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Helen Treichel
Gislaine Fongaro *Editors*

Improving Biogas Production

Technological Challenges, Alternative Sources, Future Developments

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Foreword

The use of biogas as a renewable energy source has increased significantly in the world in the last years. The main driver for this is due to initiatives for reduction of GHG emissions, fossil fuel replacement, technological and economic aspects that permit to generate biogas (or biomethane) under stable conditions.

There are a lot of alternatives of organic materials to be used as substrate for biogas generation processes. For tropical conditions, the diversity is even bigger, mainly for plant materials, owing to the climatic conditions when compared to temperate and cold countries.

One of the big challenges is to increase the yield of biogas generation in the biodigestors. This can be done using strategies to prepare substrates by different blends for codigestion and pretreat the organic materials to increase the bioavailability of organic carbon. The study and understanding of microorganism community activity and inhibition processes are also very important for the biodigestors' good performance.

In this way, the book *Improving Biogas Production: Technological Challenges, Alternative Sources, Future Developments* was very intelligently edited by Dr. Helen Treichel and Dr. Gislaine Fongaro. The topics were organized and discussed to bring us a very important contribution for science and technology advances to be applied to the biogas chain.

The 14 chapters of this book present, in detail, relevant information that can be used as a good support material for professionals and students to increase their knowledge in strategies for biogas production improvement.

Concórdia, Brazil — Airton Kunz

Contents

Abstract

Biogas production and yield represents one of the most important targets of renewable energy in the world. With an innovative and biotechnological vision, this book will present alternative sources for biogas production, such as pre-treatments of substrates, accelerators (enzyme-mediated) and inhibitors involved in the process of obtaining biogas and its yield, design specification of digester/modified digester, managing biogas plants, upgradation, microbial risk and slurry management, energy balance and positive climatic impacts relating to biogas production chain, and also the impact on human, animal, and environmental health ("One Health" concept on biogas chain).

Chapter 1
Waste Biomass and Blended Bioresources in Biogas Production

Luciane Maria Colla, Ana Cláudia Freitas Margarites, Andressa Decesaro, Francisco Gerhardt Magro, Naiara Kreling, Alan Rempel and Thaís Strieder Machado

Abstract Global energy demand is getting higher, and most of this energy is produced through fossil fuels. Recent studies report that anaerobic digestion is an efficient alternative to produce biogas. Moreover, the transformation of complex organic materials into a source of clean and renewable energy reduces the emission of greenhouse gases and can produce as by-product a high-value fertilizer for growing crops. The anaerobic co-digestion is an option to solve the disadvantages of single substrate digestion system, being the chemical composition and properties of the substrates, the operating parameters (temperature, pH, charge rate, etc.), the biodegradability, bioaccessibility, and bioavailability, important parameters to be optimized. The main materials that could be used for biogas production are waste from cities, residues from the production of other biofuels, agro-industrial waste in general, agricultural crops, straws, or microalgae biomass obtained by cultivation in wastewater. However, some of these materials, specially raw materials, need to be treated to improve the biogas production. The aim of this chapter is to review the main materials that could be used for biogas production and the factors to optimize the production.

Keywords Biogas · Co-digestion · Pretreatments

1.1 Introduction

Anaerobic digestion (AD) is a suitable, efficient method for the management of organic materials (Surendra et al. 2014; Appels et al. 2011) and an efficient alternative combining biofuel production and sustainable waste management

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(Achinas et al. 2017). However, AD is a very complex, sensitive process involving numerous microorganisms with extreme operational and environmental conditions (Hags et al. 2017). Consequently, the better use of raw materials to improve biogas production has drawn growing attention. However, the challenges of low biogas production, long retention time, and high investment costs prevent achieving the maximum performance of biogas production in anaerobic digestion systems (Patinvoh et al. 2017).

Biogas production from different organic materials depends primarily on the substrate contents that can be converted into biogas, while their chemical compositions and biodegradability are key factors in the production of biogas and methane (Amon et al. 2007). For efficient biogas production, the most appropriate raw material must be selected, but the direct use of some substrates is hampered by nutritional imbalances, lack of diversified microorganisms, and the effects of operational factors (Achinas et al. 2017). It, therefore, is often necessary to pretreat the biomass to be used again, and co-digestion is recommended to overcome nutritional deficiencies in the residues to improve biogas production in digesters (Nielfa et al. 2015). Moreover, it is worth mentioning that the profitability of the AD process is directly influenced by the cost of obtaining raw materials, which, in turn, is affected by competing interests, the possibility of controlling the supply, and the distance from the supplies to biogas production plants. This chapter describes the main residues and biomasses used in biogas production and the pretreatment necessary for efficient biogas production.

1.2 Waste Use in Biogas Production

Biogas has potential to be produced from widely available, abundant raw materials, including agricultural residues (e.g., animal manure), landfill and food waste, and aquatic biomass and lignocellulosic raw materials. However, most of these feedstocks have slow degradation rates and require long retention times. In addition, some raw materials form toxic intermediates or contain toxic compounds, which inhibit the biogas production process. However, their abundance and low cost point to the need for new strategies to better use such waste streams (Taherzadeh and Karimi 2008). Depending on the raw material used, the production of biogas must be optimized to take into account crucial parameters, such as the substrates' chemical composition and characterization, operational parameters (e.g., temperature and pH), biodegradability, bioaccessibility, and bioavailability (Hagos et al. 2017).

Theoretically, biogas can be produced from the organic fraction of any material, but today biogas is produced only from raw materials easily usable by the microorganisms that transform them into biogas. Other digestible raw materials, such as crop residues, separated municipal waste, food waste, and wastewater with high organic content, are not as plentiful or readily available, limiting biogas production from them (Patinvoh et al. 2017).

1.2.1 Urban Waste

The generation of urban solid waste, or municipal waste, increases with population growth, high economic activity, and goods production (Barros et al. 2018). These factors generate enormous amounts of waste, especially in big urban centers, residences, and small commercial establishments, such as restaurants and bakeries (Browne et al. 2014). Municipal solid waste, when well separated, can be reused for other processes, but it is necessary to maximize the value of the organic residues obtained from this source. Approximately, 46% of urban solid waste consists of organic fraction (e.g., food, garden, wood, and process waste), which can be processed by anaerobic digestion and converted into biogas (Tyagi et al. 2018). Classification and transportation of this waste are the first treatment processes, so their efficiency affects the overall efficiency of the digestion process' conversion of energy. Additionally, separation of the residue at the source permits the best use of the digestible compounds (organic fraction) and reduces the impacts associated with the use of conventional energy in mechanical separation (Morero et al. 2017). Generating a flow that has no raw material costs and only requires transportation to anaerobic digesters is also necessary if food waste is taken to landfills (Florkowski et al. 2018), where an estimated 97% of food waste ends up (Levis et al. 2010).

Anaerobic digestion serves to maximize the value of organic waste, generating energy with several applications. Consequently, it has gained recognized as an economic and environmentally friendly solution for management of organic waste flows (Clercq et al. 2016). The organic fraction of urban solid waste yields up to 200 m^3 of biogas ($\sim$400 kWh of power) per ton of treated waste (Bolzonella et al. 2006) and a methane yield of up to 330 L/kg of total volatile solids (Hartmann et al. 2002).

Plants are increasingly developed and constructed to treat food waste in cities and so far have achieved good results in biogas production (Deng et al. 2017), improving management of food and food parts whose waste is unavoidable. These residues are primarily composed of lignocellulosic materials and are rich in carbohydrate materials (Karimi and Karimi 2018), substrates potentially suitable for biogas production. The success of the anaerobic digestion of a carbohydrate-rich substrate, though, depends on the balance between acidogenesis and methanogenesis (Tyagi et al. 2018).

In 2009, the city of Chongqing, China, built biogas plants for the treatment of food waste in Heishizi in 2009. The plans apply fermentation technology to process 1000 t of food waste daily and produce 28 million m^3 of biogas per year, generating 33 million kWh of electricity (Deng et al. 2017). In Lubelskie Voivodeship in Poland, food waste used for biogas production and energy generation accounted for 1.5% of electricity consumption in the region and 0.18% of electricity generated from renewable resources nationally in 2012 (Aneks Diagnostyczny 2014).

Anyaoku and Baroutian (2018) analyzed the strategy of decentralization of solid waste management through anaerobic digestion to minimize waste transportation costs and maximize the benefits of the final product to the local community.

The decentralization of biogas processing and its almost direct use by the community contributed to a sense of waste management, acting as an incentive for households to separate their own waste. In developed economies, households are the biggest contributors to food waste (Florkowski et al. 2018). Decentralization of anaerobic digestion requires a capacity of <3000 t/a (Righi et al. 2013), which can be achieved through multiple units or smaller digester cells for batch feeding (Anyaoku and Baroutian 2018). Variations in waste composition can create an imbalance in the system through the use of inadequate carbon and nitrogen rates, which are necessary to optimize the process.

Browne et al. (2014) studied the variability of methane production as a function of urban solid waste sources. The organic waste sample was composed of household (urban and rural), commercial (restaurant), and food-processing waste (bakery and cheese), with approximately 10 kg from a global sample of each waste source. The study showed that restaurant waste samples exhibited a higher biochemical methane potential (491–535 mL CH_4g/VS in 30 days) and a larger portion of biodegradable organic material than domestic waste samples (274–368 mL CH_4g/VS in 30 days) (Browne et al. 2014). In addition, the inclusion of garden waste significantly reduced methane production when added to household waste (Browne et al. 2014). Likewise, commercial waste samples were unstable in the process due to excess ammoniacal nitrogen, causing a low carbon and nitrogen ratio (*C*/*N*). Kayhanian and Hardy (1994) considered *C*/*N* ratios of 25–30 to be excellent for anaerobic digestion of the organic fraction of urban solid waste.

Barros et al. (2018) simulated a pilot-scale landfill cell to quantify the biogas produced by urban solid waste with a similar composition to that disposed in a municipal landfill from 101.60 kg of waste (42.7 kg of organic material). It was observed that temperature had a direct relation with biogas production, with higher temperatures producing higher methane contents (0.799 m^3/t day) due to increased microbiological activity (Barros et al. 2018).

Depending on the source used in anaerobic digestion of urban waste, the raw material requires a series of pretreatments, beginning with mechanical separation of inorganic components, such as plastics and metals (Gutiérrez et al. 2018). Next, residues can be ground to reduce particle size (Levis et al. 2010) or even liquefied to increase the anaerobic digestibility of food residues (Kavitha et al. 2017). However, pretreatments require further investigation to determine their economic viability (Tyagi et al. 2018) because their aim is to accelerate hydrolysis and increase the solubilization of the residue. To this end, mathematical modeling can help select appropriate combinations of raw materials and pretreatments (Tyagi et al. 2018).

Fats, oils, and greases present another urban waste, mostly generated in the restaurant and food-processing industries. These residues are characterized by high lipid content with easily degradable organic components. Lipids can potentially produce almost twice as much biogas than carbohydrates and proteins (Gallert and Winter 2005). A sample of these dehydrated lipid residues, along with anaerobic sludge inoculum, was evaluated by Kobayashi et al. (2017), who confirmed that lipid residues have potential as promising sources for biomethane generation (767.5 mLN/gVS_{added}).

As in sanitary sewage treatment plants, biogas can be obtained from treatment of the effluent in anaerobic reactors and from anaerobic digestion of sludge produced from the treatment (Santos et al. 2018). However, the use of sludge applications as auxiliaries in co-digestion requires more detailed study of its characteristics because sludge might contribute to an environment unfavorable to the survival and activities of microorganisms responsible for anaerobic degradation. Sludge might also have toxic substances, such as surfactants and detergents, as verified by Barros et al. (2018), who reported that sludge containing these substances decreased biogas production.

The use of organic waste as an energy source, therefore, solves waste disposal problems and generates less environmental impact and lower costs than landfill disposal (Morero et al. 2017). To solve low macro- and micronutrient ratios, toxic compound dilution, pH stability, temperature, moisture content, different substrates can be used in the anaerobic co-digestion alternative. With this, challenging the control of the process (Gutiérrez et al. 2018; Tyagi et al. 2018; Morero et al. 2017) is directly related to the physical–chemical characteristics of the raw material, which can diversify across regional, seasonal, and socioeconomic contexts.

1.2.2 Waste Animal Manure

In agricultural residues, the main source of raw material for biogas production is animal manure from swine, cattle, and poultry. The use of biodigesters contributes to the integration of agricultural activities, converting manure, which usually has little or no commercial value, into energy. Animal manure is recognized as a highly favorable substrate for biogas production because it better combines energy production and nutrient recycling and reduces CH_4 and N_2O emissions more than conventional manure management (Holm-Nielsen et al. 2009; Hijazi et al. 2016). However, due to the high water concentration in animal manure, it is rarely economically feasible to operate biogas plants with only animal manure. Researchers have attempted to improve its efficiency by adding lignocellulosics to increase the dry matter and the *C/N* ratio. According to Yadvika et al. (2004), the addition of green vegetation Poaceae improved biogas yield from 18 to 40% when co-digested with animal manure, while increases of 10–80% were observed with the agricultural residues of wheat straw, rice straw, and corn stems, depending on the pretreatment conditions and dry matter contents.

