Chapter

Microbial Responses to Different Operating Practices for Biogas Production Systems

Maria Westerholm and Anna Schnürer

Abstract

Biogas production requires a number of different microbial groups that work in a synchronized and closely interacting manner. For bioreactors constructed to maximize waste treatment and energy production, it is crucial to manage this process in a way that secures the growth and activity of these microorganisms, as otherwise there is a great risk of process failure. However, the microbiome has a remarkable ability to adapt to various conditions related to substrate composition and operating conditions, thus showing high functional redundancy and robustness. In order to optimize and steer the process, it is important to have an understanding of the anaerobic microbiome, how it responds to various conditions, and its upper limits. This chapter reviews current knowledge regarding microbial responses to different operational management strategies. Microbial responses under various conditions and how the process can be operated to maintain the activity of key species are addressed. Parameters discussed include for example substrate composition, pretreatment, ammonia level, temperature and organic load.

Keywords: anaerobic degradation, microbiology, taxonomy, start-up, temperature, substrate composition, feeding, additives, bioaugmentation

1. Introduction

As the world's population continues to grow, it is necessary to find ways to develop resourceful waste treatment methods while concurrently reducing the dependency on fossil fuels. In this regard, biogas produced through anaerobic degradation (AD) is highly interesting, as it can replace fossil fuels in power and heat production, be used as feedstock for production of biochemicals, or be converted to vehicle fuel [1]. The biogas technology also enables resource sustainability when the digestion residue (digestate) is used as organic fertilizer to replace fossil energy-requiring mineral fertilizers [2].

Anaerobic digestion of organic material to biogas is a complex microbiological process requiring the combined activity of several groups of microorganisms with different metabolic capacities and growth requirements. To obtain a stable and efficient biogas process, it is important to meet the growth requirements of all microorganisms involved. The substrate is one critical parameter in this regard, contributing growth factors and macro- and micronutrients. Some organic materials can be used as the sole substrate, while others have to be co-digested with

substrates that are complementary in composition in order to provide favorable conditions for microbial growth [3]. However, addition of additives such as iron, trace metals, or buffering chemicals may be essential in certain processes in order to ensure sufficient microbial activity and to prevent process collapse [4]. In addition to the nutrient composition, operating parameters such as pretreatment method, load of input material, retention time, process temperature, and stirring are of critical importance. All these parameters have to be set at appropriate levels in order to ensure high activity and gas yield with minimized risk of inhibition or washout of critical functions and microorganisms [5–9]. Thus, many different aspects need to be taken into consideration to achieve optimal microbial activity giving a high degree of degradation and gas production. It should be borne in mind that many operating and biological parameters are interlinked, sometimes with counteracting effects.

2. Characteristics of substrates used for biogas production

The composition of substrates can vary considerably between anaerobic digesters, which bring different challenges depending on the feed characteristics combined with the parameters chosen for the specific system. For example, substrates rich in protein and fat have a high energy content and thus a high methane potential, but can sometimes cause process disturbances due to formation of inhibitory compounds or foaming [10–12]. Other materials posing a lower risk of process disturbance, such as lignocellulosic materials, can require an unfeasibly long time for degradation. In order to explain the prerequisites for microbial degradation and the challenges that exist, this section briefly describes the main characteristics of common substrates for biogas production. This provides background for a detailed description of the microbial degradation process and the responses to changes in operating parameters.

Plant-based materials, such as fruit, grains, vegetables, and root crops, are typically rich in different polysaccharides. Polysaccharides are chains of sugars linked in linear chains (cellulose and starch) or branched chains (hemicellulose, pectin, and glycogen). In the plant cell wall, hemicellulose, cellulose, and lignin are associated in the form of lignocellulose [13]. Simple polysaccharides such as starch and glycogen are easily cleaved by microorganisms into glucose units. Hemicellulose and cellulose are also relatively easily degraded but, when combined with lignin (i.e., lignocellulose) as in plants, the structure becomes relatively persistent to microbial degradation [14, 15]. Lignocellulosic materials such as straw (wheat, rice, corn, barley) and sugarcane bagasse are the most abundant renewable biomass and have high potential to contribute to expansion of worldwide biogas production [13, 16].

Protein-rich materials for biogas production include waste from animal rearing (slaughterhouse, dairy, animal manure, aquaculture sludge), ethanol fermentation (distiller's waste), food industry, and households [10, 17–21]. Proteins consist of long chains of amino acids joined by peptide (or amide) bonds and there are 20 different amino acids of various lengths. A feature of all amino acids is that they have at least one amine group ($-NH_2$). The efficiency of protein degradation depends on the structure of these compounds and their solubility [22].

Slaughterhouse waste, food waste, and grease-separation sludge are materials with a high fat content [23–25]. Fat molecules are of different lengths (saturated or unsaturated) and are hydrolyzed to long-chain fatty acids (LCFA, >12 carbon atoms) and glycerol [26]. Lipids are normally rapidly degraded in AD, whereas the conversion of LCFA can represent a rate-limiting step [27, 28].

3. The microbial degradation steps leading to methane

The microbial process comprises the main degradation steps hydrolysis, acidogenesis, acetogenesis, and methanogenesis (**Figure 1**) and this process has to be efficient and balanced in order to obtain successful anaerobic digestion. The initial step is performed by hydrolytic bacteria, and possibly also fungi, that convert polymers (polysaccharides, lipids, proteins, etc.) into soluble monomers (LCFA, glycerol, amino acids, sugars, etc.) [29, 30]. The hydrolytic reaction is mediated by extracellular enzymes secreted by bacteria to the bulk solution and/or attached to their cell wall. Cellulose is hydrolyzed to cellobiose and glucose, while hemicelluloses are degraded to monomeric sugars and acetic acid by bacteria that often have several different enzymes combined into so-called cellulosomes situated on their cell wall [16, 31]. These cellulosomes contain proteins that have the ability to bind to cellulose, which makes the degradation more efficient because the enzymes can work directly "on-site." Fungal cellulases use a different mechanism and not only bind to the surface of the cellulose, but also to penetrate inside the complex biomass materials (e.g., plant cell walls) [32].

