Chapter 4
Improved Methanogenic Communities for Biogas Production

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Abstract Last decade advances on methane microbial ecology in natural environments and man-made systems have introduced possibilities and challenges to biogas-producing processes. Mostly restricted to anaerobic environments, methanogens have also been detected in aerobic desertic soils, and their presence in extreme environments, such as hydrothermal vents, soda lakes, and Antarctic sediments, shows how ubiquitous and adapted they are to different environmental conditions. Most known methanogens belong to Euryarchaeota classes, producing methane from acetoclastic, hydrogenotrophic, or methylotrophic pathways. Recently discovered representatives in Thermoplasmata and Halobacteria classes, as well as in Bathyarchaeota and Vestretearchaeota, Phyla brought new insights on methanogenic diversity and their metabolic pathways. Biotechnological application of methanogens has been studied in bioreactors used for treatment of wastewater and waste. These bioreactors can be operated with acidogenesis and methanogenesis occurring in one stage or, with phase separation, acidogenesis followed by methanogenesis, with suspended and/or attached cells. Several factors have been studied to understand and optimize biogas production in bioreactors, such as temperature, organic load, and type of wastewater input. The biogas-producing communities received special attention following the development of metagenomics, metatranscriptomics, and single-cell genomic approaches. Coupled to the discovery of new methanogenic lineages, these methods revealed the complexity of microbial community structure and functions in both natural environments and

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bioreactors. However, a comprehensive view of these communities is still needed to improve current biogas-producing processes.

Keywords Biogas · Methanogenic archaea · Anaerobic digestion · High-rate anaerobic bioreactor · Biodiversity · Ecology

4.1 Methanogenesis: Ecology, Metabolism, and Diversity

Methanogenesis is one of the most ancient metabolisms on Earth, probably dating back 3.5 Ga (Liu et al. 2012). Biogenic methane production accounts up to 75% CH_4 total emissions to the atmosphere (Whalen 2005), most of it being produced by methanogenic archaea. It is estimated that global production of methane through biogenic anaerobic methanogenesis reaches 1 Gt of methane per year, being the final product of about 2% of net CO_2 fixed into biomass by photosynthesis (Thauer et al. 2008).

Due to the strict anaerobic nature of methanogenic archaea, methanogenesis is traditionally described to occur in anaerobic natural or man-made ecosystems, such as wetlands, paddy fields, tundra soils, sediments and monimolimnion of saline and freshwater bodies, marine sediments, permafrost, intestinal tract of ruminants and some insects, human body, wastewater treatment plants, landfills, hydroelectric power reservoirs, and hydrothermal vents (Conrad 2007; Liu and Whitman 2008; Martin et al. 2008; Saia et al. 2011; Boetius et al. 2015; Kallistova et al. 2017; Enzmann et al. 2018). However, occurrence of methanogens and/or methanogenesis in aerated soils have also been reported in different sites, indicating that methanogenic archaea are also ubiquitous in these soils and can be either readily activated when incubated under anoxic conditions (Angel et al. 2012), or may even be highly active, as described by Angle et al. (2017) in soils of a freshwater wetland. More recently, new aerobic methanogenic processes from heterotrophic bacteria (*Pseudomonas stutzeri*) and cyanobacteria using dissolved organic matter phosphonate and methylphosphonate as substrates have been discovered, explaining the occurrence of methane in concentrations above atmospheric equilibrium produced in high-sulfate, oxygenated surface waters (the marine methane paradox). These new findings raise new questions about biogenic methane production and open horizons for new biotechnological applications of methanogenesis (Repeta et al. 2016; Bizic-Ionescu et al. 2018). However, in this chapter, focus will be given only to anaerobic methanogenesis, which is the process used in biogas production.

Methanogenic archaea are ubiquitous, and the number of described methanogenic groups is rapidly increasing, especially with the advance of techniques for phylogenetic and genomic analysis. Until a decade ago, all known methanogens belonged to six orders of the Euryarchaeota Phylum: Methanobacteriales, Methanococcales, Methanosarcinales, Methanomicrobiales, Methanopyrales, and Methanocellales (Dworkin et al. 2006; Sakai et al. 2008). In 2012, a new order, Methanomassiliicoccales, belonging to Thermoplasmata class was revealed

(Dridi et al. 2012; Iino et al. 2013), and since then, with advances in phylogenetic and genomic analyses, knowledge on Archaea rapidly expanded. Today, two new classes in Euryarchaeota Phylum are being proposed (Methanofastidiosa and Methanonatronarchaeia) and genes encoding Mcr complex and for metabolism of methylated compounds were found in the Phyla Bathyarchaeota and Verstraetearchaeota (Spang et al. 2017). Many methanogens are mesophilic, such as *Methanosarcina*, most *Methanococcus* and *Methanobacterium*, but the record of growth in high temperatures belongs to a methanogen, *Methanopyrus kandleri*, able to grow at 122 °C, under high pressure (Takai et al. 2008). A new genera of an uncultured hydrogenotrophic methanogen have also been described in thawing permafrost (*Methanoflorens stordalenmirensis*) that has genes for utilization of hydrogen, formate, and formaldehyde (Mondav et al. 2014). Other methanogenic extremophiles include the halophilic *Methanosarcina mazei* (Enzmann et al. 2018) and the hyperthermophilic methylotrophic Methanonatronarchaeia (Sorokin et al. 2017).

Methane is the final product of the anaerobic digestion of organic matter, a multiphase process involving complex and diverse microbial communities and relying on syntrophic relations of anaerobic bacteria and fungi, protozoa, acetogenic bacteria, and methanogenic archaea (Thauer et al. 2008). Different from aerobic environments, where the high energetic yields of aerobic metabolism drive reactions preferentially to the use of oxygen as the terminal electron acceptor, anoxic habitats count on interactive metabolism to completely degrade the complex organic matter compounds and make their stored energy bioavailable. In this process, it is possible to identify *syntrophic primary degraders*, carrying out the breakdown of complex molecules into smaller compounds, and *consumers*, which remove released products of metabolism, thus helping to maintain their concentrations low enough to prevent inhibition of enzymes and to allow some reactions to keep exergonic (Morris et al. 2013). Thus, even though diversity of methanogenic communities may reach several thousand microbial species (Güllert et al. 2016) in different systems and environments, they share four main phases mediated by different microbial groups: hydrolysis, acidogenesis, acetogenesis, and methanogenesis.

At hydrolysis stage, bacteria and fungi break complex molecules, such as polysaccharides, proteins, and fats into their forming units (amino acids, saccharides, fatty acids, and alcohols). Time of hydrolysis may vary fro Santos, SP, Brazil m hours, as for carbohydrates to few days in the case of proteins and fats. Lignocellulose and lignin take longer to hydrolyze and are usually incompletely degraded through one of three mechanisms: (1) release of extracellular cellulases to act directly on polymer surfaces and absorb the products of degradation by aerobic or anaerobic fungi (e.g. the genera *Neocallimastigales*, frequently found in landfills) or bacteria (*Bacillus* and *Spirochaeta*); (2) production of cellulosomes, large multi exoenzyme complexes, performing hydrolysis associated to the membranes, as in Clostridia (a dominating class of hydrolytic bacteria in biogas fermenters), *Acetivibrio*, *Ruminococcus,* and *Fibrobacter*; and (3) production of polysaccharide utilization loci (PULs), which are prevalent in the phylum Bacteroidetes, very

common in cow rumen or in the gut of other studied herbivorous animals (Lynd et al. 2002; Deublein and Steinhauser 2008; Güllert et al. 2016). Hydrolysis contributes to lower the potential redox in bioreactors due to the consumption of oxygen by facultative anaerobic hydrolytic microorganisms and is closely related to acidogenesis, since the same microbial groups can carry out both types of reactions (Kallistova et al. 2017). Given the recalcitrance of hydrolysis substrates, it usually determines the degradation rates of the whole process and can be the limiting step in anaerobic digestion. For that reason, pretreatment of substrates may be necessary before anaerobic digestion (Amani et al. 2010; Ahmad et al. 2018).