The optimal proportion for biogas production reported has varied greatly. For example, according to Li et al. (2013a, b), chicken manure and corn stover mixed at 1:3 ratio produced a methane yield of 298.2 mL g^{-1} VS, higher than from chicken manure alone (291.1 mL g^{-1} VS). In another batch test, synergism could be seen in the co-digestion of chicken manure and corn stover with a 1:1 ratio.

The anaerobic digestion treatment of manure from poultry is more problematic than other animals because poultry residue contains high nitrogen levels, which can

result in ammonia inhibition (Belostotskiy et al. 2015). In the anaerobic digestion of chicken residue, the high content of uric acid and undigested proteins results in ammonia production (Abouelenien et al. 2010). In addition to toxicity, chicken residue contains a fraction of wood chips used as bedding materials, so such high NH_3 and lignocellulose residues are less susceptible to anaerobic destruction for biogas production (Costa et al. 2012; Ziganshina et al. 2014). Researchers have tested different techniques to avoid inhibition effects during anaerobic digestion of ammonia-rich organic residues. Co-digestion of chicken manure with other substrates is a strategy to control the inhibition caused by ammonia (Wang et al. 2012).

1.2.3 Industrial Waste

The use of wastewater from industrial processes for biogas production has been tested in anaerobic digestion for wastewater treatment and energy production (Hultberg et al. 2017). Research done in Scopus platform indicates that the use of industrial wastewater has increased during the past 10 years, with the number of articles published rising from 53 over 2000–2004 to 90 over 2010–2014. The most-cited articles are on organic wastewater from industrial processing of oils and bagasse (Ng et al. 2017; Mohamad et al. 2017). The use of wastewater from inorganic sources, such as chromium, has also been studied as an alternative for energy production, a more environmentally sustainable approach that avoids landfill disposal of these wastes (Agustini et al. 2018; Priebe et al. 2016).

Industrial waste and wastewater have potential uses in biogas production due to their characteristics, such as high organic load (Mannucci et al. 2010). The most common treatment for industrial waste is landfill disposal, which generates costs and environmental harms. The advantages of anaerobic treatment for solid waste applications include low energy costs, the use of organic matter as a substrate for biogas production, and reduced need for waste disposal in landfills (Appels et al. 2008). In addition to generating electricity, the biogas produced can be used in vehicles, domestic heating, and chemical industries. Brazil, in particular, had 15 biogas plants, with a total production of 114.7 MW of energy, equivalent to 0.83% of the total national biomass capacity, as of January 2017 (ANEEL 2017).

Agustini et al. (2018) evaluated the use of leather waste and silt containing chromium and vegetable tannin in biogas and methane production. Using a 25 mL:1 g ratio (sludge: residues) evaluated co-digestion using two types of sludge and three types of waste over 200 days. In experiments with 7.35 mg/L of chromium in the sludge, the presence of chromium resulted in 27.9 mL/g of volatile suspended solids. The high biogas production occurred because the enzymes and coenzymes depended on the metals for their activity, and the sludge concentration was ideal for production. The highest methane production was obtained when chromium was present (11.3 mL accumulated methane/gVSS). In experiments with residues containing 7.79% chromium, biogas production reached 21.5 mL of biogas/VSS, and methane production reached 10.7 mL of methane/gVSS

(Agustini et al. 2018). The authors reported that low chromium concentrations in the sludge were anaerobically biodegradable and had potential to produce biogas and methane.

Budiyono et al. (2018) investigated the use of wastewater from cassava processing in tapioca production, with the aim to produce biogas. Over 45 days of production, the addition of 1% tapioca residue, 0.08% yeast, 0.04% urea, and 10% bacteria was evaluated in comparison with biogas production in a tank without the addition of yeast. A higher volume of biogas production was observed when yeast was present (1400 mL of biogas in 36 days of cultivation) compared to biogas production without the addition of yeast (1000 mL in 36 days of culture) (Budiyono et al. 2018). Explaining this result, the addition of the yeast *Saccharomyces cerevisiae* promoted hydrolysis of the effluent from a polysaccharide to a monosaccharide, which could be used more quickly and efficiently by methanogenic bacteria as a substrate source, increasing biogas production (Budiyono et al. 2018).

Suksong et al. (2017) evaluated biogas production from the anaerobic digestion of solid waste from the palm oil industry. Suksong et al. (2017) used solid-state anaerobic digestion and various types of waste types (fruit bagasse and leaves) in different ratios of raw material: inoculum (2:1, 3:1, 4:1, 5:1) over 45 days of cultivation. The highest methane production (223.3 m^3 ton^{-1} VS) was obtained from the inoculum ratio of 2:1, *C/N* ratio of 40:1, and initial concentration of 16% total solids in the residue of fruit bagasse (Suksong et al. 2017). The study also proved that the thermophilic phase is more efficient than the mesophilic phase in biogas production because hydrolysis of the substrate accelerates in the thermophilic phase (Suksong et al. 2017).

Using sludge from the pulp industry, Lopes et al. (2017) evaluated biogas production from anaerobic digestion in the thermophilic phase. Three types of sludge obtained after passing water through a press filter system were evaluated. The second sludge obtained generated higher accumulated methane production (46.9 mLN CH_4/gVS) during an assay over 30 days. The primary sludge and the mix made of both primary and secondary sludge in a 2.5:1 ratio obtained cumulative methane yields of 3.5 and 3.3 mLN CH_4/gVS, respectively.

In Brazil, Santos et al. (2018) analyzed the biogas production potential of organic residues, including vinasse. This waste was produced by distilleries and the sugarcane industry and had biogas production potential of $1142{,}614.10^6$ m^3/year, preventing the emission of 0.725 $MtCO_2$/year. This waste also had the potential to generate electricity of 254,675 MW and is viable of use since Brazil is the world's largest sugarcane producer (Santos et al. 2018).

Filho et al. (2018) evaluated the potential for biogas production from waste from wine production. The experiment used grape bagasse, must, primary and secondary sludge from a wastewater treatment plant and a mixture of waste containing bagasse and primary and secondary sludge and must. The highest biogas production was obtained from must (fresh grapes not used in the wine fermentation process), at 1151.71 m^3 ton VS^{-1}, and the mixture of all residues, at 289.13 m^3 ton VS^{-1}

(Filho et al. 2018). Together, these studies demonstrated that biomethane production from industrial waste is a sustainable option for energy generation in both developed and developing countries (Prabakar et al. 2018).

1.2.4 Lignocellulosic Materials

Lignocellulosic biomass is a renewable raw material with potential for use in methane production due to the presence of highly fermentable monomers (Solarte-Toro et al. 2018). This raw material is composed of glucose, mannose, xylose, arabinose, and other organic compounds (e.g., proteins and lipids) that have high production potential and are easily degraded via anaerobic digestion (Surendra et al. 2014). Methane production from this biomass varies by the energy content of each material. However, the use of lignocellulosic materials in these production processes presents a disadvantage compared to other biomass sources (Zheng et al. 2014).

The presence of lignin in these materials hinders the action of microorganisms on the substrate in anaerobic digestion, which directly affects the yield of the process. Pretreatments in this biomass offer one way to increase the conversion rates of substrates in biogas (Fig. 1.1). The pretreatments required for

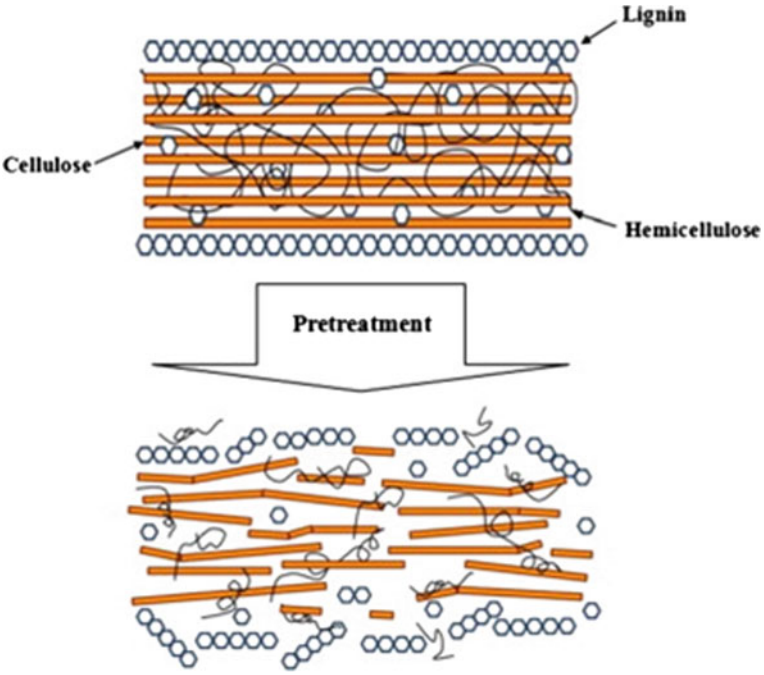

Fig. 1.1 Effect of pretreatments on lignocellulosic materials. *Source* Mood et al. (2013)

Table 1.1 Main lignocellulosic biomass for methane production, pretreatments used, and methane generation capacity

Biomass	Pretreatments used	Methane (mL CH_4/g VS_{added})
Rice straw	Chemical: alkaline	292
Wheat straw	Physical: steam explosion	273
Corn straw	Chemical: alkaline	372.4
Sugarcane bagasse	Physical: liquid, hot water	100.6
Rice husk	Chemical: ammonia	55.7
Empty fruit bunches	Chemical: alkaline	404
Straw of bean	Physical: autoclaving	440
Straw of rye	Chemical: chemical oxidation	360

Source Adapted from Raposo et al. (2012)

lignocellulosic materials are discussed in this chapter, including physical chemical, physical-chemical and biological pretreatment methods.

After pretreatment, the biomass carbohydrates are released to the next step. Hydrolysis then transforms these compounds (cellulose and hemicellulose) into simpler structure (glucose, xylose, mannose, and arabinose), later degraded through anaerobic digestion (Paudel et al. 2017; Zhao et al. 2018).

To achieve high conversion rates of these substrates to biogas, inoculums adapted to different substrates are used. These inoculums usually come from anaerobic reactors in wastewater treatment plants using animal waste (Pilli et al. 2014; Li et al. 2015). Several sources of lignocellulosic feedstock have been reported to have biomethane production potential, and these substrates vary by their origin, with most lignocellulosic feedstocks coming from cereal residues (see Table 1.1). The pretreatments commonly used are presented.

Venturin et al. (2018) investigated biogas production from corn stalks, using different pretreatments and orbital shaker agitation and pressure in the presence of sulfuric acid (H_2SO_4) in different concentrations and hydrogen peroxide (H_2O_2). The biochemical potential of biogas production was tested with pretreated corn stalk and an inoculum of mesophilic microorganisms. Biogas production using H_2O_2 as a pretreatment removed 71.6% of lignin, reduced hemicellulose by 19.3%, and increased cellulose content by 73.4%. This pretreatment increased the final volume of biogas by 22% and reduced the time to obtain this volume by almost a third.

Schroyen et al. (2015) produced biomethane from biomasses with high levels of lignin (corn stover, wheat straw, flax, hemp, miscanthus, and willow). These biomasses underwent enzymatic pretreatments with the laccase enzymes produced by *Trametes versicolor* and peroxidase produced by *Bjerkandera adusta*. Biomethane production was tested by adding an inoculum from a bovine-manure-effluent treatment plant. The results showed increased biomethane yield in all the biomasses after the enzymatic action (Schroyen et al. 2015).

The yield of methane production is variable and directly related to the physicochemical characteristics of the substrates used. Moreover, the measurements of the biogas produced and the pretreatment and inoculum types used are significant factors in yield.

1.2.5 Biomass of Microalgae

In recent years, research on biofuel generation has focused on microalgae crops. Compared to terrestrial raw materials, microalgae have advantages: They grow 5–10 times faster in beneficial conditions and have higher production rates than terrestrial biomass (Kroger and Muller-Langer 2012). In addition, microalgae can be grown in arid areas (e.g., deserts and coastal land) and nutrient-rich wastewater (Saharan et al. 2013; González et al. 2018). Microalgae contain lipids (2–90% dry matter), protein (10–60% dry matter), and carbohydrates (5–50% dry matter), whose levels vary by species. The microalgae can be grown for direct burning or specifically for production of biodiesel, bioethanol, hydrogen, and biogas, depending on their characteristics (Varol and Ugurlu 2016). Microalgae, therefore, have been shown to be a promising alternative raw material for energy production, including for biogas.

However, in addition to the intracellular constituents, the cell wall structure should also be considered because some microalgae have low biodegradability, which directly affects biogas production (Fernández et al. 2013). The cell wall structure of microalgae (e.g., *Scenedesmus obliquus*) consists of glucose, mannose, and galactose (Takeda 1996). These compounds can form cellulose and hemicellulose, which give cell walls high resistance to enzymatic hydrolysis, limiting their availability for anaerobic digestion. In a comparative analysis of biogas production of different microalgae species, *Chlorella kessleri* and *S. obliquus* had the lowest methane yield (218 and 178 mL/g VS, respectively), while other chloroplast microalgae, such as *Chlamydomonas reinhardtii* (proteins without cellulose) and *Dunaliella salina* (without cell walls), produced biomethane yields of 387 and 323 mL/g VS, respectively (Mussgnug et al. 2010). These results suggest that microalgae species with thin cell walls are more digestible and should be preferred as feedstock for biogas production from anaerobic digestion (González et al. 2018).