Through the action of extracellular enzymes (proteases), proteins are hydrolyzed into amino acids, which are subsequently degraded in the Stickland reaction or through uncoupled oxidation. In the Stickland reaction, one amino acid acts as an electron donor and the other as an electron acceptor, and the oxidation process produces a volatile carboxylic acid that is one carbon atom shorter than the original amino acid. For example, alanine with its three-carbon chain is converted to acetate [33]. Amino acids can also be fermented through uncoupled oxidation where electrons are instead released as hydrogen. This process can only occur in cooperation with a hydrogen-utilizing partner, such as methanogens, that keeps the hydrogen partial pressure low [34]. Irrespective of the degradation pathway, the amino group in the amino acid is released as ammonia and the sulfur in cysteine and methionine results in sulfide. Lipases are excreted by hydrolytic bacteria and catalyze the hydrolysis of lipids at the water-lipid interface [35], forming saturated or unsaturated LCFA and glycerol [36]. LCFAs thereafter absorb to and are transported through microbial

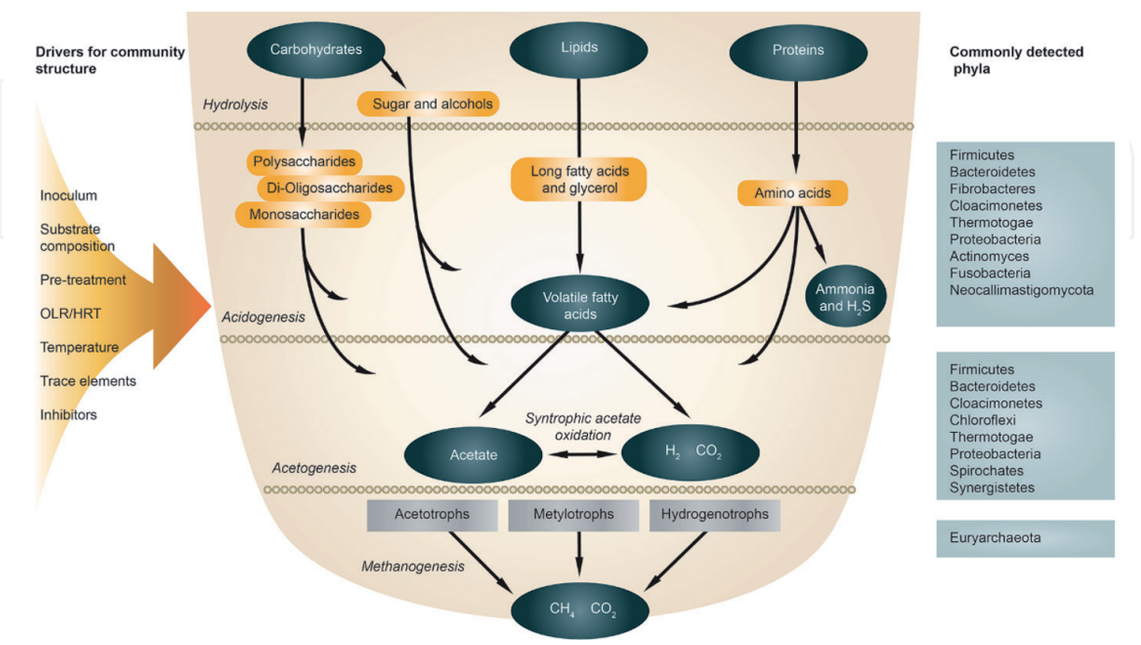

Figure 1.
Anaerobic degradation of carbohydrates, lipids, and proteins and the phyla commonly reported to be involved in the different steps. Biogas digester parameters identified as main drivers for community structure is depicted. The figure is adapted from Kougias et al. [39].

cell membranes of acetogenic bacteria, where the LCFAs are converted to acetate via beta-oxidation to acetate, carbon dioxide (CO_2), and hydrogen (H_2) [37, 38].

The soluble monomers produced in the hydrolytic and acidogenic steps are further degraded to intermediate products. These mainly comprise volatile fatty acids (e.g., acetate, propionate, butyrate, lactate, valerate, and caproate), alcohols, formate, H_2, and CO_2 [40]. During acetogenesis, the products formed in hydrolysis/acidogenesis are further converted by a group of bacteria called acetogens, generating acetate, H_2, and CO_2 as main products. During this process, various electron acceptors can be used, including CO_2, nitrate, sulfate, and protons, with the latter being most important in the biogas process [41]. Acetogens can also directly use products from hydrolysis, such as sugars and amino acids [42], or oxidize pyruvate, which is a common intermediate in anaerobic degradation reactions, to acetate [43]. For thermodynamic reasons, many reactions performed by acetogens, such as oxidation of organic acids and LCFA, can only proceed if the partial pressure of H_2 (p_{H2}) is kept low [44]. For some acids, such as propionate, the removal of acetate can also be of crucial importance [45]. The removal of the acidogenic products acetate and H_2/formate and some methylated compounds mainly proceeds through consumption by methanogens. The energetic situation for the methanogens is comparatively more favorable than acetogenesis, and thus combining these reactions allows both organisms to obtain energy for growth. This type of symbiosis, in which neither organism can operate without the other but together they exhibit metabolic activities that they could not accomplish on their own, is called syntrophy [43, 44].

In the last step, methanogenic archaea use acetate, CO_2, or methylated compounds to produce methane (CH_4) (**Figure 1**). In acetate-utilizing (aceticlastic) methanogenesis, acetate is split into a methyl group and CO_2, and the methyl group is later reduced to methane using an electron provided by the carboxyl group. CO_2 is reduced to methane by hydrogenotrophic methanogens, using H_2 or formate as primary electron donors. In methanogenesis from methylated compounds such as methanol, methylamines, and methylsulfides, the methyl group is reduced to methane. Most methylotrophic methanogens then obtain the electrons they require for reduction from oxidation of additional methyl groups to CO_2 [46, 47].