During acidogenesis phase, facultative and strict anaerobic bacteria ferment sugars, peptides, amino acids, and other products of hydrolysis to hydrogen, carbon dioxide, short-chain volatile acids (e.g., formic, acetic, propionic, and butyric acids), and alcohols. Some fermenting bacteria are also able to metabolize phenolic, nitrogenated, and sulfurated compounds (Semrau 2011). Acidogenic activity contributes to maintaining hydrolysis products at low concentrations, thus preventing the inhibition of the hydrolases. Acidogenic communities in anaerobic treatment systems are frequently highly diverse, with a high functional redundancy, a characteristic that increases the resistance of the process to variations in environmental conditions and allows the utilization of a broad spectrum of organic substrates (De Vrieze et al. 2017). Acidogenic groups in reactors and landfills include fermenting bacteria from Clostridia class, lactobacilli, and other fermenters, such as *Enterococcus faecalis*, *Pseudoramibacter alactolyticus*, *Anaerobaculum mobile,* and *Sporanaerobacter acetigenes*.

At acetogenesis stage, VFA, alcohols, amino acids, and aromatic compounds resulting from acidogenesis are oxidized, generating hydrogen, carbon dioxide, formate, and acetate. However, several acetogenic reactions are exergonic only when partial hydrogen pressures and formate are low. For that reason, syntrophic associations between hydrogen-producing acetogenic bacteria and hydrogenotrophic methanogenic archaea are common (Semrau 2011). When methanogenesis is inhibited, syntrophic acetogenic bacteria can be induced by homoacetogenic bacteria (Wang et al. 2013). Homoacetogens produce acetate using hydrogen to reduce carbon dioxide to acetic acid via acetyl-CoA pathway (Diekert and Wohlfart 1994). Removing H_2 and CO_2 from the medium homoacetogens allows the occurrence of syntrophic acetogenesis. Examples of syntrophic acetogenic bacteria include: *Pelobacter* (alcohol oxidiser); *Syntrophobacter*, *Syntrophomonas*, *Clostridium* (fatty acid oxidisers); *Syntrophus* (benzoic acid oxidiser); *Syntrophococcus* (fructose oxidiser); *Syntrophobotulus* (glycolate oxidiser) (Garcia et al. 2000).

Syntrophic associations of acetogenic bacteria and methanogens or homoacetogens involve interspecies transfer of electrons, through different mechanisms. In mediated interspecies electron transfer (MIET), soluble chemical compounds shuttle electrons between the donator and the acceptor partners by diffusion. Most common MIET carriers in methanogenesis are hydrogen and formate. In contrast to MIET, syntrophy partners can carry out direct interspecies electron transfer (DIET) through electrically conductive pili, through electrically conductive materials, and through electron transport proteins connected with outer cell surfaces

(Morris et al. 2013; Lovley 2017). DIET and MIET are of biotechnological interest, contributing to the improvement or creation of new possibilities for the development of bioeletrochemical technologies (electromethanogenesis) (Enzmann et al. 2018). In anaerobic digestion, DIET was reported to happen in an upflow anaerobic sludge blanket reactor (UASB) treating simulated brewery waste between *Geobacter* and *Methanothrix* (former *Methanosaeta*), allowing the methanogen, known to feed only on acetate, to reduce carbon dioxide using electrons transferred from *Geobacter* by an e-pili (Rotaru et al. 2014a, b). The same behavior was observed between *Methanosarcina barkeri* and *Geobacter metallireducens*. When co-cultured, aggregates were formed, and electrons were exchanged by DIET. Co-cultures with Pilin-deficient *Geobacter* were not successful, showing that the e-pili is important for DIET, but it could be compensated by the addition of activated carbon as conductive material (Rotaru et al. 2014a). Magnetite and carbon cloth are other types of material reported to promote DIET in methanogenic bioreactors, and the presence of these materials may increase anaerobic digestion efficiency (Lovely 2017). In methanogenic rice paddy soils, *Geobacter* was found to be one of the most active bacteria, even when Fe (II) reduction was not significant. *Methanothrix* was also abundant, showing high expression of carbon dioxide reduction genes, which indicated the occurrence of DIET in the soils. A similar behavior of *Methanothrix* was observed in peat soils, suggesting that this genus may have a greater contribution to methane emissions, promoting methanogenesis not only derived from acetate but also from CO_2 reduction using DIET transferred electrons (Lovley 2017).

The final stage of anaerobic digestion is methanogenesis, performed by methanogenic archaea. Methanogens are distinguished according to the group of substrates used to produce methane: hydrogenotrophic methanogens (or obligate CO_2 reducing methanogens) produce methane from CO_2 reduction from oxidation of hydrogen or formate; acetoclastic methanogenesis, from acetate; and methylotrophic methanogenesis, using methylated compounds such as methanol, methylamines, and methyl sulfides to generate methane. For the literature about methanogenic routes and energy conservation, see Thauer et al. (2008), Costa and Leigh (2014), Kallistova et al. (2017), and Yan and Ferry (2018).

In hydrogenotrophic methanogenesis, CO_2 is reduced and activated to a formyl group covalently bonded to methanofuran (MFR), with a reduced ferredoxin (Fd_{red}) being the electron donor. The formyl group is then transferred to the tetrahydromethanopterin (H_4MPT), dehydrating and reducing to methenyl-H_4MPT and to methylene-H_4MPT and subsequently reduced to methyl-H_4MPT with reduced F_{420} ($F_{420}H_2$) as electron donor. The methyl group is then transferred to 2-mercaptoethanesulfonate coenzyme M (HS-CoM), and, finally, the methyl group is reduced to methane by methyl-coenzyme M reductase complex, present in all described methanogens so far. The resulting heterodisulfide (CoM-S-S-CoB) is then reduced with hydrogen to recycle the coenzymes (Borrel et al. 2012). Formate is used by many hydrogenotrophic methanogens instead of H_2, and some groups are also able to use alcohols (ethanol, 2-propanol) as electron donors (Enzmann et al. 2018). Electron bifurcation is used as a means of energy coupling between a high- and a

low-potential substrate (the heterodisulfide-reducing step and the initial reduction of CO_2 to formyl-MFR) (Costa and Leigh 2014). The hydrogenotrophic route is considered an ancient trait, maybe older than methylotrophic and acetoclastic ones (Liu et al. 2012), and is present in almost all groups of methanogenic archaea (Methanobacteriales, Methanococcales, Methanomicrobiales, Methanopyrales, Methanocellales, and Methanosarcinales).

