab-scale reactors

Phase	1	2 (early)	2 (late)	3	4
	No stress conditions	Induction of NH_4^+-N stress	Induction of NH_4^+-N stress	Reduction of CH_4 yield	Recovery
Day	5	8	13	15	35
NH_4^+-N (%)*	100	120	183	185	143
CH_4 yield (%)*	100	100	100	70	89
ECON (%)*	100	62	35	28	48
ACON (%)*	100	50	40	10	44
VFA (%)*	100	110	150	140	110
TAC (%)*	100	110	130	135	130

*all parameters of the stress fermenters were compared to parameters of control fermenters which were set to 100%

IV. Summary

Biomass from energy crops and organic waste is one of the most important renewable energy sources and can be used for the production of biogas. Biogas formation is based on the fermentation of organic matter, e.g. energy crops and different types of manure, and is performed in biogas plants that generate electric power and heat. Many groups of microorganisms are involved in the anaerobic degradation of organic material and in the formation of biogas, which proceeds in the four interdependent steps of hydrolysis, acidogenesis, acetogenesis and methanogenesis. Thus, the synthesis of biogas depends on a variety of microorganisms and includes a huge number of biochemical reactions. However, the weakest link of the microbial anaerobic degradation chain determines the performance and the speed of the overall system. This bottle neck has to be determined to specifically optimize the biogas production process.

So far, it is only possible to analyze the overall process performance by analytical monitoring parameters, e.g. biogas formation, VFA concentration, pH, and buffer capacity. Nevertheless, the biochemical bottlenecks are not identified yet and a differential analysis of metabolic activities of the microorganism involved in biogas formation is not possible but this analysis is crucial to identify the rate-limiting step.

Therefore, the aims of this thesis focused on the development of monitoring strategies for the quantification of metabolic capacities of the microorganisms involved in anaerobic degradation of organic matter to identify biochemical bottlenecks and to improve the efficiency of biogas production.

1) In the **first chapter** an enzymatic test system is presented for the quantification of methanogenic archaea, the most important microbial group in biogas formation. The analysis of their metabolic activity was based on the heterodisulfide reductase, a key enzyme in all methanogenic pathways. Using a rapid enzymatic test system, the activity of the heterodisulfide reductase was detected in cell free extract prepared from biogas sludge. These activity measurements enabled the specific quantification of all methanogenic archaea involved in the anaerobic degradation process. In the second test system cell lysates obtained from biogas sludge were separated by ultracentrifugation. Cytoplasmic membranes and cytoplasmic fractions revealed heterodisulfide reductase activity, respectively, indicating the presence of hydrogenotrophic and aceticlastic methanogens. The different localization of the heterodisulfide reductase within these groups of organisms allowed to quantify the metabolic activity of both groups of methanogenic archaea. In hydrogenotrophic methanogens the heterodisulfide reductase is restricted to the cytoplasm of the cells and in aceticlastic methanogens the enzyme is localized in the cytoplasmic membrane. It became evident that one third of the total Hdr activity was found in the membrane fraction representing aceticlastic methanogens. The cytoplasmic fraction contained two third of the total Hdr activity that derived from hydrogenotrophic methanogens.

2) **Chapter 2** comprises the results on the analysis of the metabolic activity of all microbial degradation steps in biogas sludge to shed light on the question which group of organism

constitutes the bottleneck in the anaerobic breakdown of organic material. Biogas sludge was incubated and analyzed in anaerobic small-scale batch reactors within 24 h. These batch reactors mirrored the conditions and the performance of the full-scale biogas plant. The stability of the process was analyzed by analytical parameters such as CH_4 production, VFA concentrations, and pH. CH_4 production was examined after supplementation of biogas sludge with substrates for syntrophic bacteria (butyrate, propionate, or ethanol) as well as with acetate and H_2+CO_2 as substrates for methanogenic archaea. A significant increase of CH_4 formation was measured when sludge from different biogas plants was supplemented with acetate or ethanol. In contrast, other fermentation products such as propionate, butyrate, or H_2 resulted in only slightly increased CH_4 yields. These results led to the conclusion that aceticlastic methanogenesis and syntrophic ethanol-oxidation did not constitute the biochemical bottleneck during biogas formation, respectively.

3) Increasing CH_4 formation caused by ethanol addition was analyzed in small-scale continuous reactors filled with biogas sludge to determine the influence of ethanol on CH_4 production over longer time periods (**Chapter 3**). These reactors reflected all conditions found in full-scale biogas plants. Pulsed ethanol supplementation and continuous addition of ethanol to the reactors led to significantly increased biomethanation. The basic CH_4 production continued to take place in the small-scale reactors. However, ethanol supplementation led to the production of additional CH_4 in the biogas sludge because an increase in organic loading rate was achieved that did not influence the normal fermentation processes. Furthermore, it was also possible to increase CH_4 formation by daily addition of ethanolic solutions (e.g. beer) to the biogas sludge. The biogas formation increased directly after the addition of ethanol or ethanolic solutions. Thus, an adjustment of CH_4 production to fluctuant power demands is possible. According to that, the addition of ethanol to biogas plants can be used to ensure power supply in times of daily or seasonal peak loads.

4) The study presented in **Chapter 4** allows to quantify the metabolic performance of microorganisms involved in different digestion levels in biogas plants. A test system referred to as BEAP profile was developed which is based on the addition of intermediate substrates of prokaryotes involved in the different digestion levels. Supplementation of biogas sludge samples with butyrate (BCON), ethanol (ECON), acetate (ACON) or propionate (PCON) and subsequently the analysis of CH_4 formation in comparison to control samples without supplementation enabled to characterize the performance of the degradation process by rapid testing of metabolic activities of the microorganisms involved in biogas formation. Furthermore, a differentiation between specific BEAP profiles was possible for standard biogas plants and for biogas reactors with process incidents (beginning of NH_4^+-N intoxication, start of acidification, insufficient hydrolysis and potential mycotoxin effects). Moreover, at the beginning of NH_4^+-N intoxication the BEAP profiles function as an early warning system to predict critical NH_4^+-N concentration thresholds leading to a drop of CH_4 formation in commercial agricultural biogas plants.

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VI. List of Publications

- **Refai S., Berger S., Wassmann K., Deppenmeier U.** (2014a) Quantification of methanogenic heterodisulfide reductase activity in biogas sludge. *J Biotechnol* **180**: 66-69.

- **Refai S., Wassmann K., Deppenmeier U.** (2014b) Short-term effect of acetate and ethanol on methane formation in biogas sludge. *Appl Microbiol Biotechnol* **98**: 7271-7280.

- **Refai S., Wassmann K., van Helmont S., Berger S., Deppenmeier U.** (2014c) Increase of methane formation by ethanol addition during continuous fermentation of biogas sludge. *J Ind Microbiol Biotechnol* **41**: 1763-1772.

- **Refai S., Berger S., Wassmann K., Hecht M., Dickhaus T., Deppenmeier U.** (2017) BEAP profiles as rapid test system for status analysis and early detection of process incidents in biogas plants. *J Ind Microbiol Biotechnol*, accepted.

- **Fischer M. A., Neulinger S. C., Wassmann K., Refai S., Künzel S., Deppenmeier U., Schmitz R. A.** (2017) High ammonia concentration significantly affecting microbial community composition and transcription patters in biogas reactors. *Biotechnol Biofuels*, to be submitted

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