4. Microorganisms engaged in the different degradation steps

Organisms that are active during the hydrolysis of polysaccharides in biogas processes include various bacteria and anaerobic fungi [14, 29]. Cellulose and starch-degrading bacteria are found within the genera *Acetivibrio*, *Butyrivibrio*, *Caldanaerobacter*, *Caldicellulosiruptor*, *Clostridium, Eubacterium*, *Halocella*, *Ruminoclostridium* and *Ruminococcus* (phylum Firmicutes), *Bacteroides* and *Paludibacter* (phylum Bacteroidetes), *Fibrobacter* (phylum Fibrobacteres), Spirochaetes (phylum Spirochaeta), and *Fervidobacterium* and *Thermotoga* (phylum Thermotogae) [14, 48–57]. Identification of the genes necessary for degradation of cellulose has also led to the suggestion that members of the phylum Proteobacteria [56], candidate phylum Cloacimonetes [58] and Actinomyces [59] have this ability. Among the anaerobic fungi, representatives of the phylum Neocallimastigomycota, commonly also found in ruminants, have been suggested as promising candidates to improve biogas production from lignocellulosic material [60, 61]. Protein and amino acid degradation in anaerobic digesters has been shown to be performed by various genera within the phylum Firmicutes, such as *Anaeromusa*, *Anaerosphaera*, *Aminobacterium*, *Aminomonas*, *Gelria*, *Peptoniphilus*,

Thermanaerovibrio [62–67], *Clostridium* [68], *Proteiniborus* [69], and *Sporanaerobacter* [70]. However, members of the phyla Bacteroidetes (e.g., genera *Fermentimonas* and *Proteiniphilum*), Fusobacteria, and Cloacimonetes have also been suggested to have an active amino acid-based metabolism in anaerobic digesters [71, 72]. Less is known about bacteria involved in hydrolysis of fat. Lipolytic bacteria in anaerobic digesters has so far been proposed to belong to families Caldilineaceae (phylum Firmicutes), Bacteroidaceae (phylum Bacteroidetes) and to genera *Trichococcus* (phylum Firmicutes), *Devosia*, and *Psycrobacter* (phylum Proteobacteria) [73, 74].

Acetogenesis and syntrophic acid degradation are often performed by bacteria belonging to the genera *Clostridium* and *Acetobacterium* (phylum Firmicutes), but have also been assigned to the phylum Proteobacteria [14, 43, 75]. Bacteria identified so far that are capable of β-oxidizing LCFA in syntrophy with methanogens all belong to the families Syntrophomonadaceae and Syntrophaceae [23, 76]. Syntrophs that degrade short-chain fatty acids, such as butyrate, propionate, and acetate, in association with methanogens are phylogenetically distributed. Syntrophic propionate and butyrate degradation is performed by genera such as *Syntrophomonas*, *Syntrophospora*, *Syntrophothermus*, *Thermosyntropha*, and *Pelotomaculum* (phylum Firmicutes), or the genera *Syntrophus*, *Smithella*, and *Syntrophobacter* (phylum Proteobacteria) [77]. In addition, the phyla Cloacimonetes, Synergistetes, and Chloriflexi have been suggested to contain bacteria capable of performing syntrophic metabolism in association with hydrogenotrophic methanogens [78–80]. Bacteria capable of syntrophic acetate oxidation identified to date belong to the genera *Clostridium*, *Thermoacetogenium*, *Syntrophaceticus*, and *Tepidanaerobacter* (phylum Firmicutes) [81]. Novel syntrophic acetate-oxidizing bacteria (SAOB) candidates have been suggested within the order Clostridiales and/or Thermoanaerobacterales [82–86], *Synergistes* group 4 [87], the genus *Coprothermobacter* [88] and the phyla Spirochaetes [89], Thermotogae [83], Chloroflexi, and Bacteroidetes [90].

In terms of relative abundance, the methanogenic community generally represents a minor part (2–5%) of the total community, but methanogens have been observed to have high activity relative to their abundance [83, 91, 92]. Methanogens commonly detected in biogas digesters belong to the orders Methanobacteriales, Methanomicrobiales, and Methanosarcinales (phylum Euryarchaeota). However, the orders Methanococcales and Methanomassiliicoccales (phylum Euryarchaeota) have also been found in AD systems [30, 93]. Hydrogenotrophs are found within all methanogenic orders except for the Methanomassiliicoccales [93]. Acetate is only used by members of the families Methanosarcinaceae and Methanosaetaceae (order Methanosarcinales). Members of the Methanosarcinaceae are comparatively more versatile, having the ability to grow on several different substrates, such as acetate, hydrogen, and methanol, while members of the Methanosaetaceae use only acetate [94]. Methane formation from methylated compounds is performed by members of the Methanomassiliicoccales, Methanobacteriales, and Methanosarcinales [93]. A candidate methanogenic class, WSA2, has also been proposed and suggested to be restricted to methanogenesis through methylated thiol reduction [95].

With ongoing advances in molecular techniques and cultivation studies, the list of anaerobic microorganisms responsible for different degradation pathways is continually being updated. The complexity of the cooperation involved in degradation is further illustrated by the fact that members within one and the same genus are often able degrade chemically different compounds. In future, the introduction of omics approaches, combined with isolates of novel species, will most likely increase insights into the taxa involved [30, 96–99].

5. The impact of different operating conditions on AD microbial communities

To optimize the anaerobic digestion process and steer it in a desired direction, it is important to have knowledge and understanding of the metabolic capacities of key microorganisms. Knowledge of the level of functional redundancy within the community (how easily the microbial community adapts to operating changes) and microbial requirements for activity can also help identify operating management practices for improved process performance. In this section, the impact on the microbial community of different operating strategies is described.