Acetoclastic methanogenesis is performed by the genera *Methanosarcina* and *Methanothrix*. In this pathway, acetate is converted to acetyl coenzyme A (acetyl-CoA) at the expense of 1 ATP and then split by the CODH/acetyl-CoA synthase complex. The methyl group is incorporated into a H_4MPT (or tetrahydrosarcinapterin—H_4SPT in *Methanosarcina*) and the carbonyl group oxidized to CO_2 in order to provide electrons for the reduction of the methyl group (Costa and Leigh 2014; Enzmann et al. 2018). Acetoclastic methanogenesis is an important route in many environments, such as rice fields, freshwater ecosystems, and bioreactors, representing the most relevant fluxes of carbon to methane production in these systems (Garcia et al. 2000; Conrad 2007).

Finally, in the methylotrophic pathway, the methyl group from the methylated substrate is transferred to a corrinoid protein by a substrate-specific methyltransferase and then to HS-CoM by another methyltransferase. The resulting methyl-S-CoM is oxidized to CO_2 via the hydrogenotrophic pathway in reverse generating enough reducing equivalents to reduce three methyl-CoM to methane and also a proton-motive force. The electrons needed to reduce the methyl-S-CoM to CH_4 are donated either by hydrogen or the oxidation of another methyl-S-CoM to CO_2. (Timmers et al. 2017; Enzmann et al. 2018). The newly described methylotrophic groups *Methanomassiliicoccus*, Methanofastidiosa, Bathyarchaeota, and Verstraetearchaeota seem to produce methane by a similar but distinguished methylotrophic routes. Members of the order Methanomassiliicoccales are a hybrid of the common methanogenic groups. The pathway in this group starts with the transference of the methyl group by substrate-specific methyltransferases to 2-mercaptoethanol (HS-CoM). Methyl-CoM is then formed and reduced to methane by the methyl-CoM reductase with 7-mercaptoheptanoyl-threonine phosphate (HS-CoB) as electron donor. This reaction leads to the formation of the heterodisulfide CoM-S-S-CoB, whose reduction is still under studies. It is assumed that in the degradation of two molecules of methanol to methane, two molecules of heterodisulfide are formed. One of them is then reduced by a multienzyme complex consisting of a [NiFe] hydrogenase (Mvh) and a heterodisulfide reductase (HdrABC), with hydrogen being used as electron donor, transferring electrons to heterodisulfide and ferredoxin (Fd) in a bifurcation reaction. It is supposed that Fd_{red} is then oxidized by a membrane-bound dehydrogenase (Fpo complex), which is similar to the H^+-translocating NADH dehydrogenase from the respiratory chain of eukaryotes and many bacteria. A second heterodisulfide reductase (HdrD) then serves as electron-accepting unit and reduces the second heterodisulfide molecule. During Fd_{red} oxidation and simultaneous heterodisulfide reduction, an electrochemical gradient is settled, which is needed for ATP synthesis (Kröninger et al 2017).

Methanogenic archaea play an important role in a number of microbiomes in very different environments: freshwater and marine aquatic ecosystems, the cryosphere, hydrothermal vents, as symbionts in plants, animals, and the man, as part of biological treatment structures, as wastewater plants and landfills. Environmental parameters, biodiversity, and interactions are greatly variable in most of them, imposing challenges to the anaerobic digestion. In spite of that, Moissl-Eichinger et al. (2018) identify some important factors that tend to influence the archaeal interaction, such as energetic pressure derived from the environment, the ability in exchanging metabolites and electrons and genomic and structural adaptation capability (both for symbionts and hosts), detoxification and facilitated horizontal gene transfer, the fundamental role of syntrophy, and structural cell characteristics (formation of special cell-surface appendages, such as nanowires, cell wall, and envelope, the archaeal double membrane). In anaerobic digesters, despite the great variations between treatments and processes, profiles seem to be similar at higher taxonomic ranks (e.g., a frequent presence of Bacteroidetes and Firmicutes), indicating the occurrence of a core community taxa performing key functions throughout the phases of anaerobic digestion (Stolze et al. 2015). At the same time, the high diversity at lower taxonomic ranks allied to community redundancy seems to be the most important factor in ensuring the capacity of the reactor to overcome adverse conditions, more than resistance and resilience of the microbial community (De Vrieze et al. 2017).

4.2 Bioreactors: Biotechnological Processes for Methane Production

The anaerobic digestion is widely used in wastewater treatment for environmental protection and resource preservation since 1970s when the oil crises reduced the focus of aerobic methods redirecting efforts to energy-saving and neutral greenhouse gas emission technologies (Seghezzo et al. 1998). Nowadays, anaerobic treatment keeps on attracting the attention of engineers and decision makers due its potential of producing a useful renewable fuel, like methane (CH_4), hydrogen (H_2) (Li et al. 2018). There are many advantages in using it including simplicity, low operational costs (no nutrients and chemicals are required), low energy consumption (no aeration is needed), low sludge production, and low space requirements (Seghezzo et al. 1998; Chong et al. 2012; Mizoyan and Gross 2013; Li et al. 2018). Moreover, recalcitrant compounds can be removed using anaerobic digestion like phenol (Na et al. 2016), polychlorinated biphenyl—PCB (De Lima and Silva et al. 2018), surfactant (Delforno et al. 2014), BTEX (De Nardi et al. 2002), and antibiotics (Chatila et al. 2015). Up to date, a lot of anaerobic reactors have been built, operated, and studied. The upflow anaerobic sludge blanket (UASB), expanded granular sludge blanket (EGSB), fixed-bed reactor—the high-rate reactors are most popularly used in the world. They were designed to operate at short

hydraulic retention time (HRT) and long solid retention time (SRT) to maintain high concentration of high-activity microorganism, improving the sludge stabilization and increasing the loading capacity of the system (Von Sperling and Chernicharo 2005).

Bearing the importance and advantage of high-rate anaerobic reactor to wastewater treatment and biofuel production, this section will summarize information about the UASB, EGSB and fixed-bed operated and one-stage and two-stage anaerobic process, acidogenesis followed by methanogenesis.

4.2.1 Upflow Anaerobic Sludge Blanket (UASB) Reactor

More than 1000 upflow anaerobic sludge blankets (UASBs) are reactors installed worldwide for wastewater treatment due to the robustness, high efficiency, and simplicity to operate this high-rate anaerobic reactor (Tiwari et al. 2005). The UASB reactor is made of two important parts—a cylindrical or rectangular column and a gas–liquid–solid (GLS) separator. In the first part, there is a dense sludge bed in the bottom, in which all biological processes take place. Under certain condition, light particles will be washed out, while heavier components, such the microorganism, will retain by the GLS separator and interact with inert organic and inorganic matter aggregating in granules or flocs (Hulshoff Pol et al. 2004). Natural turbulence is caused by the upflow system and by the rising gas bubbles which provide a good transfer of substrate to the microorganisms inside the granule to be converted into biogas. The produced biogas, consisting of mainly methane (CH_4), hydrogen (H_2), and carbon dioxide (CO_2), is separated from the effluent by GLS separator (Lettinga and Hulshoff Pol 1991).

Even being designed and operated for almost 50 years, UASB has some drawbacks such as long start-up period, impure biogas (presence of hydrogen sulfide), and incomplete or insufficient removal of organic matter, pathogens, and nutrients in the final effluent, thereby failing to comply with the local standards for discharge or reuse needing a post-treatment technology (Seghezzo et al. 1998; Chong et al. 2012).