Rempel (2018) produced biomethane from the waste of bioethanol production with microalgae, in particular, using the residuals of saccharification and fermentation stages. In addition to the residues, the potential of *Spirulina platensis* biomass was evaluated without any pretreatment. The fermentation residue was the substrate with the highest biomethane production potential 422 ± 15 LN (kg SV_{add}^{-1}) (Rempel 2018). The *Spirulina* biomass test produced a value of 326 ± 2 LN (kg SV_{add}^{-1}), similar to values for anaerobic *Spirulina* digestion found in the literature. The saccharification residue had the lowest production potential value, at 296 ± 10 LN (kg SV_{add}^{-1}). *S. platensis* is a microalga with high protein content, a high growth rate, and low lipid content (4–9%), making it attractive for biogas production through anaerobic digestion (Bruton et al. 2009).

Another means to improve the performance of anaerobic digestion is co-digestion of complementary substrates in the same reactor. In this case, the objective is to balance the substrate composition, including the *C*/*N* ratio, to promote the best microbial growth. The *C*/*N* ratio plays an important role in the stability of anaerobic digestion, and values of 15–30 have been shown to have a positive effect on methane yield (Sorensen 2000). Lower *C*/*N* ratios can result in inhibition by ammonia, while higher *C*/*N* ratios can cause nitrogen deficiencies for biomass synthesis. Co-digestion of different substrates thus creates a synergistic effect, alleviating the imbalance of nutrient concentrations and attenuating the effects of the potential inhibition of the use of individual substrates (Uggetti et al. 2017).

1.3 Pretreatments of Some Raw Materials for Biogas Production

Raw materials might not be ideal for biogas production for several reasons: (a) They cannot be digested by microorganisms; (b) digestion by microorganisms is very difficult; (c) digestion is possible but very slow; and (d) inhibitors are present in the raw material, or inhibitory compounds are produced during microbial degradation. The purpose of pretreatment is to facilitate the digestion process by removing these barriers and making the organic contents of the substrate easily accessible and usable by the microbial community.

Waste conversion into biogas can be hampered by the complex structure of many raw materials, such as lignocellulosic materials. The accessible surface area, cellulose crystallinity, and lignin content of lignocellulosic matter limit its digestibility (Hendriks and Zeeman 2009). Pretreatment before anaerobic digestion is necessary to overcome the limitations imposed by the hydrolysis rate (Taherzadeh and Karimi 2008). Pretreatment assists in the hydrolysis of lignocellulosic polysaccharides in soluble monosaccharides that can be readily used by microbial biocatalysts during anaerobic digestion (Monlau et al. 2012; Barua et al. 2018). Pretreatments can be classified as physical, chemical, biological, and various combinations (Chen et al. 2017; Patinvoh et al. 2017). The compatibility of raw materials, enzymes, and organisms should be considered in the choice of pretreatment.

1.3.1 Physical Pretreatments

Physical pretreatment methods do not use chemicals or microorganisms (Zheng et al. 2014). The main functions of physical pretreatment of raw material are to increase the surface area and size of the pores, rupture the structure of the biomass, and decrease the crystallinity. High energy consumption and frequent equipment

repair are the economic challenges (Wang et al. 2016). Common physical methods include mechanical size reduction, mechanical comminution, milling, irradiation (microwave and ultrasound), extrusion, pyrolysis, freezing, and steam explosion. Pretreatment with mechanical size reduction, mechanical comminution, or milling is the first step of pretreatment (Kumari and Singh 2018), commonly applied before other methods to facilitate and make the process more effective (Zubrowska-Sudol and Walczak 2014). The method of mechanical fragmentation includes dry and wet crushing, milling (e.g., two-roller milling, ball milling, hammer milling, disk milling, and colloidal milling), and compression. The method selected depends on the moisture content of the raw material (Zheng et al. 2014).

Reduction of particle size may alter the biomass's inherent structure, increase the feedstock's contact surface area with subsequent acid or enzyme application (Chen et al. 2017; Patinvoh et al. 2017), and reduce cellulose crystallization and polymerization to improve digestibility (Kratky and Jirout 2011). The type of physical pretreatment used determines the final particle size (Kumari and Singh 2018). Excessive reduction of biomass particle size may decrease biofuel production and result in the overproduction of inhibitory volatile fatty acids, which disrupt methane production during anaerobic digestion (De la Rubia et al. 2011). Combining size reduction with other pretreatments is more effective.

Irradiation is a physical pretreatment that involves microwave and ultrasound (Zheng et al. 2014). Pretreatment by microwave irradiation is the most conventional alternative technique due to its simple heating process. This technology has a simple operation, high uniformity and selectivity, and good energy efficiency because it has a short process time and lower power requirements than traditional heating (Kumari and Singh 2018). It can also improve the accessibility and reactivity of cellulose because it alters the biomass's cell wall structure and decreases cellulose crystallinity. Researchers have found that after microwave treatment, the lignocellulosic feedstock's adaptability to the enzymes increases, as does the subsequent effect of the enzyme activity (Chen et al. 2017). Microwave pretreatment has not been used separately to treat biomass but is generally applied to provide heat to assist acid or alkaline pretreatment (Cheng and Liu 2010). However, the disadvantage of pretreatment using microwaves is the high cost of equipment.

Ultrasound pretreatment can disrupt the cell wall structure, increase specific surface areas, reduce the degree of polymerization, open the crystalline regions of cellulose, decompose lignin molecules, and significantly improve the accessibility and chemical reactivity of cellulose, leading to increased biodegradability (Zheng et al. 2014). Ultrasonic pretreatment generates monolithic cavitations, resulting in physical and chemical effects on liquid solutions. The combination of these physical and chemical effects can destroy the cell wall structure. However, it has limited effect on the fine structure of cellulose. Ultrasonic treatment can decompose the hemicellulose, decreasing the ratio of the fiber area to the surface area, which negatively influences the subsequent enzymatic hydrolysis. Nevertheless, some studies have reported that ultrasonic pretreatment of biomass can improve cellulose saccharification (Yachmenev et al. 2009). Ultrasound pretreatment has been extensively studied and found to increase the biogas yield from the sludge.

Extrusion pretreatment is considered to be thermophysical because it involves mixing, heating, and cutting material, resulting in physical and chemical alterations (Zhan et al. 2006). The raw materials are fed into one end of an extruder and then transported along the length of the barrel with a drive screw (Ravindran and Jaiswal 2016). As the material moves, it is subjected to friction heat, mixing, and vigorous cutting after the release of pressure at the finishing end. The method is used for heating and shearing of humid biomass containing more than 15–20% moisture (Kumari and Singh 2018). This method is considered to be advantageous because it requires less energy than mechanical comminution, and the high mechanical shear ruptures the biomass structure, resulting in defibrillation and shortening of the fiber. Extrusion causes depolymerization of cellulose, hemicellulose, lignin, and protein (Karunanithy and Muthukumarappan 2010). Depending on the stress intensity of the extrusion screw, extrusion can also cause thermal degradation of sugars and amino acids, resulting in the degradation of slowly degradable compounds and even non-degradable ones. The extrusion process, under certain conditions, especially high pressure, can produce inhibitors (e.g., furfural and phenolic compounds) due to the degradation of sugar and lignin, which decreases biogas production (Williams et al. 1997). Care, therefore, should be taken with the treatment conditions to avoid or mitigate this problem.

Pyrolysis is a less energy-consuming endothermic process in which the lignocellulosic biomass is treated at a temperature higher than 300 °C (Kumari and Singh 2018). During pyrolysis, cellulose can decompose rapidly, resulting in the release of gaseous products and the production of coke-like residue. The coal residue is treated with weak acid and leached with water. The main component of the water–leachate is glucose, which can serve as a carbon source for biofuel production (Chen et al. 2017).

Freezing is a recently developed physical pretreatment of biomass. It has the capacity to significantly increase the digestibility of the lignocellulosic biomass enzyme. The method has specific characteristics, including low environmental impacts, high productivity, and the application of less hazardous chemicals. However, it has a very high cost, so it has not been applied to many studies (Kumari and Singh 2018).

Steam explosion is one of the most common pretreatment methods for lignocellulosic biomass. In this method, the biomass particles are heated with high-pressure saturated steam for a short period of time, and then the pressure is rapidly reduced to terminate the reactions, causing the biomass to undergo explosive decompression. Typical pretreatment temperature, pressure, and time are within the range of 160–260 °C and 0.69–4.83 MPa for a few seconds or a few minutes, respectively (Sun and Chen 2002). Under these conditions, the hemicellulose is hydrolyzed into its constituent monomers, and the lignin is transformed to a certain degree, making the pretreated biomass more degradable (Zheng et al. 2014). Steam explosion is considered to be one of the most effective pretreatment technologies for pilot and commercial scale applications, especially for wood and agricultural waste (Marousek 2012).

Given the advantages and disadvantages of different pretreatment methods, a successful physical pretreatment must be able to: (1) improve the digestibility of the raw materials for microorganisms; (2) avoid degradation or loss of carbohydrates; (3) prevent formation of inhibitors; (4) require minimal, low-cost chemicals or water; (5) avoid costly pretreatment reactors; (6) need limited size reduction; (7) require low energy input (heat or power); (8) avoid the need for waste disposal; and (9) be cost-effective and environmentally sound (Taherzadeh and Karimi 2008). In addition, the type of biomass should be considered in the choice of pretreatment technology.

1.3.2 Chemical Pretreatments

Chemical pretreatments are widely used with lignocellulosic biomasses and are generally viewed as satisfactory. Compared to physical and biological methods, chemical pretreatment has received more attention because it is generally less expensive and is faster and more efficient at increasing the degradation of complex organic materials (Song et al. 2014; Zhou et al. 2012). However, it also requires special equipment and generates more severe pollution (Chen et al. 2017). Chemical pretreatment can be used with lignin-rich biomasses that could not otherwise be digested (Rodriguez et al. 2017). Commonly used chemical pretreatments are acid, basic, oxidation, ionic liquid, and organosolv processes.

In acid pretreatment, inorganic acids (sulfuric, nitric, hydrochloric, and phosphoric acids) and organic acids (formic, acetic, and propionic acids) are used (Chen et al. 2017; Pierre et al. 2015; Gámez et al. 2006; Martínez et al. 2015; Aslanzadeh et al. 2014). Its primary functions are to separate and remove lignin and hydrolyze plant fibers. Here, hydrolysis refers mainly to the hydrolysis of cellulose and hemicellulose. Although cellulose has a crystalline structure and high acid resistance, these compounds are commonly used to solubilize hemicellulose and are not as efficient at solubilizing the lignin present in the biomass. Acid pretreatment makes hemicellulose more available for enzyme attacks (Zheng et al. 2014).

Acid pretreatment can be carried out using concentrated acids (30–70%) and low temperatures (around 40 °C) or diluted acids (around 0.1%) and high temperatures (230 °C). Concentrated acid is highly effective at cellulose hydrolysis but is extremely toxic, corrosive, and hazardous and requires expensive equipment, such as nonmetallic materials and specialized alloys, for the construction of reactors. In addition, for economic reasons, the acid must be recovered after treatment of biomass because the process has high energy consumption and costs. Consequently, diluted acid is favored over concentrated acid for pretreatment of lignocellulosic biomass and has become one of the most commonly applied and extensively studied chemical pretreatment methods. Diluted acid pretreatment hydrolyzes up to 100% hemicellulose in its sugar components (erg xylose, arabinose, and galactose), depending on the pretreatment conditions. It can also disrupt lignin to a high degree but is not effective at dissolving lignin in most cases. The main function of diluted

acid pretreatment is to significantly increase cellulose susceptibility to microbial degradation and enzymatic hydrolysis (Rodriguez et al. 2017; Chen et al. 2017).

Alkaline pretreatment is more efficient than acid treatment at solubilizing lignin, depending on the biomass's lignin content. The most commonly used bases are sodium, ammonium, calcium, and potassium hydroxides. By removing cross-links, alkaline pretreatment increases the biomass's porosity and inner surface area, causes structural swelling, decreases polymerization and crystallinity, ruptures the lignin structure, and breaks down lignin and other polymers (Zheng et al. 2014; He et al. 2009). Sodium hydroxide is the most popular base used in alkaline pretreatment and has improved the biogas yield from lignocellulosic biomass in numerous studies (Cho et al. 2013). The residual base remaining in the pretreated biomass can help prevent decreased pH during acidogenesis (Cho et al. 2013; Behera et al. 2014).