5.1 Start-up strategies

The inoculum used for starting up a biogas process has been shown to be of importance for the degradation rate, specific methane yield, and stress tolerance, possibly depending on differences in the composition of the microbial community [52, 100–104]. In addition, chemical parameters, such as presence of trace elements needed for microbial activity, have been suggested to be important [105]. Inocula, most commonly applied in practice, can be categorized as originating from one of the following three sources: wastewater treatment plants, agricultural biogas plants, and plants treating various biowastes, such as municipal and industrial food waste [101]. Microbial analyses of biogas plants belonging to these different groups have clearly shown separation based on microbial community structure [102, 103, 106, 107]. This separation is believed to be caused by the substrate characteristics and operating conditions, with temperature and ammonia being strong regulating parameters [106]. It has been suggested that wastewater sludge is most optimal as the inoculum for biomethane potential (BMP) tests, due to its diverse and highly active community [101]. However, Koch et al. [108] found that inoculum originating from a plant degrading similar substrate to that evaluated in the BMP test gave the best results, suggesting that a substrate-adjusted microbial community is more suitable. Choosing inoculum from a well-functioning biogas process degrading similar substrate and operating under the parameters planned for the new process has also been shown to reduce the period for start-up and avoid initial instability during continuous operation [52, 100]. It has been suggested that methanogenic activity and abundance are appropriate parameters for assessing the suitability of an inoculum and for achieving high rates and yields in BMP tests, as well as for operation of a continuous biogas process [100, 109]. Another factor that can be favorable for the process is to use an inoculum with high microbial diversity, which is considered to correlate with high functional redundancy. One hypothesis to explain this is that having a large number of species provides potential for failing species to be easily replaced by other species performing similar functions, with little impact on the overall process [110].

Evaluations of different inocula during semi-continuous operation using the same substrate have been made for mesophilic processes operating with maize silage [103], a mix of manure and grass [52], cellulose [102], and a mix of waste-activated sludge and glycerol [100]. These studies have produced some contradictory results with regard to the composition of the microbial community over time. Han et al. [102] found that the inoculum source was determining for methane yield, pH, and volatile fatty acid (VFA) production using cellulose as a substrate, both during start-up and after reaching stable operation. Different steady state community patterns were also obtained in the different reactors started with different inocula. Moreover, reactors characterized by high VFA levels and low pH had comparatively

low levels of Methanosarcinales, highlighting the importance of this methanogen for efficient biogas production. In line with this, high levels of Methanosarcinales have also been shown to be important for efficient start-up and revival of a thermophilic process suffering from high acetate levels [111]. In contradiction to the results reported by Han et al. [102], a study employing three different inocula for start-up of parallel processes using a manure-grass mix as substrate found that the overall microbial community and process performance became similar in the parallel processes after three hydraulic retention times (HRT) of operation [52]. However, a clear difference in performance was seen during the initial phase after start-up in that study, with poor performance when using an inoculum from a high-ammonia process. Less efficient start-up using a high-ammonia inoculum was also seen in a study by de Vrieze et al. [100] on AD with sludge and glycerol. High-ammonia levels usually impact microbial richness and cause significant shifts in both the bacterial and methanogenic community [82, 106]. This possibly explains the less efficient start-up performance when using substrate with a comparatively low nitrogen level [100]. A negative correlation between ammonia level and cellulose degradation efficiency was also found in the abovementioned study by Liu et al. [52]. Interestingly, when processes started with different inocula and unified in performance and microbial community were supplemented with an additional substrate in that study, the processes again diverged in both performance and microbiology. These results illustrate that choice of inoculum can influence long-term performance of biogas processes [112]. Moreover, even when the same inoculum and operating parameters are used during start-up, different process performances and microbial communities can evolve [102, 113]. This illustrates that stochastic factors play an important role in the microbial community assembly in biogas reactors. It also highlights the need for further research on the impact of inoculum source and operating conditions on long-term effects and optimized performance.

5.2 Temperature

Temperature strongly affects the microbial community structure and thus also process performance and stability [5, 92, 106, 107, 114–118]. When choosing the operating temperature, other operating parameters such as substrate, feeding strategy, and presence or possible formation of inhibitory compounds should be taken into account. The temperatures normally used for digestion in industrial biogas processes are not only mesophilic (37–40°C) or thermophilic (50–55°C), but also psychrophilic (<25°C) and temperatures between mesophilic and thermophilic (41–45°C) have been shown to be achievable [57, 118–122]. Some studies investigating AD at 41–45°C have even reported higher methane production compared with the more commonly used mesophilic or thermophilic range, with associated microbial shifts [57, 119, 121]. In general, metabolic rates and biochemical processes increase with increasing temperature [115, 123, 124]. However, thermophilic conditions can also make the process more sensitive to disturbances and inhibitory compounds [115, 125] and cause less efficient degradation of some inhibitory compounds [126]. Shifts in microbial community in response to temperature change can take time and involve periods of instability. It is therefore recommended to allow the community to adapt to the temperature change by a slow increase/decrease (±1°C per day) [5, 127–130]. In order to avoid process collapse, temperature changes should be carefully monitored, both when increasing and decreasing the operating temperature. A temporary reduction in feed rate and prolonged retention time can be required in the event of disturbance during step-wise temperature changes [5]. Another important aspect to consider during AD operation is that the microbial community, specifically the methanogens, is sensitive to long-term

temperature variations. Experience from large-scale operations shows that constant temperature fluctuations should not exceed ±2–3°C in order to avoid instability [131].

One quite consistent effect of operation at thermophilic instead of mesophilic temperature is a higher level of Firmicutes compared with Bacteroidetes/Proteobacteria [5, 57, 116, 118, 121, 132–136]. A high Firmicutes to Bacteroidetes ratio in mesophilic AD has been shown to correlate positively with high methane yield [137, 138]. However, an increase in this ratio has also been suggested to decrease the richness of predicted lignocellulolytic enzymes in biogas digesters, an effect attributed to lower hydrolysis in comparison with natural anaerobic systems [139]. Whether similar correlations arise in comparisons between mesophilic and thermophilic biogas communities has yet to be determined. Another characteristic feature of thermophilic communities is a higher dominance of the phylum Thermotogae [91, 114, 117, 123, 133, 135, 136, 140–143]. Members of the Thermotogae degrade polysaccharides to ethanol, acetate, CO_2, and H_2 [72, 144], but can also be involved in degradation of alcohols to CO_2 and H_2 in syntrophic association with a hydrogen-consuming partner [72, 145].