The microbial community and the abundance of microorganisms related to the methanogenesis process in UASB reactor depends on operational conditions (pH, temperature, hydraulic retention time) and substrates. Li et al. (2018) studied microbial community structure of two UASB reactors operated at 37, 45, and 50 °C using ethanol as substrate in one and glucose in other. *Methanobacterium*, *Methanosaeta*, *Methanosarcina*, and *Methanomassiliicoccus* were the dominant methanogens in all reactors. As the temperature increased from 37 to 50 °C, the abundance of *Methanobacterium* decreased and the abundance of *Methanosaeta* became higher. Furthermore, in the reactor fed with ethanol as substrate, the abundance of the *Methanosaeta* was higher than the reactor fed with glucose (from 1.37% at 45 °C to 19% at 50 °C in ethanol reactor and from 0.76 to 2.36% in glucose fed reactor). Lu et al. (2018) studied different relations of organic matter

and sulfate (COD/SO_4^{-2}) in UASB reactor and observed that decreasing COD/SO_4^{-2} ratio, the microbial community shifted. The Syntrophobacterales were substitute to Desulfovibrio, which co-worked with *Methanosaeta* while suppressing *Methanobacterium,* thereby altering starch bioconversion routes. Propionate accumulated when the abundance of Syntrophobacterales was reduced with a slight process upset. Delforno et al. (2017) observed the abundance of the acetotrophic genus Methanosaeta in the microbial composition from a full-scale UASB reactor applied to poultry slaughterhouse wastewater treatment. Genes related to the acetotrophic methanogenesis pathways were more predominant than methylotrophic and hydrogenotrophic. Moreover, these authors identified a variety of metabolic genes involved in sulfur, nitrogen, iron, and phosphorus cycles, with many genera able to act in all cycles, present at microbial community of UASB reactor (Delforno et al. 2017).

4.2.2 Expanded Granular Sludge Bed (EGSB) Bioreactor

The expanded granular sludge bed (EGSB) bioreactor was developed as a modified reactor of the traditional UASB, where a high relation between height and diameter resulted in high superficial velocity (>4 m h^{-1}) and in optimal internal mixing, eliminating dead zones observed in UASB reactor. Consequently, EGSB reactor has a better substrate–biomass contact within the treatment system, by expanding the sludge bed and intensifying hydraulic mixing (Seghezzo et al. 1998; Zhang et al. 2017).

Many researchers have studied on EGSB in such areas as flow pattern, kinetics, toxicity inhibition, and start-up and operation characteristics. Moreover, EGSB reactors have been successfully applied to treat many kinds of wastewater, such as brewery wastewater, starch wastewater, molasses alcohol slops, domestic and municipal wastewater, and so on (Seghezzo et al. 1998; Zhang et al. 2017).

Microbial diversity in EGSB reactor can be assessed using different molecular tools (PCR-DGGE, 16S rRNA high-throughput sequencing, and sequencing of the bamA gene). The richness and the abundance of microorganisms related to the methanogenesis process in EGSB reactors depend on operational conditions (pH, temperature, hydraulic retention time) and substrates. Centurion et al. (2018) observed a microbial stratification along the sludge bed, and the microbial community had high diversity and richness when 16.1 mg L^{-1} of LAS (linear alkylbenzene sulfonate) was presented in the commercial laundry wastewater. These authors observed predominance of the genera *Bellilinea*, *Syntrophus*, *Syntrophobacter*, *Cytophaga*, *Bacteroides,* and *Synergistes* for the Bacteria domain and the genera *Methanosaeta* and *Methanolinea* for the Archaea domains. These microorganisms have genetic potential for the aromatic ring cleavage under anaerobic conditions, removing surfactant from wastewater. Meng et al. (2017) operated two EGSB reactors to evaluate the effect of cefalexin (CFX) on the performance of the system and microbial community structure. The addition of CFX

caused a negative effect on the removal of organic matter, but this phenomenon was recoverable. Moreover, these authors observed high diversity of bacterial and archaea communities in the system treating CFX and they considered as a response against the toxicity substrate environment. The hydrogenotrophic methanogens were the main pathway for methane generation, and the fungi genera *Trichosporon* and *Phoma* and the bacterial genera *Gelria* and *Syntrophorhabdus* played an important role on degradation of complex organic pollution in the EGSB re*actor.*

4.2.3 Horizontal-Flow Anaerobic Immobilized Biomass (HAIB) Reactor

Anaerobic fixed-bed reactors have been searched to treat domestic sewage and industrial wastewater. The main contributing factors for this are long cellular retention times and high biomass *concentrations* (Lima et al. 2005). The configuration of horizontal-flow anaerobic immobilized biomass (HAIB) reactor was proposed by Foresti et al. (1995) as an innovative fixed-bed reactor for wastewater treatment. This reactor offers a potential alternative for full-scale application, as shown previously by the high performance of a bench-scale reactor treating paper industry effluent (Foresti et al. 1995), glucose-based substrate (Zaiat et al. 1997), and toxic substances such as phenol, benzene, toluene, ethylbenzene, xylenes, formaldehyde, and pentachlorophenol (De Nardi et al. 2002; Oliveira et al. 2004; Saia et al. 2007). In this kind of reactor, the support utilized to immobilize the biomass plays an essential function, and it is directly associated with the cellular retention time, biomass concentration, and microbial diversity. Polyurethane foam has been studied for the adhesion of anaerobic microorganisms and has shown promising results (Ribeiro et al. 2003; Saia et al. 2007). This support material provides a suitable environment for the adhesion of a mixed consortium of anaerobic microorganisms necessary for methanogenesis. For example, Saia et al. (2007) detected cells of *Methanosarcina* and *Methanosaeta* in HAIB reactor feed with PCP showing that although methanogens are not directly implicated in PCP dechlorination, they are obligate members of the consortium degrading organic matter until methane, driving the flux of electron donors to PCP dehalogenation. However, this type of reactor is randomly packed and this type of packing often causes hydrodynamic problems, such as channeling within the bioreactor or pressure drops, which occur when the bioreactor becomes clogged with accumulated biomass and/or solids from the influent (Mockaitis et al. 2014). This occurs more frequently under acidogenesis condition when the reactor is fed with domestic sewage (Lima et al. 2005), restricting its application to wastewater that contains toxic or recalcitrant compounds.

4.2.4 Fixed-Structure Bed Reactor (ABFSB) Reactor

To *overcome* the common problems of randomly packed-bed anaerobic bioreactors such as the HAIB reactor, a fixed-structure bed reactor (ABFSB) was developed by Mockaitis et al. (2014). This technology combines the advantages of immobilized cell growth, such as lower sensitivity to environmental variations (i.e., pH, temperature and OLR) and higher substrate conversion rates, with higher bed porosity, preventing the accumulation of extracellular polymeric compounds and suspended solids. The higher void index allows for designing more compact units than conventional packed-bed systems. Moreover, the ABFSB reactor requires lower energy input than second-generation sludge blanket reactors (i.e., expanded and fluidized-bed systems), as the biomass is attached throughout the entire length of the reactor, and thus, sludge expansion is eliminated (Mockaitis et al. 2014; Camiloti et al. 2014; Fuess et al. 2017). This reactor has been employed, in laboratory scale, for the treatment of vinasse (Aquino et al. 2014; Fuess et al. 2017), and wastewater containing sulfate (Camiloti et al. 2014) showing that this reactor is a suitable configuration for the development and retention of anaerobic microbiota involved directly and indirectly on methanogenesis. Camiloti et al. (2014) operated the reactor with synthetic wastewater with different COD/[SO_4^{-2}] ratios: 0.72, 1.7, 3.5, and 6.1. The ABSFB was suitable for the simultaneous organic matter and sulfate removal, especially at COD/[SO_4^{-2}] ratio of 1.7, but demonstrated a stable and efficient process in all conditions studied. Aquino et al. (2014) operated ABSFB reactor, under methanogenic condition, with increasing organic load of vinasse of 2.4; 3.8, and 5.5 g COD L^{-1} day^{-1} for 135 days. The reactor showed organic matter removal by of 89%. Clogging of bed was not observed.