Wet oxidation is a pretreatment in which water and an oxidizing agent (e.g., air, oxygen, and hydrogen peroxide (H_2O_2)) are added to the raw materials before pretreatment under a high temperature (125–300 °C) and high pressure (0.5–20 MPa). The treatment time is the most critical factor in wet oxidation and varies from a few minutes to hours. Insulation of oxygen can increase reaction rates and production of free radicals. Although faster reaction rates can be obtained with high oxygen concentrations, the use of pure oxygen results in high operating costs. Air is usually used as an oxidizing agent in wet oxidation pretreatment. This process is exothermic, so in most cases the heat produced by the reactions is sufficient to maintain the desired temperature once pretreatment has begun, eliminating or minimizing power inputs. This process can be carried out at a relatively lower temperature because it generates heat. The water content is critical for the process, and water should be added to dry biomass, such as wood and straw. During wet oxidation, the main reactions include electrophilic substitutions, oxidative cleavage of aromatic nuclei, displacement of side chains, and cleavage of alkyl aryl ether linkages (Zheng et al. 2014).

Wet oxidation can effectively increase the biological accessibility of the cellulose fraction to microorganisms and enzymes by removing lignin and hemicellulose. In the case of lignocellulosic biomass, all three major fractions are affected. Hemicellulose is generally broken into monomeric sugars and degraded in organic acid, cellulose is partially degraded, and lignin undergoes cleavage and oxidation (Hendriks and Zeeman 2009).

Pretreatment ionic liquids include *N*-methylmorpholine-*N*-oxide monohydrate (NMMO), 1-*n*-butyl-3 methylimidazolium chloride (BMIMCl), 1-allyl-3-methy limidazolium chloride, 3-methyl-*N*-bytylpyridinium chloride (MBPCl), and benzyldimethyl (tetradecyl) ammonium chloride. In the pretreatment of lignocellulosic biomass, these compounds can improve enzymatic digestibility (Liu and Chen 2006). The mechanism of cellulose dissolution in ionic liquids involves the oxygen and hydrogen atoms of the hydroxyl groups of cellulose, which form complexes donor or receptors of electrons that interact with ionic liquids (Feng and Chen 2008). After the interaction between the hydroxyl groups of the cellulose and the ionic liquids, the hydrogen bonds are broken, which opens the hydrogen bonds

between the molecular chains of the cellulose and results in the dissolution of the cellulose (Zhu 2008). The solubilized cellulose can be precipitated rapidly with antisolvents, such as ethanol, methanol, acetone, and water.

The organosolv method involves pretreatment of lignocellulosic materials with organic solvents, such as low-boiling alcohols, to chemically break down the lignin fraction through cleavage of ether bonds and their subsequent dissolution (McDonough 1992). The organic solvent partially hydrolyzes the lignin fraction and the bonds between the lignin and the carbohydrates, removing the main barrier to an enzymatic attack. Organic solvents, such as low-molecular-weight alcohols and organic acids, used in lignocellulosic pretreatment do not inhibit methane-producing microorganisms because microorganisms in the anaerobic digestion system can use these readily degradable compounds (Kabir et al. 2015). The main advantages of the organosolv method are the easy recycling of the solvent by distillation and the recovery via precipitation of a highly pure lignin fraction, that consists an economically valuable by-product with various applications in fuel and chemical products industries (Ostovareh et al. 2015).

1.3.3 Biological and Enzymatic Pretreatments

Pretreatment improves the accessibility of cellulose, which can increase biogas production. A main objective of biological pretreatment, therefore, is to minimize carbohydrate loss and to maximize lignin removal, both efficiently accomplished by the anaerobic digestion process (Zheng et al. 2014). The biological pretreatment of biomass to increase biogas production in anaerobic digestion mostly uses fungi, microbial consortium, and enzymes (Rodriguez et al. 2017).

Fungal pretreatment studies have primarily evaluated fungi that selectively degrade lignin and hemicellulose while using low cellulose, which is more recalcitrant to fungal attack than other components. Degradation of lignin and hemicellulose increases cellulose digestibility, which is preferred to anaerobic digestion processes. Several classes of fungi, including brown, white, and soft-rot fungi, are used to degrade lignocellulosic biomass. White and soft-rot fungi attack cellulose and lignin, while brown rot attacks mainly cellulose. White rot has been shown to be the most effective fungus at degrading lignocellulosic biomass (Sun and Chen 2002). The efficiency of delignification heavily depends on the production of lignolytic enzymes, such as lacasse, lignin peroxidase, and manganese peroxidase. *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *T. versicolor*, *Flammulina velutipes*, and *Ceriporiopsis subvermispora* are among the selective lignin degradation fungi used to reduce the recalcitrance of the lignocellulosic biomass (Sindhu et al. 2016) and to increase methane generation (Lalak et al. 2016; Amirta et al. 2006; Zhao et al. 2014).

Pretreatment with microbial consortium is performed by microorganisms selected from natural environments whose substrate is the lignocellulosic biomass. Whereas fungal pretreatment mainly attacks lignin, a microbial consortium generally has high

degradability of cellulose and hemicellulose. In addition to consortia selected from natural environments, complex microbial agents in a lyophilized powder, containing a mixture of pure strains of yeast and cellulolytic bacteria, are also used in biological pretreatment (Zhang et al. 2011). In most cases, sterilization of lignocellulosic feedstock is not necessary when using a microbial consortium for pretreatment, an advantage over fungal pretreatment (Zheng et al. 2014).

Enzymatic hydrolysis is another biological pretreatment. Although enzymes are already present in digesters produced by digestion microorganisms, an enzyme or mixture of enzymes can be added to increase the degradation of the biomass. Cellulosic, hemicellulosic, and starch-degrading enzymes are most frequently used for lignocellulosic feedstock. Pretreatment by enzymatic hydrolysis may provide an alternative to energy-demanding thermal and mechanical pretreatments and to chemical pretreatments because enzymes are safer compounds than chemicals (Agbor et al. 2011). In most cases, enzymes increase biogas production only minimally and have high costs, so the application of enzymatic pretreatment has been limited. However, a study using mushroom compound extract with laccase activity and carboxymethylcellulose to pretreat cellulose and paper sludge increased methane production by 34.2% (Lin et al. 2010). The success of enzyme pretreatment is a function of enzyme type, enzyme stability, dose, incubation conditions (e.g., temperature, pH, and time), inhibitors, and many other factors (Bonilla et al. 2018). Compared to enzymatic pretreatment, fungi and microbial consortium pretreatment produce much better results in the anaerobic digestion process due to its greater functional diversity and tolerance of environmental factors, such as temperature and pH (Shrestha et al. 2017).

Compared with physical and chemical pretreatment, biological pretreatment requires much less energy and does not generate any inhibitors (phenolic, furfural, and hydroxymethylfurfural compounds) during anaerobic digestion (Mosier et al. 2005). Biological pretreatment can be conducted in milder environmental conditions, so few inhibitors can adversely affect anaerobic digestion (Alexandrovoulou et al. 2016; Taherzadeh and Karimi 2008). However, most biological pretreatments are not as effective as chemical pretreatments, and the required prolonged treatment time ranges from one to several weeks. Before biological pretreatment is feasible for application in commercial biogas production, additional research is needed to address key issues, such as cost, selectivity, and efficiency.

1.4 Final Considerations

Biogas production through anaerobic digestion can be performed using a wide variety of residues. However, for an efficient process, it is necessary to evaluate the nutrients' bioavailability and biogestibility characteristics and the proportions of the available compounds. These issues, when properly evaluated, may favor the development of anaerobic digestion, supporting the use of this biological process in treatment of a wide range of biomass raw materials for biogas production.

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Chapter 2
Physical, Chemical, and Biological Substrate Pretreatments to Enhance Biogas Yield

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Abstract Anaerobic digestion is an environmentally friendly technology for the stabilization and recovery of biodegradable organic waste, both agroindustrial and urban. Hydrolysis is the first and one of the main steps of the anaerobic digestion process, as it determines the overall biodegradation rate of the substrates. Fibrous materials, for example, although rich in carbon, present sugars protected by lignocellulosic structures, which hinders their biodegradability. Lipid residues present a great energetic potential; however, they are hydrophobic, which hinders their hydrolysis. Residues that have coarse granulometry tend to exhibit long periods of biodegradation due to their small surface areas and difficult solubilization. In this regard, the present chapter will discuss the application of pretreatments of substrates for anaerobic biodigestion by physical, chemical, and biological methods. The aim is to facilitate the hydrolysis and increase the energy and nutritional use of the residues in shorter time intervals, increasing the yield and optimizing the biogas production chain.

Keywords Anaerobic biodigestion · Hydrolysis · Biodegradability

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2.1 Introduction

Anaerobic digestion (AD) is a biotechnology that combines the stabilization of organic matter with the recycling of energy and nutrients contained in biodegradable organic substrates through biogas production. Biogas is considered to be a renewable energy source with the potential to complement energy matrices in order to reduce dependence on fossil fuels, thereby minimizing climate change related to greenhouse gas emissions and reducing energy security problems through diversification of the matrices. Relevant information on the biogas production process and its benefits have been reported in the literature for years. However, one of the current major challenges is to maximize bioenergy production from recalcitrant substrates. AD is a technology with proven efficiency, being widely used in the stabilization of industrial wastewater, urban solid waste, animal manure, and sewage sludge.

The biochemical and sequential conversion phases involved in AD are classified as hydrolysis, acetogenesis, acidogenesis, and methanogenesis. Of these four phases, it is believed that the global rate-limiting step is hydrolysis, especially when dealing with substrates of difficult degradation. Methanogenesis, in turn, limits the rate of digestion of more biodegradable substrates.

In practice, processes of AD are often operated below their ideal performance, mainly due to a limited degradation of recalcitrant substrates. Thus, the use of a pretreatment to improve biodegradability is a prerequisite for AD of lignocellulosic biomass, for example. Most agricultural biomass contains lignocellulosic compounds, but the relative amount of these compounds in the biomass varies.

Since the available substrates for AD have different properties, the use of a specific pretreatment is of extreme importance in order to increase digestibility and biogas production. Figure 2.1 shows a schematic summary of different pretreatment techniques that can be used on different substrates. It is of extreme importance to point out that many of the pretreatments can be used together or in sequential processes in order to further increase the conversion of the substrates into bioenergy.

The purpose of this chapter is to present the fundamentals and the state of the art of different types of pretreatment for AD of several biomasses, reporting some advantages and disadvantages of each method. In addition, several researches that obtained promising results for increasing biogas production using pretreatment techniques were compiled.

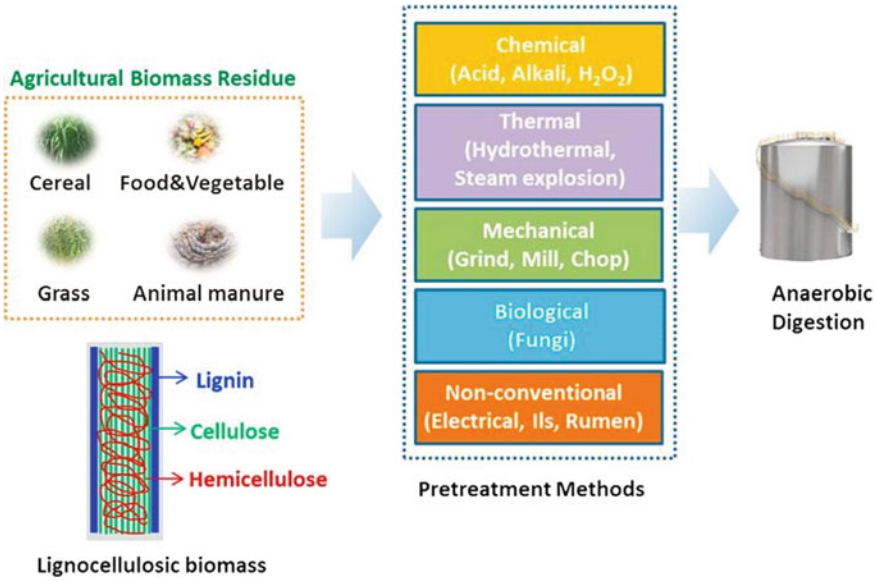

Fig. 2.1 Scheme of the different pretreatments that can be used to increase biogas production. *Source* Adapted from Paudel et al. (2017)

2.2 Physical Pretreatments

2.2.1 Mechanical Pretreatment

Mechanical pretreatments are widely diffused, since they generally do not require sophisticated technologies and, therefore, present low operating costs (Mata-Alvarez et al. 2000). Among the mechanical pretreatments, the sieving and grinding stand out due to their simplicity.

The purpose of mechanical pretreatments is to reduce the granulometry of the substrate. These procedures, in addition to avoiding possible operational problems of the biodigester, such as clogging, increase the specific surface and solubility of the substrate, in order to facilitate the microbiological attack and, therefore, its conversion into biogas.

According to Meena et al. (2011), mechanical pretreatments catalyze enzymatic hydrolysis and increase methane yields by reducing particle size. In addition, mechanical treatment does not produce toxic or inhibitory substances, and does not yield complex molecules that are difficult to digest (Menardo et al. 2012).