Another aspect to consider during operation at thermophilic temperature is that ammonia inhibition occurs more quickly at higher temperature, as the equilibrium between ammonium and ammonia shifts towards the latter when the temperature rises [146]. Irrespective of temperature, methanogens performing the last step in AD are among the least tolerant to ammonia and reduced methane yield, and accumulation of fatty acids is a common consequence of microbial inhibition of this group [81]. Methanogenic community changes related to temperature, often combined with increasing ammonia levels, have been reported to include positive correlations between high temperature and enhanced relative abundance of Methanobacteriales (often *Methanothermobacter*) and/or Methanomicrobiales (often *Methanoculleus*) [5, 106, 116–118, 133, 136, 140–142, 147–150]. The shift in methanogenic community often also involves a shift in acetate degradation pathway from aceticlastic methanogenesis to syntrophic acetate oxidation (SAO) [81]. However, in AD processes that seldom reach high-ammonia levels, such as AD of wastewater sludge, instant temperature changes without associated instability have been shown to be possible [143, 151, 152].

5.3 Pretreatment

Wastes rich in lignocellulose (e.g., forestry by-products, straw) or keratinase (e.g., waste from poultry, meat, and fish industries) and wastewater sludge have significant biogas potential [15, 16, 153, 154]. However, the complex floc structures of microbial cells in sewage sludge and the recalcitrant structure of lignocellulose make hydrolysis the rate-limiting step in AD systems [16, 155, 156]. Pretreatment is a well-proven approach to improve degradation of such waste. Common pretreatment strategies comprise physical (e.g., heat/pressure, irradiation, ultrasonic), chemical (e.g., acids/bases, ozonation, oxidation), and biological (addition of fungi/bacteria/enzymes under aerobic or anaerobic conditions) methods [16, 157]. The general concept of pretreatment is that it should improve the accessibility of the material to microbial degradation by disrupting the structure, changing the biomass porosity, and reducing the particle size to enhance the surface area that can be attacked. Many studies have investigated the effect on methane yield of pretreatment of various materials and many methods have shown improved process efficiency following pretreatment [16, 157, 158]. However, fewer studies have examined the influence of pretreatment on microbial communities and relationships to the increase in methane yield, and most of the studies performed to date have been on AD of waste-activated sludge, with differing results. For example, during mesophilic AD of sewage sludge, some studies have found no

responses in the microbial community following thermophilic aerobic digestion or ultrasonic or alkaline pretreatment [159–161]. However, in other studies investigating mesophilic AD processes, ultrasonic, microwave, and electrokinetic pretreatments have all been shown to increase the relative abundance of *Clostridiales* (phylum Firmicutes) and Cloacimonetes and decrease the relative abundance of Proteobacteria [162, 163]. Moreover, in mesophilic AD of microalgae biomass, thermal pretreatment has been found to increase the relative abundance of the families Rikenellaceae (phylum Bacteriodetes) and Anaerolineaceae (phylum Chloroflexi) and decrease the relative abundance of the phylum Proteobacteria [164]. Using metatranscriptomic analysis, Xia et al. [165] found that low-frequency ultrasonic treatment of sludge during thermophilic digestion increased the hydrolytic activity of representatives of the phyla Bacteroidetes and Cloacimonetes and increased motility and chemotaxis in members of the phylum Thermotoga. Another noteworthy finding in that study was that, among the bacteria involved in cellulose degradation, members of the order Bacteroidales were more active than members of the Clostridiales. Both these groups contain well-known cellulose-degrading bacteria, but members of the Bacteroidales typically do not possess the cellulosomes often seen in Clostridiales. Xia et al. [165] concluded that low-frequency ultrasonic pretreatment allows enrichment of a community with high hydrolytic activity without attachment to its substrate.

For substrates other than sludge, Wang et al. [166] reported a weak effect on the microbial community structure during digestion of thermal pretreated distilled grain waste in thermophilic solid AD. Thermal and thermochemical pretreatment approaches are the most commonly used methods for lignocellulosic materials used for bioenergy production purposes [167]. Such methods are often efficient in breaking the carbohydrate polymers to soluble sugars and improving the accessibility of the substrate to microbial degradation, thus increasing the biogas yield. However, these pretreatments can also release inhibitors such as furfural, 5-HMF, vanillin, and other phenolic compounds [167]. Depending on concentration, these lignin-derived compounds have been found to be inhibitory to methanogen and to result in decreased hydrolytic activity, and major shifts have been shown to occur in both archaeal and bacterial populations (see reviews [167, 168]). However, adaptation and degradation of these compounds is possible and is suggested to involve members within the families Syntrophorhabdaceae and Synergistaceae, combined with hydrogenotrophic methanogens [167–169]. For optimized degradation of phenolic compounds, thermophilic pretreatment has been suggested [126].

The combined results from studies performed to date suggest that pretreatment mostly causes minor structural adjustments in the prevailing AD microbial community, but still impacts the activity. It is likely that the effect of pretreatment depends strongly on the prevailing operating conditions (e.g., substrate and temperature) and the activity of the microbial community. It can be anticipated that the response in microbial community structure is also linked to the physical effects of the pretreatment on the substrate. Thus, if the pretreatment enhances the solubilization of all components in the substrate, the impact on community structure will be lower than if the pretreatment increases the solubilization of one particular compound (i.e., proteins, carbohydrates, or lipids).

5.4 Loading rate and retention time

The hydraulic retention time (HRT) or solid retention time (SRT), i.e., the average time that the biomass is maintained in the digester, and the organic loading rate (OLR) are of great importance for the microbial community. A short HRT and a high OLR are often desirable in commercial biogas production plants, since they

allow for high-quantity waste treatment and high biogas production (if the AD can maintain efficiency). However, SRT should exceed the microbial doubling time of prevailing microorganisms, in order to avoid washout of the consortium and thus process collapse. Immobilization of microorganisms through inclusion of support material or by allowing the formation of granular sludge, flocks, or biofilms is a strategy used in high HRT systems to support and maintain organisms with lower growth rate than the solid retention time [170, 171].