As discussed in Sect. 4.1, a complex microbial community promotes hydrolytic, fermentative, and syntrophic processes in methanogenic environment, while methanogenic populations are generally responsible for the last steps of anaerobic organic matter degradation. Microbial populations that promote hydrolytic and fermentative process have environmental and physiological requirements as well as growth kinetics different from methanogens. Thus, phase separation, i.e., acidogenic bioreactor followed by methanogenic bioreactor have been searched (Ferraz et al. 2016; Fuess et al. 2017). The hydrolysis step tends to be enhanced in the acidogenic phase, and improvements in the biodegradability of wastewaters, as well as higher energy yields, should be observed in combined acidogenic–methanogenic processes (Fuess et al. 2017). This is a direct consequence of a more stable methanogenesis, arising from the ready availability of acetate either directly by the fraction of acetic acid from acidogenesis or indirectly by the prompt conversion of propionic and butyric acids to acetate by the acetogenic bacteria (Luo et al. 2011). Among the wastewaters potentially suited to two-phase systems, particular attention has to be given to sugarcane vinasse, the primary wastewater from ethanol production due to its high organic and nutritional content (Ferraz et al. 2016). Among the different configurations of reactors, ABFSB is a suitable technology due to the characteristics described above. Fuess et al. (2017) published the first report on

applying two-phase ABFSB reactor and acidogenic followed by methanogenic and acidogenic ABFSB reactor followed by methanogenic UASB rector on the treatment of vinasse. Both systems were operated under thermophilic condition with OLR increasing from 15 to 30 COD m^{-3} day^{-1}. The authors demonstrated the feasibility of applying the anaerobic process with phase separation and a structured-bed reactor, specifically as the methanogenic reactor, to the treatment of sugarcane vinasse. Global average COD removal values exceeded 80%, in association with an energetic potential of 181.5 MJ for each cubic meter of sugarcane vinasse from both hydrogen and methane when using ABSFSB reactors. However, the UASB reactor yielded severe performance losses of COD removal, leading to the accumulation of volatile fatty acids for every increase in the OLR. Molecular analyses indicated low numbers of unique operational taxonomic units for both methanogenic reactors, and five of eight identified genera *Anaerobaculum, Methanosarcina*, *Syntrophaceticus*, and *Thermodesulfovibrio* were observed in both reactors. Thus, the observed performance discrepancies likely resulted from design and operating aspects of the systems.

4.3 Application of Molecular Biology and Bioinformatics in the Improvement of Knowledge of Methanogenic Processes

Methanogenic populations play an important role in both natural and engineered environments, such as anaerobic digester bioreactors. As discussed above, complex microbial communities promote hydrolytic, fermentative, and syntrophic processes in these systems, while methanogenic populations are generally responsible for the last steps of anaerobic organic matter degradation. Although methanogenic processes are important for wastewater treatment, biogas production, and other biotechnological applications, the detailed understanding of how methanogens interact with their environment and with other organisms remains a black box for microbiologists and engineers. Despite years of efforts dedicated to understanding methanogenic processes in several systems, their complex dynamics still need further investigation.

In the last decades, molecular biology approaches (i.e., culture-independent) began clearing the path of complex microbial communities, enabling a more comprehensive view of how microbial and functional diversity takes place in different systems. Most of these investigations used genetic information of microbial populations in order to identify which species exists in the system and which metabolisms are being active along the processes.

Molecular tools used to characterize microbial communities rely upon detection and sometimes sequencing of DNA molecules extracted directly from microbial cells. These approaches have an important advantage over growing microorganisms

in culture media—since each cell has specific DNA sequences, one could detect and quantify the presence of individual microbial populations without the need of developing a culture medium. It is well known that less than 1% of the microorganisms in environmental samples could be grown in culture media (Amann et al. 1995). In other words, only a small fraction of the cells visible in a microscope could really grow under laboratory conditions, leaving a huge portion of the microbial community mostly unexplored. This phenomenon, known as "the great plate count anomaly" (Staley and Konopka 1985), emerges from the fact that we do not know the specific nutritional demands for each microbial species. Therefore, molecular methods, such as DNA sequencing, could overcome this problem essentially because every cell has a specific DNA that could be detected and identified on a sample.

In general, molecular methods are able to capture the "big picture" of a microbial community in a given time. Most of them rely on the amplification of specific DNA markers such as the 16S rRNA gene, which is considered a gold standard for identification of Bacteria and Archaea in the environment. Since each microorganism has a specific 16S rRNA gene sequence, it is possible to acquire a broad view of how the microbial community is structured—which species exists in the system and how abundant each species is in comparison with each other.

A phylogenetic marker is a DNA sequence that is specific to a group of microorganisms and could be used to detect the presence of this group in a sample. As mentioned above, the 16S rRNA gene is the mostly used phylogenetic marker for the detection of bacterial and archaeal species (Amann et al. 1995). This gene has about 1500 nucleotides and encodes the small subunit of the ribosomal RNA; therefore, it is present in all prokaryotic cells. The 16S rRNA gene has highly variable as well conserved regions, which are useful for inferring phylogenetic relationships. The conserved regions are used for designing specific primers that will match the nucleotidic sequence of taxonomic groups (from species to domain). On the other hand, the variable regions are different in each species, and thus, they are used for the detection and identification of specific microbial populations. The comparison of 16S rRNA gene sequences from two or more microbial cells is used to determine whether they belong to the same species or genus using a conventional threshold of 3 and 5% dissimilarity, respectively.

While most studies use 16S rRNA gene for studying the general microbial composition, the precise detection of methanogenic populations could also be achieved using methanogenic-specific phylogenetic markers, such as genes encoding enzymes from the methane generation pathway. Since the late 1990s, the use of PCR to amplify methyl-coenzyme M reductase (MCR) genes has become a usual choice for both environmental and bioreactor microbial communities. MCR enzymatic complex catalyzes the reduction of methyl groups bound to coenzyme M, with subsequent release of methane (Ellermann et al. 1988). Two isoenzymes of MCR exist in methanogens: the MCR-I, which is coded by the mcrABCDG operon and occurs in all methanogens; and the MCR-II, which is coded by the mrtABDG operon and was only been detected in the orders Methanobacteriales and Methanococcales (Bonacker et al. 1993; Lueders et al. 2001; Luton et al. 2002).

The mcrA gene from the MCR-I isoenzyme has between 490 and 555 nucleotides (Nölling et al. 1996; Luton et al. 2002) and is highly conserved among methanogens (Hallam et al. 2003), and therefore, it has been selected as standard for the detection of methanogens with PCR-based methods. Also, the comparison of 16S rRNA-based and mcrA-based phylogenies has shown that tree topologies are largely consistent (Springer et al. 1995; Lueders et al. 2001). Using specific primers to amplify DNA fragments that exist only in methanogens increases not only the precise quantification of this group, but also the sensibility of detecting rare (less abundant) populations.