2.2.1.1 Sieving

In anaerobic digestion, the unitary sieving process consists of the separation of solid (coarse solids) and liquid fractions. This simple technology can be very attractive because the granulometry of the substrate particles will be, at most, the size of the mesh of the sieve used. Therefore, the smaller the mesh, the smaller the particles in the liquid fraction. This makes it possible to reduce sludge formation and hydraulic retention time of the substrate in the biodigester. In addition, it can also improve the biogas production because the slower biodegradability materials are retained in the sieve mesh, while the solubilized and more easily solubilized substances enter the biodigester.

In this way, sieving can be a good strategy for the recovery of substrates/residues of confined animal production systems and slaughterhouses (red lines), since such activities produce large volumes of wastewater.

do Amaral et al. (2016) studied the influence of the separation of fractions of raw swine wastewater on biogas production. The fraction separation was performed using a 2-mm mesh sieve. Thus, a solid fraction retained in the mesh (grain size > 2 mm) and a liquid fraction were obtained. The liquid fraction was subdivided into supernatant and total suspended solids after 1 h of decanting. The authors concluded that the fractions showed different yields of biogas and methane. The supernatant fraction presented organic matter more bioavailable than the other fractions and reached the highest biogas and methane production. Although the sediment sludge fraction had higher concentrations of volatile solids than the other two fractions, it had lower biogas yields and slower degradation kinetics.

Sieving, in fact, allows the separation or concentration of coarse solids, which can contribute substantially to the yield of biogas and methane production. However, if the separation of the liquid fraction for the energy recovery is performed, other biological processes must be integrated to the anaerobic digestion in order to stabilize the organic substrates of the coarse solids. Composting and/or vermicomposting may be attractive alternatives for the agronomic valorization of the nutrients contained in the solid fraction of the substrates (de Costa et al. 2016).

2.2.1.2 Grinding and Milling

Grinding and milling pretreatments are unitary operations which purpose is to reduce the size of larger particles. This occurs through the application of forces of impact, compression, and abrasion. These pretreatments can be performed using different mills (disks, knives, hammers, rolls, balls) and propeller and jaw crushers (Appels et al. 2008).

Among the advantages of particle size reduction is the increase in surface/volume ratio, uniformity and solubility of the processed material, making subsequent operations more efficient, such as heating, dehydration, cooling, biological degradation (Elliot and Mahmood 2012), among others. For these reasons, often the pretreatment of grinding or milling precedes or is combined with other

pretreatments, such as thermal, ultrasonic, acidic, basic, among others pretreatments. These efforts are undertaken to improve the rate of hydrolysis in order to maximize methane production (Esposito et al. 2011).

Grinding of wheat straw and rice straw with a size of 0.75 mm increased methane production by 38.7% (Chandra et al. 2015). Maceration is also effective in reducing the size of recalcitrant fiber waste in animal manure. Maceration of manure fibers to size 2 and 0.35 mm increased methane production by 16 and 20%, respectively (Angelidaki and Ahring 2000). In addition to increased methane production, milling can reduce the incidence of operational problems of large-scale reactors. In real plants, grinding or milling of fibrous substrates and/or of substrates with larger granulometry is necessary to avoid possible pipe obstructions (Carrère et al. 2016).

Izumi et al. (2010) studied the effect of particle size of food residues submitted to anaerobic digestion and reported that the reduction in diameter from 0.843 to 0.391 mm improved the total chemical oxygen demand (COD) solubilization by 40%, and the reduction of particle size from 0.888 to 0.718 mm increased methane production by 28%. However, it was observed by the authors that granulometry less than 0.5 mm caused an accumulation of organic acids of short molecular chain, which can inhibit the methanogenic activity and decrease the anaerobic digestion performance.

Therefore, it is possible to state that the inversely proportional relation between the granulometry of the substrate and the methane production is valid up to a certain limit, varying from substrate to substrate and according to the operating conditions of the biodigester. In such cases, sieving may be effective in selecting the desired particle size after pretreatment or can be used to reduce the concentration of solids added in biodigesters in order to decrease the chances of alkalinity and acidification problems occurring.

2.2.2 Ultrasound

The effect of ultrasonic pretreatment is based on the monolithic cavitation process, that is, sound waves excite the water molecules present in the substrate, causing them to vibrate and move at high speeds. This movement causes reduction of the pressure of the medium, causing the formation of bubbles. When they pass through areas with higher pressures, these bubbles implode, releasing a shock wave that may be strong enough to shear organic macromolecules (Carrère et al. 2016).

In this way, this technique can have physical and chemical impacts on the substrates. According to Gronroos et al. (2004), the implosion of the cavitation bubbles during sonification modifies the chemical structure by the creation of free radicals. This disintegration leads to increased digestibility and, consequently, higher microbial activity which, in turn, improves biogas yield (Kwiatkowska et al. 2011).

Zeynali et al. (2017) used a sonotrode of 38 mm in diameter, operating at 20 kHz and amplitude of 80 μm, to study the effect of ultrasonic pretreatment on shredded vegetable residues (5 mm in diameter) with 0 (control), 9, 18, and 27 min of sonification. The highest biogas yield was achieved with 18 min of ultrasound. Methane production after the ultrasonic pretreatment was 80% higher than that obtained with the control, which compensates for the energy required for sonication at laboratory scale (3.7 kJ g^{-1} SV).

The impact of ultrasound has been extensively investigated within the scope of anaerobic digestion of municipal sludge and wastewater. However, there are few reports in the literature on the application of ultrasound to solid substrates, because in order to increase the effectiveness of pretreatment, the solids are generally ground and diluted in water.

Viéitez and Ghosh (1999) examined the effect of ultrasonic pretreatment on biogas production from kitchen waste and stated that sonication time and density (W mL^{-1}) have significant effects on biogas yield.

Although the positive effect of ultrasound on biogas yield is proven, the efficiency of this pretreatment in terms of net energy yield is still little discussed. The main concern in the practical application of ultrasound is its high energy consumption. The energy needed for sonication is related to potencies and time; however, it is already known that ultrasonic powers have more effect than exposure time (Zhang et al. 2008; Carrère et al. 2016).

However, it is noteworthy that studies have indicated that the noise of ultrasonic devices can cause negative symptoms in exposed operators, such as dizziness, tinnitus, excessive fatigue, nausea, ear fullness, and headache. Therefore, it has been suggested to control ultrasonic pollution using steel or even glass frames, along with acoustic blankets to coat the machine enclosure to reduce noise (Smagowska and Pawlaczyk-Luszczynska 2013).

2.2.3 Thermal Pretreatment

The thermal pretreatments applied to the anaerobic digestion substrates consist in the use of thermal energy to cause intense molecular agitation, in order to promote the hydrolysis and, consequently, to cause an increase in the methanogenic production in a shorter period of time. In addition, heat can also be employed to eliminate resistant pathogens, such as viruses, for example, by dehydrating protein substrates, such as animal carcasses. In these cases, the energy costs from the heat source must be compensated by the increase in methane production (Bougrier et al. 2006).

A wide temperature gradient was studied in order to improve the digestibility of the substrates (Bordeleau and Droste 2011). Thermal pretreatments usually use temperatures ranging from 60 to 180 °C, since temperatures above 200 °C can form refractory, inhibitory, or toxic compounds (Rodriguez-Abalde et al. 2011).

As the temperatures of the thermal pretreatment can be high, the heat is generally combined with pressure. Depending on the type of heating method, the thermal pretreatment can be called hydrothermal, steam explosion or thermobaric (Rajput et al. 2018).

Very high temperatures can incite Maillard reactions and have a reverse effect. Such chemical reactions convert carbohydrates and amino acids into melanoidins—recalcitrant compounds that hinder biological degradation (Rodriguez-Abalde et al. 2011; Liu et al. 2012).

Thermal pretreatments may also induce a release of inhibitory products, such as large concentrations of ammonia and soluble inert organic matter, that hinder the anaerobic digestion process by being toxic to methanogenic archaea (Phothilangka et al. 2008).

According to Choi et al. (2018), pretreatment of activated sludge at 180 °C for 76 min (optimum observed condition) provided increases in the solubility of proteins, carbohydrates, and volatile acids in the order of 1.4, 3.3, and 10.1 times, respectively, when compared to the untreated substrate. Such increases in solubility from thermal hydrolysis caused a 17% increase in methanogenic yield, without showing evidence of refractory compounds formation.

Rajput et al. (2018) subjected wheat straw to thermal pretreatment using temperatures of 120, 140, 160, and 180 °C and reported that temperatures above 160 °C promoted changes in the lignocellulosic structure and increase in the cellulose content of the straw. This higher availability of sugars caused by the pretreatment increased the biogas yield by 53% when compared to the yield of raw straw.

When using temperatures below 100 °C, there is a need to extend the time of the thermal pretreatment to achieve good results. This is because in the thermal range close to 60 °C, for example, molecular agitation has no significant effect, and the hydrolysis of the substrate is carried out by hydrolytic thermophilic microorganisms, which require more time to produce the enzymes needed (Carrère et al. 2016).

2.3 Chemical Pretreatments

In this group are the pretreatments that are purely initiated by chemical reactions that modify the structure of the biomass. They are most often used for lignocellulosic substrates and improve the biodegradability of biomass components (Zheng et al. 2009).

Chemical pretreatments consist in the use of different acids, bases, or oxidizing agents to extract or decompose the organic compounds present in the biomass (Ariunbaatar et al. 2014). The main function of most biomass chemical pretreatments is the destruction of rigid and/or complex structures.

2.3.1 Acid Pretreatment

Strong concentrated acids are used for the treatment of lignocellulosic materials, as they are powerful agents for the hydrolysis of cellulose (Sun and Cheng 2002). They can hydrolyze hemicellulose and solubilize lignin. However, solubilized lignin, which precipitates in a short time, reduces the digestibility of organic matter (Frigon and Guiot 2010).

The use of sulfuric and nitric acid seems to decrease biogas production and methane concentration in the produced biogas because other gases, such as sulfites and nitrite ions, are produced (Castelli 2011; Venturin et al. 2018). Mussoline et al. (2013) reported that treatment with strong acids such as H_2SO_4, HNO_3, H_3PO_4, and HCl tend to inhibit the anaerobic digestion process through the production of undesirable by-products such as furfural and its derivatives.

Strong acids cause excessive degradation of the complex substrates, resulting in loss of volatile solids. From an economic point of view, they tend to negatively impact the digestion process (Kumar and Murthy 2011; Taherzadeh and Karimi 2008). Although they are highly efficient in cellulose hydrolysis, the concentrated acids are extremely corrosive, requiring high-cost materials to build the reactor, such as specialized nonmetallic materials or alloys (Zheng et al. 2014). In addition, they are highly toxic and dangerous, posing risks to the operator. Thus, it is more appropriate to adopt pretreatments with diluted acids (<4% w/w). Dilute acid pretreatment is often associated with high temperatures (>100 °C), becoming a thermochemical pretreatment (Agbor et al. 2011). Gu et al. (2011) combined the effects of acid treatment and thermal process, achieving positive results in terms of methane production (67% increase).

Thus, the use of dilute acids associated with high temperatures is a more suitable technique for pretreatment of lignocellulosic materials when compared to the use of concentrated acids. According to Zheng et al. (2014), the pretreatment with dilute acid hydrolyzes up to 100% of the hemicellulose depending on the granulometry of the material. However, in most cases, it is not effective for the solubilization of lignin. Thus, the main objective of using an acid pretreatment is to make cellulose more susceptible to microbial degradation and to the action of hydrolytic enzymes.

2.3.2 Alkaline Pretreatment

Although acid pretreatments are available to improve the biodegradability of organic wastes (Ariunbaatar et al. 2014), basic pretreatment is the most appropriate because it provides better operating conditions for anaerobic digestion. The presence of a small amount of alkaline residues in the pretreated material can prevent the pH drop during the acidogenesis process, for example (Li et al. 2012; Taherdanak and Zilouei 2014).

The main effect of alkaline pretreatment is the removal of lignin from the lignocellulosic biomass, improving the biodegradability of the remaining polysaccharides. The reaction is based on the saponification of intermolecular ester bonds (Castelli 2011; Sun and Cheng 2002). Several alkaline solutions of NaOH, KOH, $Ca(OH)_2$, and NH_3 can degrade the lignin and disrupt the binding between lignin and carbohydrates, leading to a change in structure. There is also swelling and increase of the specific surface of the organic matter (Carlsson et al. 2012). The substrate becomes more accessible to the microbial enzymes, favoring anaerobic digestion (Modenbach and Nokes 2012; Torres and Lloréns 2008; Yao et al. 2018).

The execution of a basic pretreatment is interesting when substrates composed of large amounts of lipids are used, such as residues from slaughterhouses, restaurants, among others. The oils, fats, and greases present in the substrate are insoluble, less dense than water and present slow biodegradation. During AD, lipids are hydrolyzed by extracellular lipases forming glycerol and long-chain fatty acids (LCFA). The limiting step of the degradation process of these compounds is the mass transfer from the solid to the liquid fraction, as well as the biodegradation of the LCFAs by the microorganisms (Battimelli et al. 2010). Thus, conversion of free lipids and LCFAs to soluble soaps by alkaline pretreatment can improve the contact between the substrate and the microorganisms, enhancing their biodegradability.