The response by the microbial community to change in OLR and HRT has been shown to vary depending on operating conditions such as temperature and composition of substrate [5, 6]. The prevailing microbial community at the time of OLR/HRT change is also important for the overall response [172]. Moreover, the feeding approach, i.e., continuous or discontinuous feeding, can be determining for community changes [173]. Changes in OLR/HRT have been shown to cause a response in most phyla dominating in AD, such as Actinobacteria, Bacteroidetes, Firmicutes, Chloroflexi, Thermotogae, Cloacimonetes, and Euryarchaeota [172–176].

In the case of increasing load, bacteria associated with hydrolytic and acidogenetic activity, such as members of the Firmicutes or Bacteroidetes, have been shown to be enriched, in parallel with accumulation of accumulation of fatty acids [172, 176–178]. Typically, acetate accumulates first and propionate accumulates if the process disturbance continues, which is assumed to be caused by limited methanogenesis and excess levels of hydrogen [5, 172, 179]. In high-solid mesophilic AD, an increase in OLR has been found to decrease the relative abundance of Firmicutes and increase that of Bacteroidetes and Candidate division WS6 [174]. During increasing OLR of protein-rich waste (blood, casein) in mesophilic AD, the order Thermoanaerobacteriales, harboring several known SAOBs (e.g., *Caldanaerobacter* and *Alkaliphilius*), has been shown to increase, while the relative abundance of *Bacillus* (Bacteroidetes) decreases [10]. In thermophilic AD of lignocellulose, decreasing the retention time from 20 to 3 days has also been shown to increase the levels of Firmicutes, while Thermotogae and Chloroflexi decrease in abundance [175]. During mesophilic AD of food waste at increasing OLR (3–7 g volatile solids L^{-1} d^{-1}) and HRT (15–20 days), a dynamic succession has been seen in different bacterial phyla (Firmicutes and Actinobacteria), while the abundance of Euryarchaeota, specifically families Methanosarcinaceae and Methanosaetaceae, increases [172].

The frequently reported increase in the genus *Methanosarcina* in response to increasing OLR has been attributed to its efficient acetate degradation capacity and robustness to stress [94]. Several studies also suggest that members of the *Methanosarcina* are important for maintained and efficient methane production under increasing OLR [172, 180]. However, members of the Methanobacteria, Methanomicrobiales, and/or Methanomassiliicoccaceae have also been observed in certain processes with a high load, depending on prevailing conditions [5, 120, 176, 179–181]. During loading by pulsed feeding, the hydrogenotrophic Methanomicrobiales have been shown to increase, favoring the consumption of propionate, most likely through hydrogen utilization. These methanogens have also been detected in high-ammonia processes operating at high OLR [179]. Ferm et al. [182] and Xu et al. [172] suggest that acetate-utilizing methanogens are critical for efficient methane production during stable performance at increasing OLR. However, with "overload" and acidification, hydrogenotrophic methanogens, such as representatives of the orders Methanomicrobiales and Methanobacteriales, become more important and dominant.

5.5 Changes in substrate composition and feeding strategies

Substrate composition is another parameter that strongly impacts the microbial community. It is well-known that co-digestion of different materials often achieves a more balanced nutrient level and improves the process performance and biogas yield

[3, 183, 184]. However, the substrate availability for a commercial biogas plant may not always be optimal and the availability can also change over time. When changing substrate composition or choosing a substrate for a new AD process, the estimated energy yield and the nutrient value of the digestate generated have to be balanced against possible problems associated with different substrates, such as ammonia inhibition, acidification, and foaming. This section reviews the microbial communities commonly observed in processes fed with protein-, carbohydrate- or fat-rich material and the microbial responses to operating challenges that often occur in these processes.

5.5.1 Protein-rich substrate

Proteins are energy-rich and contribute nutrients to the digestate, but a possible effect of ammonia inhibition has to be considered in the processing. Ammonia (NH_3) and ammonium (NH_4^+) are formed by the microbial degradation of proteins and in particularly the unionized NH_3 is toxic to microorganisms [185]. NH_3 and NH_4^+ exist in equilibrium and higher temperature and pH shift the ratio toward a higher level of ammonia. Thus, in addition to the nitrogen content, temperature and pH should be taken into account in prediction of inhibition following a change in substrate composition [186]. The aceticlastic methanogens (*Methanosaeta* sp. and certain *Methanosarcina* sp.) are considered to be most sensitive to ammonia, but if an ammonia-tolerant community is allowed to persist in the digester, the process can cope with substantially higher ammonia levels than an unadapted process [19]. An ammonia-tolerant community often includes methane formation from acetate via SAO [120, 187–193]. In SAO, acetate-oxidizing bacteria and hydrogenotrophic methanogens work in a syntrophic manner to generate methane. Bacteria species currently known to be capable of SAO belong to the genera *Thermacetogenium* [194], *Pseudothermotoga* [145, 195], *Tepidanaerobacter acetatoxydans* [196], *Clostridium* [197], and *Syntrophaceticus* [198]. Methanogenic partners in SAO are suggested to be members of the hydrogenotrophic Methanobacteriales and Methanomicrobiales (often the genus *Methanoculleus*) [81]. *Methanosarcina* is moderately ammonia-tolerant and can use both the hydrogenotrophic and aceticlastic pathways for methane formation, and can thus possibly act as a hydrogen scavenger in SAO [81, 94] or mediate the entire process, i.e., both acetate oxidation and subsequent methanogenesis [199, 200]. An increased level of protein can also affect degradation steps other than the syntrophic and methanogenic steps. For example, an increased level of protein in AD of food waste has been demonstrated to increase the abundance of the families Porphyromonadaceae, Actinomytaceae, Lactobacillaceae, and Caldicoprobacteraceae, suggesting their direct or indirect involvement in protein hydrolysis [82]. In AD of animal manure, higher protein content has been shown to increase the genera *Desulfotomaculum* and *Eubacterium* [82, 201]. High levels of ammonia have also been shown to be negatively correlated with degradation of cellulose and with some potential cellulose degraders [112].