Molecular methods (Table 4.1) could be divided into two basic categories: molecular fingerprinting and sequencing approaches. Molecular fingerprinting allows a rapid and inexpensive comparison of microbial communities over space and time, while sequencing approaches (especially the "-omics" techniques) provide a deeper insight into microbial diversity and functionality. Nevertheless, the choice on which technique is suitable to use from the broad range of available methods depends on the questions to be answered. Discussion of each molecular approach that could be applied in methanogenic community studies would be an exhaustive and nearly impossible effort. Therefore, the most frequent and recent techniques used in the investigation of methanogenic archaea are discussed below.

4.3.1 Molecular Fingerprinting

The standard approach to analyze microbial communities from natural anaerobic environments to wastewater-fueled bioreactors is the use of fingerprinting methods. These methods involve the use of the polymerase chain reaction (PCR) to amplify universal phylogenetic marker genes (e.g., 16S rRNA gene, ITS region, etc.) from the entire microbial community of a given sample, followed by the analysis of the amplified DNA in a gel electrophoresis. In the case of methanogenic populations, the *mcrA* gene has become a standard choice of methanogen-specific genetic marker for fingerprinting methods. Among the most commonly used (and cost-effective) fingerprinting approaches applied on *mcrA* genes are the terminal restriction fragment length polymorphism (T-RFLP) and the denaturing gradient gel electrophoresis (DGGE) methods.

The T-RFLP method is based on the profile resulted from an enzymatic cleavage of PCR fragments. The PCR is performed using a standard pair of primers (e.g., those that amplify *mcrA* genes) that includes a fluorescent label at the 5′ end of one of the primers. Many fluorescent dyes are available such as 6-carboxyfluorescein (6-FAM), carboxytetramethylrhodamine (TAMRA), and hexachlorofluorescein (HEX). The fluorescent labeled PCR products are cut with a restriction enzyme, and the size of the fluorescent subproducts is analyzed in a chromatograph. The presence or absence of restriction sites, as well as the lengths of the resulting fragments, creates a T-RFLP profile for each microbial group. The final T-RFLP graph, or

Table 4.1 List of the most common methods used to study methanogenic populations in both natural and engineered environments. Broad generalizations are presented as sensibility sensors to detect rare organisms in the community, phylogenetic resolution, diversity coverage, and typical costs

Method	Sensitivity to rare organisms	Phylogenetic resolution	Diversity coverage	Cost	Comments
Culturing	Moderate	High	Low	Low	Coverage could be enhanced by new culturing strategies
Fluorescent microscopy (FISH)	Moderate	Low–high	High	Low–moderate	Resolution depends on probe specificity
DGGE	Moderate	High	High	Low–moderate	Interpretation depends on gel and PCR quality. Quantitative analysis may be problematic
T-RFLP	Moderate	Moderate–high	High	Low–moderate	Taxa are missed if restriction site is near the primer
16S rRNA cloning	Moderate–high	High–moderate	High	Low–high	Allows identification of "unknown" organism through phylogenetic trees. High cost for thousands of sequences
qPCR	High	High	High	Moderate	Quantitative results only. Universal or specific primers needed for diversity analysis
Stable isotope probing (SIP)	High	High–moderate	High	Moderate–high	Possible examination of microbial food webs and ecological succession under conditions approaching those observed in situ
16S rRNA metagenomics	High	High	High	Moderate–high	Short reads could limit phylogenetic resolution
Functional metagenomics (WGS)	Moderate	Moderate	High	High	Usually needs high computational effort

electropherogram, has peaks that indicate the presence of different taxa, while the peak intensity is interpreted as the taxa abundance in the sample.

The first investigations of methanogens in environmental samples using T-RFLP date back to 1999 when Chin et al. (1999) used a combined 16S rRNA cloning and T-RFLP approach to evaluate the influence of temperature on the methanogenic community in rice field soils. Later on, Lueders et al. (2001) used T-RFLP over *mcrA* genes to specifically detect methanogens in those rice field soils, showing that all methanogens in the samples were detectable and clearly discriminated by distinct terminal restriction fragments. The choice of *mcrA* instead of 16S rRNA for T-RFLP analysis via group-specific *Sau*96I restriction sites avoided some shortcomings. For example, using *Taq*I restriction enzyme to cleave Archaeal 16S rRNA amplicons, members of the Methanosarcinaceae family and other non-methanogenic archaea (e.g., RV-VI terrestrial mesophilic Crenarchaeota) share the same restriction sites and will produce the same terminal restriction fragments, impairing the precise identification of those groups (Lueders et al. 2001). T-RFLP on *mcrA* soon became a common strategy to study a wide variety of environments, including hypereutrophic lakes (Earl et al. 2003), lake sediments (Banning et al. 2005; West et al. 2012), permafrost (Barbier et al. 2012), agriculture soils (Ma et al. 2012; Liu et al. 2018), among others. This approach was also applied to analyze methanogens in bioreactors fueled with a variety of substrates, such as maize (Lv et al. 2014; Lucas et al. 2015), grass silage (Popp et al. 2015), dried distiller grains (Nikolausz et al. 2013), swine manure (Zhang et al. 2014), and wastewater (Cheng et al. 2018). In all these examples, the methanogenic community was successfully described using T-RFLP. Moreover, novel methanogenic groups were discovered (Lueders et al. 2001; Barbier et al. 2012), showing that T-RFLP on *mcrA* genes is a powerful approach for understanding methanogenic communities. At the present time, with the increasing amount of data of *mcrA*, simple T-RFLP protocols and databases are available for cost- and time-effective profiling of methanogens (Bühligen et al. 2016).

The DGGE is another fingerprinting method widely used to investigate the microbial community diversity. This method is based on the separation of PCR-amplified fragments after a gel electrophoresis containing increasing amounts of a denaturing agent, usually formamide and urea. Initially, the total DNA of a sample is extracted and submitted to a PCR amplification using special DGGE primers: One primer has an additional 40 nucleotides GC-rich sequence (also known as "GC clamp") at the 5′ end, while the other is an conventional primer (Muyzer et al. 1993). The PCR product will contain a mixture of the amplified DNA fragments recovered from the sample, being all these fragments nearly the same size but with a relatively different nucleotide sequence. The PCR product is submitted to a polyacrylamide gel electrophoresis containing denaturants which will remove the hydrogen bonds between nucleotides. Since single-stranded DNA, double-stranded DNA, and partially single-stranded DNA migrate at different speeds in the gel electrophoresis, the DGGE is able to separate DNA fragments of the same length but with different nucleotide compositions. The GC clamp present in all PCR-amplified products will form a stable and partially melted DNA

fragment, avoiding the formation of two single-stranded DNA that could differ in mobility and could confound the analysis. At the end, the DGGE will generate a band profile for each sample, where each band virtually represents a single microbial population. Interpreting the DGGE usually goes by comparing band profiles in terms of amount of bands (total number of species) and the band intensity (relative abundance of each species). However, biases exist, and caution should be taken when considering band intensities into account (Araújo and Schneider 2008). Calculation of similarity indices such as Jaccard or Bray–Curtis is also a common practice for interpreting the DGGE data, which could be further used to build similarity dendrograms or submitted to a multivariate statistical test (e.g., principal component analysis—PCA).