2.3.2.1 Calcium Hydroxide and Sodium Hydroxide

The use of calcium or sodium hydroxides during the alkaline pretreatment forms salts that can be incorporated into the biomass. These salts can disrupt the subsequent steps, so they need to be removed or recycled (González et al. 1986). The process conditions are relatively mild, temperature around 40 °C, but with a long reaction time (Sambusiti et al. 2013). These mild conditions prevent condensation of the lignin, resulting in high solubility of this compound, especially for biomass with low lignin content, such as grasses. The addition of air or oxygen to the reaction improves the delignification process (Chang and Holtzapple 2000). According to Chandra et al. (2012a, b, c), NaOH treatment is particularly advantageous in wheat and rice straw, resulting in an increase in methane production of 112% and 124, respectively, and an average increase in biogas yield of 87.5%.

2.3.2.2 Ammonia

Pretreatment of biomass with aqueous ammonia at elevated temperatures reduces the lignin content and removes some hemicelluloses. Ammonia pretreatment techniques include ammonia fiber expansion (AFEX), ammonia recycle percolation (ARP), and soaking in aqueous ammonia (SAA) (Kim et al. 2003, 2008; Kim and Lee 2005).

The cost of ammonia, and especially its recovery, increases pretreatment costs (Holtzapple et al. 1991; Holtzapple et al. 1994). However, the global economy of

the process is positive because of the high yields achieved. Using ammonia as an alkaline pretreatment in wheat straw, some authors reported positive effects, increasing biogas production by 40% (Li et al. 2015).

2.3.3 Oxidative Pretreatment

Delignification can also be achieved by pretreating the biomass with an oxidizing agent, such as hydrogen peroxide, ozone, oxygen, or even air. The efficacy in delignification can be attributed to the high reactivity of oxidizing agents with the aromatic ring present in the biomass. In addition to the effect on lignin, oxidative treatment can also attack the hemicellulose of the lignocellulosic complex. This pretreatment is associated with electrophilic substitution, displacement of side chains, cleavage of alkyl-aryl-ether linkages or oxidative cleavage of aromatic nuclei.

Oxidizing compounds should be used with caution because they are not selective in relation to lignin, so that hemicellulose and cellulose can also be lost, reducing the volatile solids content and, consequently, decreasing the final volume of biogas produced (Castelli 2011).

2.3.3.1 Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) has the ability to remove lignin and hemicellulose from the biomass, resulting in an increase in cellulose content (Hendriks and Zeeman 2009). Pretreatment with H_2O_2 should occur at high pH, about 11.5, which is pKa for H_2O_2. Hydroxyl (HO•) and superoxide (O•) radicals are formed, which are extremely reactive, readily attacking the lignin, resulting in low molecular weight compounds. There is no generation of by-products and chemical inhibitory residues.

Venturin et al. (2018) studied the pretreatment of corn stem with alkaline hydrogen peroxide and reported a 22% increase in the volume of biogas generated. Sun et al. (2015) reported a positive effect for cotton stalks, with an increase in methane production of 25%.

Song et al. (2013) reported positive effects of an oxidative process on rice straw, so that the treated material produced 88% more methane than the untreated straw. Michalska et al. (2012) also obtained satisfactory results combining the oxidative treatment of straw with biological processes.

2.3.3.2 Ozone

Ozone treatment promotes an increase in the digestibility of the treated material without producing toxic residues (Kumar et al. 2009). Lignin degradation occurs

through the attack and cleavage of aromatic ring structures, whereas hemicellulose and cellulose are hardly decomposed. It can be used to degrade the structure of different lignocellulosic materials, such as cotton straw, wheat straw, bagasse, pine, peanut, and poplar sawdust (Sun and Cheng 2002). Heiske (2013) used the ozonolysis process to treat wheat straw, which increased biogas production by 45%.

Some advantages of the ozone pretreatment are that it can occur at room temperature and at atmospheric pressure. The disadvantage of this process is that a large amount of ozone is needed, increasing the cost of this pretreatment.

2.3.3.3 Wet Oxidation

The wet oxidation operates with oxygen or air in combination with water at elevated temperature and pressure (McGinnis et al. 1983). It was presented as an alternative to the steam explosion, which has become the most widely used pretreatment method. Industrially, the processes of wet oxidation with air have been used for the treatment of wastes with high content of organic matter. It is used in the oxidation of soluble or suspended materials, using aqueous phase oxygen at high temperatures (150–350 °C) and high pressure (5–20 MPa) (Jorgensen et al. 2007).

2.3.4 Ionic Liquids

Ionic liquids (ILs) are salts that are in the liquid phase at a temperature as low as room temperature. There is a wide variety of ILs; however, they share a common feature; they are usually composed of an inorganic anion and an organic cation of very heterogeneous molecular structure. The difference in molecular structure makes ion binding weak enough for the salt to behave as liquid at room temperature (Xie et al. 2017).

However, due to their polarity and in general their unique properties, they can function as selective solvents of lignin or cellulose. This results in the separation of lignin and in the increase of cellular accessibility in environmental conditions, increasing methane production. This technique avoids the use of acid or alkaline solution and the formation of inhibitory compounds (Gao et al. 2013).

2.4 Biological Pretreatments

In biological treatments, microorganisms are used to hydrolyze complex organic chains, such as protein polymers, lipids, and carbohydrates into simpler molecules: amino acids, long-chain fatty acids, and sugars, respectively. The hydrolysis reaction affects the downstream process, influencing the conversion of the biomass in the desired product (Jain et al. 2015). Substrates with high levels of

lignocellulosic biomass are limited to direct use in anaerobic digestion, because lignin, present in this type of biomass, blocks the access of microorganisms to cellulose and hemicellulose. In addition, lignin causes nonspecific binding of enzymes, reducing the enzyme activity for the hydrolysis of cellulose (Croce et al. 2016).

2.4.1 Fungi

Pretreatment with fungi is an alternative to overcome the recalcitrance of the lignocellulosic biomass by the degradation of lignin and to improve the yield of the biogas. *Ceriporiopsis subvermispora* is a fungal species that selectively degrades lignin, although its selectivity varies with the type of biomass and the time of harvesting. The fungal pretreatment of giant cane with *C. subvermispora* increased glucose yields by 20 and 22% in November and December harvests, respectively, and increased biogas methane contents by 63–66% in four days of anaerobic digestion (Liu et al. 2016).

Mustafa et al. (2016) subjected rice straw to fungus pretreatment using *Pleurotus ostreatus* and *Trichoderma reesei* to improve their biodegradability and methane production via solid-state anaerobic digestion. The fungus *P. ostreatus* significantly degraded lignin (33.4%) with selectivity (lignin/cellulose removal ratio) of 4.30 (optimal value). The fungus *T. reesei* degraded lignin (23.6%) to an optimum selectivity of 2.88. Selectivity value of 1.0 indicates that both lignin and cellulose were lost during pretreatment (Saha et al. 2016).

Pretreatment with *P. ostreatus* and *T. reesei* resulted in a 120 and 78.3% increase in methane production, respectively. The increase in methane production showed a strong direct linear correlation with the value of the selectivity and a weak relation with the degradation of the lignin during the pretreatment. Therefore, to improve methane production using this pretreatment, both lignin degradation and high selectivity during the process must be guaranteed (Mustafa et al. 2016).

Pretreatment of rice straw with *P. ostreatus* causes degradation in the fibrous structure: The lignin fibers are damaged and the secondary cell wall is exposed, leading to an increase of the surface area (Fig. 2.2c), while the nontreated straw shows a compact and rigid structure (Fig. 2.2a) and the control (autoclaving) damages the fibrous structure (Fig. 2.2b), forming micropores on the surface of the lignocellulosic material (Mustafa et al. 2016).

The increase in the surface pore allows enzymes to migrate through the cell wall during the early stages of degradation (Wan and Li 2010) and may also facilitate enzymatic hydrolysis in a subsequent step.

Saha et al. (2016) investigated the pretreatment of corn straw by white rot fungi under solid-state conditions for the production of fermentable sugars after enzymatic hydrolysis using three commercial enzymes (cellulase, β-glucosidase, and hemicellulase). In the production of biogas, the enzymes are used to increase the solubilization of sugars and biogas yield and also to decrease the viscosity of the

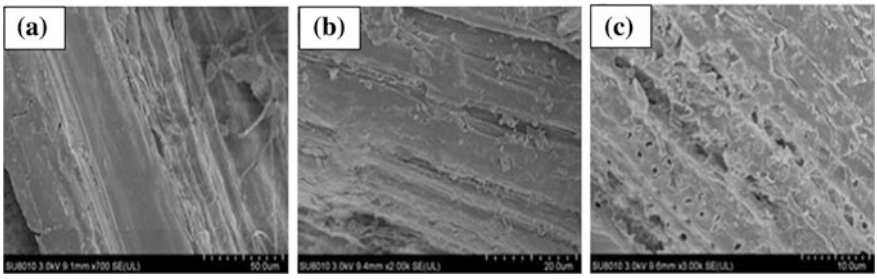

Fig. 2.2 SEM image for nontreated rice straw (**a**), autoclaved rice straw (control) (**b**), and rice straw after pretreatment with *P. ostreatus* during 10 days with 75% humidity (optimal condition) (**c**). *Source* Adapted from Mustafa et al. (2016)

fermentation medium or the substrate (Carrere et al. 2016). The pretreatment proposed by Saha et al. (2016) favored the degradation of the lignin, which provided a more accessible surface area to the cellulases and hemicellulases and, consequently, improved the enzymatic hydrolysis in terms of sugar yield of the pretreated corn stalk.

Pretreatment with fungi has shown to be a promising process, as it can efficiently degrade lignin, in addition to providing less aggressive hydrolysis products compared to thermal pretreatments. Its main disadvantage is the loss of organic matter during pretreatment, which is avoided in the enzymatic processes (Rouches et al. 2016).

2.4.2 Enzymes

Enzymatic pretreatments are more investigated at laboratory scale, and their efficiency, relative to biogas production, is evaluated through biochemical methane potential (BMP) tests. The most studied enzymes for improving the efficiency of biogas production include cellulases, cellobiases, endoglucanase, xylanase, pectinases, and lignilolytic enzymes such as laccases, manganese, lipases, and versatile peroxidases, as well as α-amylases and proteases for urban solid waste (Carrere et al. 2016).

Mahdy et al. (2015) evaluated the enzymatic pretreatment with commercial protease to hydrolyze the cell wall of the microalgae *Chorella vulgaris* aiming to increase biogas production. After pretreatment, the soluble organic matter increased from 2.5 to 45%. The reactor fed with the pretreated biomass resulted in 128 (mL CH_4 g COD in^{-1}), 56% total COD removal and 94% soluble COD removal. The reactor fed with the pretreated biomass resulted in a biogas yield 2.6 times higher when compared to the production obtained in the reactor fed with nontreated biomass. The authors also observed that 77% of the organic nitrogen was mineralized during anaerobic digestion, which caused a slight inhibition of ammonium.

The effect of copper (added as $CuCl_2$) on co-digestion of Phragmites straw and cow manure was studied by Hao et al. (2017) in a pilot experiment, evaluating biogas production. The results showed that 30 and 100 mg/L of Cu^{2+} increased the biogas yield by up to 43.62 and 20.77%, respectively. In the presence of Cu^{2+}, the degradation of volatile fatty acids and organic molecules was increased, and the concentrations of ammoniacal nitrogen were more stable than in the control. The levels of lignin and hemicellulose in the substrate also decreased with the presence of Cu^{2+}, which provided more biodegradable molecules for biogas production. These authors also studied the activities of cellulase and coenzyme F_{420} during the anaerobic digestion and verified that there was no significant relationship between the activities of the enzymes and the biogas production after the addition of Cu^{2+}.

However, some methanogenic microorganisms and their enzymes can be stimulated by Cu^{2+}. Copper (Cu) can replace nickel (Ni) in acetyl CoM, which divides acetyl CoA into CO, CoA, and a methyl group bound to CoM (Kretsinger et al. 2013). The addition of Cu^{2+} in the anaerobic digestion, in these cases, interferes in the function of other necessary metals.

When enzymatic hydrolysis is used for subsequent anaerobic digestion, there is a risk that the released sugars will be consumed by endogenous microorganisms; therefore, sterilization is required in order to eliminate these microorganisms. However, in large-scale biogas plants, the sterilization process is hampered, so enzymes are introduced directly into the digester (Carrere et al. 2016). Schimpf et al. (2013) added pectinases directly into the digester (volume of 2000 m^3) and verified a low yield of additional biogas (up to 4.7%), while the same laboratory-scale experiment increased biogas yield by 15%.

However, in some cases enzymes are essential to ensure the conversion of by-products into biogas. By-products of animal origin, for example, have a high organic content (proteins and fats) and can be used in anaerobic digestion. However, efficient recovery of methane from these by-products is not easy to achieve because the rate of biodegradability of lipids is slow and the decomposition of protein and long-chain fatty acids generated by lipid hydrolysis leads to ammonia buildup, which causes inhibition (Carrere et al. 2016).

To overcome these limitations, some strategies have been proposed, including enzymatic pretreatment steps to increase lipid bioavailability for anaerobic microorganisms or lipid removal prior to biodigestion. Among these strategies, the use of enzymes (lipases) has been highlighted due to strict environmental regulations and the possibility of producing significant amounts of biogas (445 ± 29 mL), besides the removal of high organic matter content (78.2%) (Mendes et al. 2006).