5.5.2 Carbohydrate-rich substrate

Carbohydrate-rich materials are difficult to use in mono-digestion for biogas, since the C/N ratio becomes too high for microbial activity. Carbohydrates are thus typically co-digested with more nitrogen-rich materials. However, complex carbohydrates can pose additional challenges, such as low degradability of lignocellulosic materials, while easily accessible carbohydrates undergo fast acidogenesis that can cause acidification [202, 203]. Animal manure and sludge are commonly used in co-digestion with straw (corn, rice, tobacco, wheat) and in these processes the two orders Clostridiales (phylum Firmicutes) and Bacteroidales (phylum Bacteroidetes) often dominate. However, the phyla Proteobacter, Chloroflexi, and Fibrobacteres also often increase in response

to addition of lignocellulosic materials, with some variation depending on co-digestion material and prevailing environmental conditions [118, 202, 204–208]. The microbial community structure in AD of rice straw has been shown to be influenced by temperature, with a higher ratio of Firmicutes to Bacteroidetes being reported at higher temperature [208]. In mesophilic AD of rice straw, Bacteroidetes is reported to be the most prevalent group and the abundance is not influenced by increased OLR, whereas the second most abundant Firmicutes decreases slightly [209]. Metagenomic studies have confirmed the involvement of the phyla Proteobacteria, Firmicutes, Chloroflexi, and Bacteroidetes, but also Actinomycetes, in the degradation of lignocellulose by demonstrating the existence of CAZymes (Carbohydrate-Active Enzymes) in consortia adapted to lignocellulosic materials [59, 202].

Interestingly, similar community profiles as described above are often seen in AD of material containing comparatively high levels of easily accessible carbohydrates. For example, in co-digestion of fruit and vegetable waste with pig manure, the phyla Firmicutes, Bacteroidetes, Chloroflexi, Proteobacteria, and Actinobacteria have been found to dominate, but the numbers of Firmicutes decrease when the fraction of fruit and vegetable waste (with the highest levels of carbohydrates) decreases [210]. In mesophilic AD of potato and cabbage waste (alone or in combination), members of the phyla Spirochaete, Bacteroidetes, Firmicutes, and Proteobacteria vary in numbers depending on the substrate combination [203]. In a study examining addition of cellulose and xylan to wastewater sludge, it was found that this increased the relative abundance of the bacterial genus *Clostridium* (phylum Firmicutes), whereas the levels of the bacterial phyla Thermotogae and Bacteroidetes decreased [211]. In thermophilic AD of cattle manure involving addition of easily degraded carbohydrates in the form of glucose, the genus *Lactobacillus* (class Bacilli) has been shown to increase [201]. The methanogenic communities identified in various studies on carbohydrate-rich material show diverging structures and appear to be primarily shaped by the co-substrate and prevailing environmental conditions. For example, during straw co-digestion with cow manure or digestion of straw alone, *Methanosarcina* or *Methanosaeta* often dominate [204–207, 208, 209]. However, with increasing nitrogen level, temperature, OLR, and/or carbohydrate accessibility, the contribution of hydogenotropic methanogenesis increases, involving *Methanoculleus, Methanothermobacter*, and *Methanobacterium* [201–203, 208, 209].

5.5.3 Lipid-rich substrate

Lipids are energy-rich and different fat-rich substrates are often used to boost biogas production from sewage and manure [212–215]. Degradation of fat results in glycerol and LCFA, with the latter being a known microbial inhibitor [23]. The bacteria *Syntrophomonas* (family Syntrophomonadaceae) is commonly enriched in mesophilic co-digestion of lipid-rich materials [216–224] and even represents as much as 30–40% of the total bacterial community during degradation of LCFA [218, 225]. Moreover, it has been reported [218] that pulse feeding of oleate, instead of continuous feeding of oleate, increases the conversion rates of oleate and acetate and induces greater metabolic flexibility within the LCFA-degrading community dominated by *Syntrophomonas* population [76]. In thermophilic degradation of animal manure, addition of oleate has been shown to increase the relative abundance of the glycerol- and inositol-fermenting *Megamonas* (phylum Firmicutes) [201], whereas in mesophilic AD increased levels of glycerol/glycerin enrich the phyla Cloacamonas [226] and Thermotogae in AD of wastewater sludge [227] and the genus *Trichococcus* and family Syntrophomonadaceae in AD of brewery wastewater [228]. *Methanoculleus*, *Methanobacterium*, and *Methanospirillum* have been proposed as important hydrogen-utilizing partners for LCFA-degrading bacteria, whereas *Methanosarcina* has been suggested to act both

as a hydrogen and acetate consumer [216, 229, 230]. However, in pulse feeding of oleate, *Methanosaeta* increases in importance relative to *Methanosarcina*, along with higher abundance of *Methanoculleus* compared with *Methanobacterium*. This was suggested by the authors to be a consequence of higher acetate affinity and tolerance for LCFA by *Methanosaeta* and higher affinity for hydrogen by *Methanoculleu*s [218]. In another study, an increased level of the hydrogenotrophic *Methanoculleus* and *Methanobrevibacter* was linked to increased methane production from oleate, driven by enhanced concentration of sulfide [224]. In addition to aceticlastic methanogensis, acetate degradation has also been shown to proceed via syntrophic acetate oxidation during LCFA conversion, which is likely linked to high-ammonia level [216].

5.6 Addition of trace elements

Trace element deficiency can severely limit microbial activity and cause accumulation of fatty acids, process instability and decreased methane yield from food waste [21, 120, 231, 232], slaughterhouse waste [233, 234], crop material [235], stillage [236], and animal manure, when used as a single substrate or as co-substrate [237, 238]. In this regard, it is important to consider the level of sulfide, which is primarily formed through protein degradation. Sulfide forms complexes with metals, which decreases the bioavailability of trace elements essential for microbial activity [239–241]. In addition, temperature has been suggested to impact nutrient bioavailability and nutrient requirements [242, 243]. However, the actual impact of different temperatures on the availability of trace metals has yet to be established.