Since the mid-1990s, DGGE has been extensively used for studying microbial community structure over a wide range of natural and engineered environments. Most of these studies applied DGGE with 16S rRNA genes amplified directly from environmental samples, turning this approach into a traditional practice to assess the unculturable portion of microbial communities. Samples with naturally occurring methanogens were studied using DGGE with PCR-amplified 16S rRNA, including agricultural soils (Jensen et al. 1998; Wang et al. 2010), abandoned coal mines (Beckmann et al. 2011), Antarctic sediments (Karr et al. 2006; Nakayama et al. 2011), domestic wastewater (Boon et al. 2002), and bioreactors operating with several types of organic load (Calli et al. 2003; Casserly and Erijman 2003; Keyser et al. 2006; Tanikul et al. 2016). The DGGE primers used to amplify the 16S rRNA were designed with nucleotide degenerations in order to match a broad range of microorganisms, sometimes called "Universal" primers. For example, the popular DGGE primer 338FGC-518R (Amann et al. 1990) will cover ~90% of the Bacteria domain but will not match the Archaea. On the other hand, the DGGE primer pair 1100F-1400R (Kudo 1997) matches the 16S rRNA from Archaea but will not amplify the same gene from Bacteria. Therefore, DGGE band profiles using a Universal approach do not guarantee that methanogens are present in the samples. In fact, the studies cited above focused not only on methanogens, but tried to profile the whole microbial community structure, and therefore, the use of Universal primers for this DGGE analysis is suitable.

In order to study methanogens using DGGE, most authors rely on two strategies. First, DGGE analyses are accompanied with other detection methods, such as fluorescent *in situ* hybridization (FISH) using methanogenic-specific probes (Calli et al. 2003; Tabatabaei et al. 2009), or the methane production is accurately quantified in the environment or the bioreactor from where samples were collected (Ganzert et al. 2007; Beckmann et al. 2011; Nakayama et al. 2011). These complementary analyses facilitated the interpretation of DGGE profiles based on 16S rRNA gene amplifications, associating the microbial diversity with methanogenic activity. The second strategy is to run a DGGE analysis on PCR products amplified from specific methanogen gene markers, such as the *mcrA* gene (Antony et al. 2012; Kymäläinen et al. 2012; Yu et al. 2014; Morris et al. 2016; Banach et al. 2018). This strategy not only allows for a precise analysis of the methanogenic community structure on several environments, but also has the advantage of *mcrA*

being a functional gene directly related to the synthesis of methane. The use of a functional gene as molecular marker is a strong approach for the validation of methanogenesis, especially when coupled with methane emission analysis (Garcia-Maldonado et al. 2012; Banach et al. 2018).

Several strategies were developed to improve the DGGE for environmental analysis, such as optimization of PCR amplification protocols, design of new sets of primers, and band excision for further sequencing. The later consists on cutting out the DNA bands (200–700 pb) from the DGGE gel, purifying to remove polyacrylamide and the denaturing agents, cloning or PCR-amplifying the excised DNA, and finally sequencing the DNA for a precise identification of the chosen band. This method turns DGGE into a powerful tool for rapid and ease identification of uncultured microorganisms associated with the experimental variables. For example, DGGE band sequencing was used to describe the methanogenic community from an anaerobic digester under mesophilic (35–37 °C) and thermophilic (55–57 °C) conditions for biogas production (Yu et al. 2014). After realizing that biogas production was higher on the thermophilic process, the DGGE band sequencing revealed that uncultured (or not-yet cultured) members of the archaeal orders Methanobacteriales, Methanosarcinales, and Methanothermobacter were responsible for methane production. Similarly, DGGE band sequencing was used to investigate uncultured methanogens from both 16S rRNA (Karr et al. 2006; Keyser et al. 2006; Wang et al. 2010; Beckmann et al. 2011) and *mcrA* sequences (Garcia-Maldonado et al. 2012; Kymäläinen et al. 2012; Morris et al. 2016).

T-RFLP and DGGE could be used as rapid and cost-effective methods for profiling methanogens in natural or engineered environments, giving also a quantitative and semiqualitative picture of the microbial community structure of a given sample. However, both T-RFLP and DGGE have inherent limitations that make reproducibility difficult, such as the very high technical expertise required, primer dimers, choice of appropriate restriction enzymes (T-RFLP only), and improper staining (DGGE only). Also, if sequencing data is of particular interest, both methods do not provide a deep throughput of species information. Current investigations prefer to use metagenomics for a more complete description of the microbial community structure, including the vast uncultivated methanogenic groups.

4.3.2 Next-Generation Sequencing

In parallel with fingerprinting analyses of the late 1990s, the development of new cloning techniques combined with Sanger DNA sequencing has rapidly become a popular culture-independent approach for studying microbial diversity. Despite this method been regularly applied for describing the microbial community structure in several ecosystems, including methanogen-rich environments (Marchesi et al. 2000; Skillman et al. 2006; Yadav et al. 2015) and biogas production systems (Liu et al. 2002; Klocke et al. 2008; Nettmann et al. 2008), the cloning and sequencing

procedure is very laborious, excessively time-consuming and usually limited by cost of sequencing (Zhou et al. 2015). Since the mid-2000s, the rapid advance of new sequencing technologies, also called "next-generation sequencing" (NGS) techniques, has overcome all these limitations and settled a milestone on microbial diversity studies.

Current NGS platforms allow the high-throughput sequencing of DNA molecules in parallel—in other words, up to billions of short reads (50–300 nucleotides each) are sequenced at once from environmental DNA extracted with routine laboratory protocols or commercial kits. Several NGS platforms are available, such as the 454 Pyrosequencing (Qiagen), Illumina MiSeq and HiSeq (Illumina Inc.), SOLiD (Life Technologies), Ion Torrent (Thermo Fisher) and MinION (Oxford Nanopore Tech.), each one differing on sequencing outputs (read lengths, quality, and number). Nevertheless, all these NGS platforms could be applied to microbial diversity studies in the new emerging field of metagenomics.

Metagenomics is defined as the analysis of the collective (meta-) microbial genomes contained within an environmental sample (Riesenfeld et al. 2004). The original metagenomics studies focused on increasing the number of 16S rRNA sequences obtained from traditional cloning efforts for a more deep view on the "real microbial diversity." Later, metagenomics efforts were applied to functional expression analysis and quickly evolved to direct sequencing of random shotgun sequencing of environmental DNA (Thomas et al. 2012). These applications not only showed the great potential of NGS, but also revealed an enormous taxonomic and functional diversity in the microbial world.

The use of metagenomics for taxonomical studies provided novel insights into the diversity of methanogenic communities in both natural and engineered environments. Metagenomics have been used to detect methanogenic archaea in a wide range of natural habitats, such as soils (Meyer et al. 2017), lake sediment (Vavourakis et al. 2018), marine sediments (Carr et al. 2018), hydrothermal vents (Reveillaud et al. 2016), landfills (Song et al. 2015), rice fields (Hernández et al. 2015), and animal gut tract (Gill et al. 2006; Kamke et al. 2016; Chew et al. 2018). Hence, metagenomics revealed that methanogenic archaea are ubiquitous, and a huge diversity of uncultured lineages exists in the biosphere (Adam et al. 2017).