Sun et al. (2017) applied three lipases obtained from different sources (*Aspergillus*, *Candida,* and Porcine pancreatic) to hydrolyze animal fat (AF), vegetable oil (VO), and floatable grease (FG) present in food residue, in order to improve the performance of anaerobic digestion. The authors obtained an increase of biomethane production in 80.8–157.7%, 26.9–53.8%, and 37.0–40.7% for AF, VO, and FG, respectively.

These studies suggest that lipids pretreated with lipases can improve the efficiency of anaerobic digestion of residues and effluents with high fat contents (Mendes et al. 2006; Sun et al. 2017).

Enzymatic treatment is more efficient, in some cases, when combined with chemical, physical, and/or mechanical treatments. Mechanical treatment (milling) increases the available surface area for the enzymatic attack and consequently improves the biodegradability of lignocellulosic material, which can increase the methane yield (Hartmann et al. 2000). The combination of physical (steam) and chemical (with NaOH) treatments followed by enzymatic treatment with the commercial enzyme laccase Novozym 51003 increased the methane yield by 34% when compared to the untreated process (Bruni et al. 2010).

Some studies have turned their attention to pretreatments with microbial consortia containing yeasts, bacteria, and fungi. Shen et al. (2018) pretreated rice straw and swine manure with a consortium of cellulolytic microorganisms and obtained 0.64 L CH_4/(L d) of methane, yield 62.4% higher than in the control (without pretreatment). Tuesorn et al. (2013) also evaluated a microbial consortium, obtained from the microflora present in cane bagasse compost, to pretreat swine manure, and the results showed an increase of 55% in biogas production in comparison to the control experiment.

2.4.3 Partial Composting and Silage

Biological pretreatments also include partial composting and silage. Although the use of silage is reserved for agricultural scale storage of biomasses such as maize, sorghum, or grass before anaerobic digestion (Rouches et al. 2016), some studies have shown that it can help improve biogas production.

In silage, the biomass size is reduced by milling and, later, the biomass undergoes anaerobic lactic fermentation (Carrere et al. 2016) that converts sugars into acids (lactic and acetic acid) and ethanol (Rouches et al. 2016). The conservation of energy and nutrients by the silage process is guaranteed by the maintenance of acidic and anaerobic conditions.

According to Williams and Shinners (2014), grass silage allowed the recovery of 97% of cellulose and hemicellulose in relation to the initial mass when maintained under anaerobic conditions. When the silo is opened, exposure to air triggers the growth of aerobic microorganisms, which consume organic substrates. Aerobic deterioration reduces silage storage efficiency, causing loss of organic matter (29.3%) and loss of methane yield (40.7%) (Zhang et al. 2018). Therefore, to ensure efficiency in biogas production from ensiled substrates, they should be kept under anaerobic conditions and parameters such as moisture content, particle size, and additives (chemical or enzymatic) should be optimized (Rouches et al. 2016).

Partial composting can be used as a pretreatment for dry anaerobic digestion, aiming to increase the temperature of the substrate, reducing the heat requirements for the beginning of the anaerobic digestion. However, partial composting leads to degradation of the organic matter, causing a decrease in the digester performance (Carrere et al. 2016).

In order to choose the ideal treatment, the energy used to increase the biogas production, as well as the costs with the chemicals or enzymes, should be considered (Bruni et al. 2010). Therefore, many techniques studied are not viable in a large-scale system (Rouches et al. 2016).

2.5 Conclusions

Based on the literature review of the different pretreatment types available, it is clear that the effectiveness of pretreatment depends on the characteristics of the raw materials—particularly on the lignocellulosic biomass contents. Larger amounts of lignocellulose require a more severe pretreatment, such as alkaline, thermal, and even thermochemical methods. However, these methods can produce negative impacts to AD if they are not implemented correctly. This way, biological pretreatments may be an interesting alternative since they present the positive characteristics of traditional pretreatments and, at the same time, they can overcome some of the disadvantages.

The use of recalcitrant substrates is a promising energy alternative, capable of generating biogas when certain steps are performed to improve its productivity. Simple solutions allow biogas production to be increased; however, depending on the substrate used and on the process characteristics, more complex pretreatments are required sometimes. Not all pretreatments can be considered efficient and those that are, need to be evaluated based on their complexity, operational cost and increase in biogas production.

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Chapter 3
Enzyme-Mediated Enhanced Biogas Yield

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Abstract Enzymes are biocatalysts present in all living cells and have main function to perform the processes of breaking down complex nutrients into simple nutrients for cellular assimilation. Enzymatic catalysis has advantages over chemical catalysis due to high enzymatic specificity and moderate reaction conditions. Of great industrial interest, the enzymes can be applied in increasing the yield of compound production or in the degradation of unwanted by-products and these characteristics make the knowledge of enzymatic catalysis in biogas production extremely relevant, since the traditional method of biogas production is based on the biodegradation of organic matter by anaerobic digestion, which is produced by the action of a variety of microorganisms and enzymes. In the production of biogas, enzyme-mediated degradation may be the key to a higher quality final product, acting in the steps of hydrolysis, acidogenesis, acetogenesis and methanogenesis, and in the identification of by-products of enzymatic catalysis that may inhibit the process. In this context, the present chapter will be addressed: (i) introduction of enzymes in anaerobic biodigestion; (ii) enzymes as a mediator of biogas yield; (iii) inhibition of biogas production and biodegradability.

Keywords Bioprocess · Biotechnology · Anaerobic digestion · Biogas upgrading

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3.1 Introduction

Enzymes are protein biopolymers formed in all living cells and are responsible for catalyzing reactions, conducting and coordinating various cellular functions. The molecular structure of the enzymes, reaction kinetics and high specified in relation to different substrates is associated with the infinite combination and sequences of amino acids that form them and that also determine their biological activity (Abedi et al. 2011). The sources of enzymatic production are generally microbial cells that excrete high concentrations of extracellular enzymes (Sanchez and Demain 2017).

In many industrial applications, enzymatic catalysis has shown promise in relation to chemical catalysis, offering competitive processes, such as moderate reaction conditions, high substrate specificity and environmentally correct processing (Abedi et al. 2011; Choi et al. 2015). The application of enzymatic biocatalysis in industrial processes starts with the search for enzymes from a wide variety of biological sources, and the microbial cells are the most used, since the adaptability of these cells in the most diverse environments, with extreme conditions and variable pH and temperature (Abedi et al. 2011).

Of great industrial interest, the enzymes can be applied in increasing the yield of compound production or in the degradation of unwanted by-products. These characteristics make the knowledge of enzymatic catalysis in biogas production extremely relevant, since the traditional method of producing biomethane is based on the biodegradation of organic matter by anaerobic digestion. This process involves a range of microorganisms which, during the degradation process of the substrates, excrete enzymes which convert the compounds into products of easy assimilation to the subsequent step (Kolbl et al. 2017).

The identification of the enzymes involved in the biogas production stages is extremely relevant for studies to improve the quality of the gas produced, reduce inhibitors or intensify other products involved in the process. Therefore, the identification of the microbiological community present in the anaerobic reactors is essential for the knowledge of the enzymes excreted into the medium.

In this sense, this chapter focuses on recent biogas production research aimed at identifying the enzymes involved in the process, as well as their performance on the substrates present in the reactors. Also, they will be treated on possible by-products generated from the enzymatic reactions, capable of acting as inhibitors of the biogas production process or reducing the yield of the processes.

3.2 Enzymes as a Mediator of Biogas Yield

The identification of the enzymes that act in the process of methane production is extremely important for the improvement of the gas-produced quality. This is possible by identifying the biological community present in the reactor and the enzymes excreted by these microorganisms.

In this sense, a detailed study of the stages of biogas production was carried out, searching for the possible enzymes in this process, in order to facilitate the understanding of the enzymatic catalysis that occurs in the reactors.

3.2.1 Hydrolysis

The first step of anaerobic digestion for the biogas production comprises hydrolysis. This process is based on depolymerization of insoluble polymers, such as lipids, proteins carbohydrates and cellulose, liquefying them into monomers as sugars, amino acids and fatty acids (Yatawara 2015). The occurrence of this depolymerization is due to different enzymes which are secreted by innumerable species of microorganisms (Christy et al. 2014).

During the hydrolysis process, the substrate contacts the hydrolytic microbial cells that release the enzymes. The kinetic hydrolysis is the rate at which hydrolysis occurs in time and depends on the type of substrate to be hydrolyzed. It can be described in two steps, the first phase deals with the colonization of hydrolytic bacteria to the surface of the macromolecules. The bacteria that are near or on the particle surface release enzymes and produce useful monomers for itself and even for other types of bacteria. Then, in a second moment, the organic matter will be degraded to a region constant depth per unit of time (Vavilin et al. 1996).

The composition of biomass for the biogas generation is very diversified. Various types of waste can be used for the generation of energy, each of which will require different microorganisms that need distinct environmental conditions to produce specific enzymes for the degradation of this matter (Al Seadi et al. 2008; Bharathiraja et al. 2018). Table 3.1 shows different biomasses that can be used in the biomethane production and their basic composition, the microorganisms that act for it decomposition and the enzymes produced from substrates decomposition, besides the methane yield from different substrates.

Cellulase, cellobiase, amylase, xylanase, lipase and protease are some hydrolytic enzymes secreted by hydrolytic bacteria to hydrolyze polysaccharide, lipids and proteins, common substrates present in waste, converting them into noncomplex and soluble compounds (Al Seadi et al. 2008; Weiland 2010).

Cellulose and starch are long-chain molecules already used as a substrate in the production of biogas. These polysaccharides can be hydrolyzed in monosaccharides by the action of enzymes such as cellulase and amylase, produced by microorganisms present in the anaerobic biodigestor. Most of the cellulases produced by microorganisms as *Bacillus* and *Micrococcus* are composed of three species: endo-3-1,4-glucanases, exo-β-1,4-glucanases and cellobiase or *p*-glucosidase. These three species of cellulase act simultaneously on the cellulose in order to hydrolyze the crystals of the molecule producing glucose (FAO Agricultural Services Bulletin—128 1997; Hussain et al. 2017).

The microbial hydrolysis of starch into glucose occurs due to the action of an enzyme called amylase. The amylolytic activity for the hydrolysis of the starch

Table 3.1 Main substrates used to produce biogas and their composition, microbial community present in the substrate and enzymes involved in degradation

Substrate	Microorganism[a]	Organic content[b]	Enzymes[c]	Biogas yield per ton fresh matter (m^3)	Source
Swine manure	*Peptostreptococcus* *Eubacterium* *Bacteroides* *Lactobacillus* *Peptococcus* *Clostridium* *Streptococcus* *Enterococci* *Staphylococcus* sp.	Carbohydrates Proteins Lipids	Cellulase Protease Lipase Amylase	11–25	Iannotti et al. (1982) Zhu (2000) Al Seadi et al. (2008) Li et al. (2011) Achinas et al. (2017)
Cattle slurry	*Psychrobacter* sp. *Pseudomonas* sp. *Clostridium* sp. *Bacillus* sp. *Corynebacterium* sp. *Lactobacillus* sp.	Carbohydrates Proteins Lipids	Cellulase Protease Lipase	55–68	Al Seadi et al. (2008) Zhao et al. (2013) Gupta et al. (2016) Achinas et al. (2017)
Poultry slurry	*Nitrosomonas* *Nitrobacter* *Azotobacter*	Carbohydrates Proteins Lipids	Cellulase Protease Lipase	126	Nodar et al. (1992) Al Seadi et al. (2008) Achinas et al. (2017)
Food waste	*Bacteroides* *Syntrophomonas* *Sedimentibacter* *Petrimonas*	Carbohydrates Proteins Lipids	Cellulase Protease Lipase	110	Al Seadi et al. (2008) Li et al. (2015) Achinas et al. (2017)
Palm oil mill effluent	*Lachnospira* sp. *Arcobacter* sp. *Coribacteria* sp. *Cellulosilyticum* sp. *Clostridium* sp. *Bacillus* sp.	Cellulose Hemicellulose Lignin Xylose Lipids	Cellulase DyP-type peroxidase Xylanase Lipase	20	Chotwattanasak and Puetpaiboon (2011) Gonzalo et al. (2016) Prasertsan et al. (2017)

[a]Microorganism present in different substrates
[b]Substrate composition
[c]Enzymes that hydrolyze the substrate
Source Author

requires the combination of five amylase species: *p*-amylases that exocleave α ± 1–4 bonds, α-amylases that endocleave α ± 1–4 bonds, amyloglucosidase that exocleave α ± l–4 and α ± l–6 bonds, maltase acting on maltose and liberating glucose and debranching enzymes acting on α ± l–6 bonds. The α^2-endo-xylanase and

α^2-xylosidase are enzymes that hydrolyze xylanase producing xylose (FAO Agricultural Services Bulletin—128 1997). Yatawara (2015) even cite *Clostridium*, *Acetivibrio*, *Cellulitis* and *Staphylococcus* as microorganisms producing extracellular hydrolytic enzymes for the degradation of cellulose and starch.