The trace elements such as cobalt, nickel, iron, molybdenum, and tungsten are essential trace elements, especially for acetogenic and methanogenic microorganisms [244–246]. So far, mainly methanogenic abundance has been shown to be influenced by trace element addition in AD, while less is known about the response in the bacterial community. Thus, it is not clear whether the improved degradation of LCFA and VFA with trace element addition is caused solely by improved activity of methanogens or also improved activity of the syntrophic community. Trace elements have demonstrated to have a pronounced effect on the methanogenic community, including increased abundance or predicted stimulatory effects on the genus *Methanoculleus* [120, 247] and increased abundance of the order Methanosarcinales [200] and the genus *Methanobrevibacter* (order Methanobacteriales), all in mesophilic AD [247]. *Methanoculleus* has also been proposed to have a more efficient strategy than *Methanosarcina* for stabilizing its energy balance, and thus can cope more successfully with trace element limitation [248, 249]. Interestingly, despite improved VFA conversion following trace element addition, SAO-dominated AD processes are reported to show no or decreased abundance of the known syntrophic acetate oxidizers *S. schinkii*, *T. acetatoxydanse*, and *C. ultunense* [120, 200].

5.7 Bioaugmentation

The approach of adding microorganisms to the anaerobic process is based on the belief that slow degradation is due to the absence or low abundance of efficient populations responsible for the particular degradation step. Bioaugmentation could thus shorten the time of microbial adaptation to certain environmental conditions/inhibitors and/or improve methane yield from specific substrates. Since the hydrolytic and methanogenic steps generally appear to be bottlenecks in AD systems, bioaugmentation efforts to date have most commonly been directed at enhancing these two steps. However, bioaugmentation has also been evaluated for improving the transition to psychrophilic temperature, to overcome inhibition of ammonia and reduce the time following overload [250].

For the degradation of lignocellulosic material in the biogas process, bioaugmentation with cellulose-degrading bacteria, hydrolytic enzymes, and anaerobic fungi has been suggested as a promising method to increase methane production from lignocellulosic materials [251–254]. Microorganisms that have so far shown positive results on methane yield include the cellulolytic bacteria *Clostridium cellulolyticulm*, *Acetobacteroides hydrogenigenes*, and *Caldicellulosiruptor lactoaceticus* (*Caldicellulosiruptor*) and the fungus *Piromyces rhizinflata*. A mix of cultures of different *Clostridium* sp. and different hemicellulose and cellulolytic bacteria has also been shown to produce positive results [250], while a mixed consortium with high endoglucanase activity has been found to result in increased biogas production from maize silage [255]. For addition of enzymes, investigations have shown mixed effects, ranging from no effect at all on rate or yield, to increased biogas yield only, or increased rate only (summarized in [252]). A likely explanation for the nonconclusive results from addition of enzyme/organisms is differences in the environmental conditions prevailing in the digester, such pH and ammonia level, which vary greatly depending on substrate. For example, a clear correlation between inefficient cellulose degradation and high-ammonia levels has been demonstrated [53]. The amount of added microorganisms has also been suggested to be of critical importance [250]. For enzyme addition, another possible reason behind the variation in results is that the hydrolytic enzymes investigated so far have mainly originated from nonbiogas environments and have a very short activity lifetime (<24 h) in the biogas process, which restricts the hydrolytic activity within these systems [256]. However, a study investigating the effects of addition of enzymes or microbes retrieved from a specific biogas environment has found promising results [252]. In that study, these enzymes were found to be active and stable in the environment and had a profound effect on both the biogas production rate and yield from forage ley [252]. Moreover, Azman et al. [257] have demonstrated that addition of hydrolytic enzymes to a cellulose and xylan-fed digester operating at 30°C can counteract the inhibitory effects of humic acid on hydrolysis efficiency.

The degradation of fats has been shown to be stimulated by the addition of hydrolyzing enzymes (lipases) or fat-degrading bacteria (*Syntrophomonas zehnderi* and *Clostridium lundense*) [250, 258], whereas addition of a co-culture of *Syntrophomonas zehnderi* and *Methanobacterium formicicum* is reported to have no effect in AD of fat-rich wastewater [259]. For protein, bioaugmentation with *Coprothermobacter proteolyticus* has been shown to improve hydrolysis and fermentation in waste-activated sludge [260]. Another factor to consider when attempting to improve the degradation of fat and protein is increased release of LCFA and ammonia. For example, high concentrations of lipases have been shown to inhibit the process, probably due to the release of LCFA. Moreover, LCFA and ammonia have been shown to have additive effects, so that the process becomes more severely inhibited if both are present at relatively high concentrations [205].

Previous attempts to increase the stability and activity of the methanogenic community have included addition of *Methanosarcina* sp. during start-up [111]. Moreover, bioaugmentation with syntrophic-acetate degrading co-cultures and with ammonia-tolerant *Methanoculleus bourgensis* has been tested with the aim of preventing ammonia inhibition of the process [189, 261, 262]. Test results in that case revealed that addition of syntrophic co-cultures did not facilitate a dynamic transition from aceticlastic methanogenesis to SAO, whereas addition of ammonia-tolerant *M. bourgensis* improved adaptation to gradually increased ammonia concentrations under mesophilic conditions.

6. Conclusions

Biogas production through anaerobic digestion enables recovery of renewable energy and of nutrients from various organic waste materials and is thus highly important for the transition to a more sustainable society. The performance and stability of the biodigestion process is highly dependent on an array of different microbial groups, and their networks and functions are in turn influenced by substrate characteristic and operating parameters. With recent advances in molecular techniques, knowledge about anaerobic microorganisms and their response to various operating conditions has increased tremendously. This knowledge has enabled the development of more controlled management and monitoring approaches, to ensure high process efficiency and stability. However, with increasing knowledge about the microbiology of biogas processes, it has also become evident that the microbiota involved is even more complicated and difficult to visualize than initially thought, particularly as many members within a particular genus are often able to degrade chemically very different compounds. Moreover, many organisms belong to candidate phyla or are even unknown, and remain to be isolated and characterized for full understanding of their role in the biogas system. Thus, in order to establish effective operating policies to achieve maximum biogas process performance, it is important to improve understanding about microorganisms and their functions and to further develop a predictive understanding of the interplay between microbial community structure and operating parameters and performance.

Conflict of interest

None declared.

Author details

Maria Westerholm and Anna Schnürer*
Department of Molecular Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden

*Address all correspondence to: anna.schnurer@slu.se

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