In the context of biogas production, the first metagenomics reports come from a production-scale biogas plant in Germany fed with grain crops and chicken manure (Krause et al. 2008; Schlüter et al. 2008; Kröber et al. 2009). These studies used a 16S rRNA metagenomics to understand how the microbial community structure is shaped in order to promote biogas production. Analysis of the genetic content and phylogenetic classification of 16S rRNA sequences revealed a dominance of Bacteria over Archaea, with order Clostridiales being the most abundant in the biogas plant. Also, the metagenomics analysis showed Methanomicrobiales as the dominant order among the plant methanogenic community (Krause et al. 2008; Schlüter et al. 2008). Several other anaerobic digesters working at production scale (Jaenicke et al. 2011; Yang et al. 2014; Stolze et al. 2015; Güllert et al. 2016; Luo et al. 2016) or laboratory scale (Rademacher et al. 2012; Kovács et al. 2013; Li et al. 2013; Wong et al. 2013; Solli et al. 2014; Nolla-Ardèvol et al. 2015;

Wirth et al. 2015; Gryta et al. 2017; Park et al. 2018) were studied using 16S rRNA metagenomics. Most of these reports also found Methanomicrobiales as the dominant archaeal order, usually followed by Methanosarcinales and Methanobacteriales. Interestingly, on production-scale anaerobic digesters, the prevalence of Methanomicrobiales was observed even in those feed with different loads such as cattle manure, sewage sludge, or industrial wastewater, with the exception of a wastewater treatment plant in Hong Kong that Methanosarcinales was dominant (Yang et al. 2014). On laboratory-scale bioreactors, the dominant methanogenic taxa varied between Methanomicrobiales (Kovács et al. 2013; Solli et al. 2014; Nolla-Ardèvol et al. 2015) and Methanosarcinales (Wirth et al. 2015).

Next-generation sequencing approaches could also reveal useful information on functional diversity and gene expression at community level. Generally, two types of methods are used: whole genome shotgun (WGS) and metatranscriptomics. The WGS consists in sequencing short fragments of DNA (50–250 bases) obtained from chemical or physical sheared environmental DNA (shotgun). In contrast with the 16S rRNA (PCR-amplicon) metagenomics, WGS will sequence the entire genetic content from all the microbial community, including dead or dormant cells. Current high-end sequencing platforms such as Illumina HiSeq provide up to 2 billion of 150 bp paired-end sequences from a given sample, but other platforms like Illumina MiSeq and Ion Torrent will provide 2–5 million of sequences per sample, which may be sufficient for a routine WGS analysis. On the other hand, metatranscriptomics consists on sequencing extracted mRNA from the whole community, i.e., the total transcribed RNA. In this case, metatranscriptomics will capture the living portion of the microbial community, including the mRNA transcribed by uncultured species. Since sequencing platforms use DNA as a template, the community mRNA must be transformed into a complimentary DNA (cDNA) before sequencing. In summary, both WGS and metatranscriptomics are capable of unveiling functional information of the microbial community. However, the WGS will reveal the metabolic potential of the cultured and uncultured community members, while the metatranscriptomics will show the metabolic potential and a quantitative snapshot of the expressed genes by living cells.

Several WGS studies described the diversity and genetic potential of methanogenic populations in bioreactors (Li et al. 2013; Park et al. 2018; Soares et al. 2018) and other biogas-producing systems (Chojnacka et al. 2015; Luo et al. 2016; Delforno et al. 2017). For example, Li et al. (2013) used 454 Pyrosequencing to investigate the methane-producing microbial community in two mesophilic solid-state biogas reactor. The sequencing effort resulted in about 2.8 million sequences with an average length of 283 bp, assembled (joined by overlapping nucleotides) into 118,433 sequence contigs (about 37,000 of these were >500 bp long). This approach showed that *Methanosarcina*, *Methanosaeta,* and *Methanoculleus* are the most abundant methanogenic genera in the bioreactor. Interestingly, the WGS revealed that an uncultured *Anaerococcus* (domain Bacteria, phylum Firmicutes) was the second most abundant organism in the whole community, suggesting an important role of this bacterium on biogas production. Since the metagenomic sequences were similar only to other uncultured *Anaerococcus*,

WGS was important for predicting the metabolic pathways from this bacterium. WGS analysis indicates that the bioreactor *Anaerococcus* has the enzyme acetate kinase (EC: 2.7.2.1) and plays an important role on acetate fermentation to acetyl-CoA, which is the first step of methane production by methanogens (Singh-Wissmann et al. 1998).

Metatranscriptomics analysis on biogas reactors began shortly after the first metagenomics studies in these devices. In fact, the same biogas plant in Germany described earlier (Krause et al. 2008; Schlüter et al. 2008) was used for RNA extraction and metatranscriptome sequencing (Zakrzewski et al. 2012). Transcripts analysis revealed a high abundance of methane-related enzymes, indicating that methanogenesis pathway was more active than previously deduced from 16S rRNA data. In another bioreactor study, metatranscriptomics was used to evaluate transcriptional dynamics of the methanogenic community after shifts in organic loading rates (Kouzuma et al. 2017a, b). Metatranscriptomic profiles observed in this study revealed that hydrogenotrophic methanogens growing in the reactor can adapt to environmental changes by regulating the expression of methanogenesis-related genes (*fwd*, *mtd*, *mer*, and *frt* genes) at the transcriptional level.

Recently, a WGS and metatranscriptomics combined approach was used to investigate the influence of temperature on microbial dynamics of biogas-producing reactors (Grohmann et al. 2018). The WGS revealed that 80% of the recovered sequences belong to only 20 microbial genomes, which indicates a high dominance of few organisms in the studied bioreactors. Firmicutes (65% of all genomes) and Bacteroidetes (17.8%) were the dominant bacterial Phyla, while Archaea presented only 4 groups (3.5% of all identified genomes): two *Methanoculleus*, one *Methanosarcina,* and one completely new archaeon candidate of Phylum *Euryarchaeota* "Eu03." Their metatranscriptomics analysis indicated that the acetoclastic *Methanosarcina* and the unknown EU03 lineage were responsible for bulk methane production. Moreover, the initial operation temperature of the reactors (35 or 41 °C for 16 days, followed by 41 °C until day 84) was relevant for the methanogenic activity. The expression of acetogenotrophic methanogenesis-related genes was three times higher in the reactor operating at 35 °C compared to 41 °C. This study linked metagenomics and metatranscriptomic results to give experimental evidence on how methanogenesis responds to environmental factors (e.g., temperature and acidification). The combination of different NGS methods for studying microbial community dynamics and functional activity is shown as powerful strategy for future optimizations on biogas production systems.

4.4 Final Remarks

In the last decade, knowledge in the area of microbial ecology has undergone a great leap. Studies on natural ecosystems and bioreactors are revealing that the diversity of methanogenic archaea and methanogenic pathways is greater than we expected. Methanogenesis is ubiquitous, occurring even in aerobic environments,

and can also be performed by aerobic bacteria. Methanogenic metabolism was reported in two new Phyla (Bathyarchaeota and Verstraetearchaeota). In Euryarchaeota Phylum at least one new order was discovered, and two new classes of methanogens were proposed, all of them producing methane through distinguished methylotrophic routes. The DIET strategy for interspecies electron transfer has also shown us that methanogenic groups can enlarge their metabolic possibilities and opens new possibilities for biotechnological application. Finally, the development of high-rate reactors represented a major breakthrough for anaerobic digestion technology. What is still ahead is how to keep the microbial communities active in the reactor and prevent bed-fouling. In this sense, the development of structured-bed reactors has been allowing the treatment of wastewater of organic load with great potential for methane generation.

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