Lipases are enzymes that transform lipids into fatty acids and glycerol. *Clostridium, Micrococcus* and *Staphylococcus* are genera of bacteria known to secrete this enzyme, since many of its species are responsible for the production of lipase (Yatawara 2015).

The proteins present in the waste that are used for biogas production are normally hydrolyzed to amino acids by the enzymes called proteases. These enzymes act on the cleavage of naturally occurring α-peptide bonds of amino acids and are produced by *Bacteroides, Butyrivibrio, Clostridium, Fusobacterium, Selenomonas* and *Streptococcus* (FAO Agricultural Services Bulletin—128 1997; Otín and Bond 2008). The amino acids generated in the hydrolysis phase and originated from a wide range of substrates are only possible to be transformed into methane from the syntrophic association with hydrogenotrophic methanogens that use the hydrogen of the medium produced in the acid phase. Otherwise, the methane production would be energetically impossible (Chojnacka et al. 2015).

Studies show that the dominance of some microorganisms as *Clostridium, Symbiobacterium* and *Bacteroidetes* in the anaerobic decomposition process is due to their capacity to metabolize innumerable substrates present in waste (Yi et al. 2014).

Hydrolytic bacteria have a faster growth when compared to microorganisms of the methanogenic phase (final phase of the biogas production process), but the bacteria of the first stage have a greater sensitivity to changes in their environment as temperature and pH. For substrates of difficult decomposition such as those with lignin, hydrolysis is generally the limiting phase of the biogas production process. The particle size, enzyme production and diffusion and absorption of enzymes in the substrate are others factors that influence the rate of hydrolysis (Venkiteshwaran et al. 2015). There are mechanical, chemical and biological processes to increase substrate decomposition in the hydrolytic phase, as discussed in Chap. 2, but recent researches show the bacterial enzyme performance in the breakdown of lignin, a polymer formed by through various ether and carbon–carbon bonds (Gonzalo et al. 2016).

Considered the most renewable and abundant biomass of the Earth, a vegetal biomass is a rich source of energy. Its main composition is lignin, cellulose and hemicellulose (Gonzalo et al. 2016). The latter two compounds of vegetable biomass are degraded by the enzyme cellulase produced by bacteria such as *Bacillus* and *Micrococcus* (Hussain et al. 2017). For lignin degradation, there are two classes of bacterial enzymes most known that are capable of modifying them, DyP-type peroxidases and laccases, and these enzymes are produced by bacteria such as *Escherichia coli K-12* and *Streptomyces* species that can be found on some substrates on bioreactor. In contrast, studies have shown that bacterial DyPs have lower lignin oxidation power than fungal Dys and fungal laccase also are more known (Gonzalo et al. 2016). Therefore, the inoculation of fungi that produce these

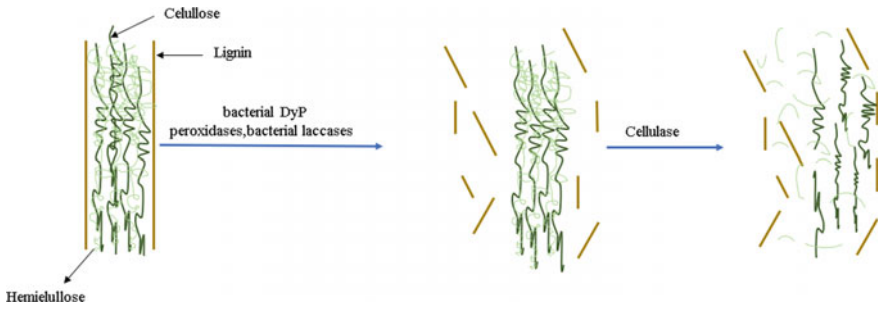

Fig. 3.1 Degradation of lignocellulosic material by enzymes

enzymes in the bioreactor seems to be a good alternative for lignin hydrolysis, eliminating the pretreatments of biomass, which sometimes make the process economically inviable. Figure 3.1 shows how the hydrolysis of lignocellulosic occurs via enzymatic action. Subsequently, the products generated in the hydrolysis phase will be decomposed by other microorganisms for use in their own metabolic process (Al Seadi et al. 2008).

3.2.2 Acidogenesis

The stage following hydrolysis is the acid fermentation or acidogenesis. In this phase, the products generated by the hydrolysis—as simple sugars, amino acids and fatty acids—form a substrate of less complex monomers, which are then degraded by acidogenic bacteria in acetates, carbon dioxide, hydrogen, volatile fatty acids (VFA) and alcohols (Al Seadi et al. 2008). By-products such as NH_3, CO_2 and H_2S are also generated during acidogenesis (Zhang et al. 2014). The main short-chain VFAs formed in the degradation of an organic compound are acetic acid, propionic acid, valeric acid and butyric acid (Buyukkamaci and Filibeli 2004).

According to Shah et al. (2014), due to the effect of various populations of microorganisms, acidogenesis can be bidirectional being divided into hydrogenation and dehydrogenation. The basic path of hydrogenation is to transform the products of the previous hydrolysis in acetates, CO_2 and H_2 which can be directly used by methanogens as an energy source. On the other hand, the alternative path—dehydrogenation—represents the accumulation of electrons from compounds such as volatile fatty acids, lactates and ethanol when there is an increase in hydrogen concentration in the solution. These products must be necessarily converted by bacteria that produce hydrogen in a posterior process called acetogenesis, thereby generating the ideal substrates to be metabolized by methanogenic organisms.

Rincón et al. (2013) and Seon et al. (2014) detected the presence of several microorganisms in the bioreactor during the acidogenesis of several products. The main genera were *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Megasphaera*, *Anaeroglobus*, *Lactobacillus* and *Corynebacterium*, *Pseudomonas*.

The acidogenesis may prevail in different forms (Fig. 3.2). Kandylis et al. (2016) report that the basic pathway for the production of all organic acids follows common metabolic processes such as the Embden–Meyerhof–Parnas pathway that converts the hexoses generated in the hydrolysis phase into pyruvate and NADH and then to acids organic compounds such as acetate, propionate, butyrate, lactate, ethanol, propanol, H_2 and CO_2 (Chen et al. 2013). The proportions of pyruvate depend on the substrate used, the environmental conditions and the properties of the strains, as well as the soluble products distribution in the final phase, and reflect the metabolic pathways that have been predominant (Zhou et al. 2017). Parallel to this process, enzymatic activities are also involved in the degradation of lipids and proteins (Kandylis et al. 2016).

Figure 3.2 represents the predominant metabolic pathways during acidogenesis, relating to some of the many microorganisms that coordinate this step and that were detected in the bioreactor by several authors. It is also worth noting that some of the mentioned microorganisms can produce different products from the same substrate, depending on process conditions and also the characteristics of the substrate in which they act.

In the fermentation route called acetate–ethanol fermentation [Fig. 3.2 (A pathway)], the products generated are considered the most popular intermediates during acidogenic fermentation and often bind to the formation of hydrogen (Liu et al. 2006). Acetate can be derived from pyruvic acid via acetyl-CoA pathway and also from the synergistic oxidation of ethanol or longer-chain fatty acids, such as propionate and butyrate (Zhou et al. 2017). The high production of acetate adjacent to this metabolic pathway is strongly associated with functional enzymes in acetyl-CoA by means of syntrophic oxidation (Müller et al. 2010). *Corynebacterium kroppenstedtii* presents as a saccharolytic microorganism that acts in this way producing ethanol, butyrate and acetate at pH 7.0 and temperature of 37 °C (Collins et al. 1998).

In the butyrate production pathway [Fig. 3.2 (B pathway)], pyruvic acid is converted to acetyl-CoA by pyruvate dehydrogenase and sequentially by butyryl-CoA from various enzymatic catalysts (Chaganti et al. 2011). The final step of butyrate production is mediated by phosphotransbutyrylase and butyrate kinase enzymes or also by butyryl-CoA: acetate-CoA transferase (Vital et al. 2014). Microorganisms such as *Corynebacterium butyricum* are notable for producing butyric acid at low concentrations of propionic acid and H_2 (Chen et al. 2006). In the final part of the production of butyrate, there is a metabolic shift in the butanol formation promoted by *Clostridium acetobutylicum*. This point has attracted enormous attention because *C. acetobutylicum* shares the same intermediary point (butyryl-CoA) and provides a competition between the butyrate formation pathways with butanol (Sillers et al. 2008). *C. acetobutylicum* has two homologous genes encoding the butyrate kinases and phosphotransbutyrylases mutants involved in the last step of butyrate formation (Huang et al. 2000; Yoo et al. 2017). This pathway requires a stable metabolic state with neutral pH and glucose consumption (Girbal et al. 1995). Likewise, with respect to the conversion of butyraldehyde dehydrogenase to butanol, the metabolic state is set at low pH with glucose

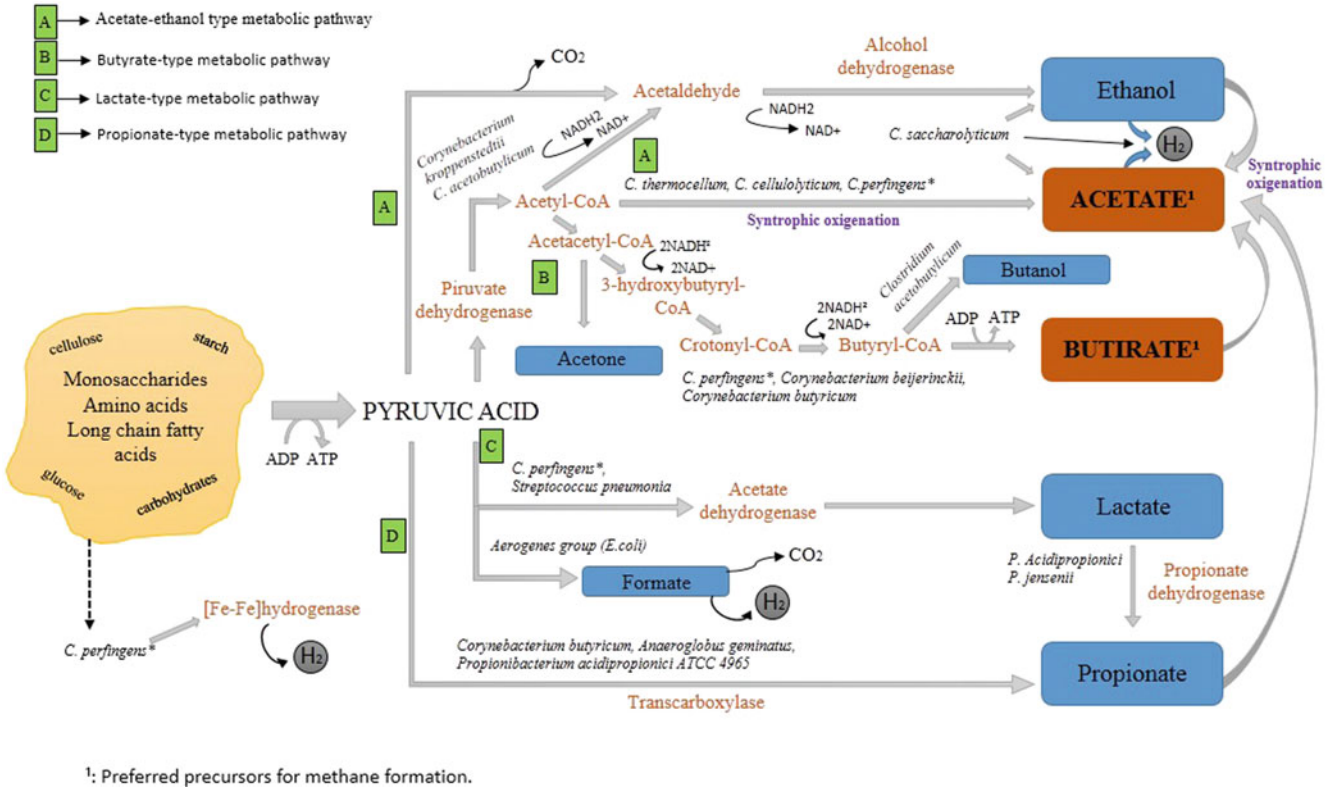

Fig. 3.2 Acidogenic metabolic pathways and some of the principal microorganisms involved in the production of the compounds. *Database* Murray et al. (1982), Svensson et al. (1992), Kaji et al. (1999), Hoskins et al. (2001), Hwang et al. (2001), Carlier et al. (2002), Guedon et al. (2002), Chen et al. (2006), Coral et al. (2008), Liu et al. (2008), Yen et al. (2011), Seon et al. (2014), Vital et al. (2014), Bensaid et al. (2015), Kandylis et al. (2016), Ahmadi et al. (2017) and Zhou et al. (2017)

consumption, and when added at neutral pH under high availability of NAD (P) H, also butanol and ethanol are formed but not acetone (Girbal and Soucaille 1994).

Lactate fermentation [Fig. 3.2 (C pathway)] is the metabolic pathway that mainly converts glucose and other organic materials to lactic acid by bacteria such as *Lactobacillus acidophilus*, *Lactobacillus casei* and *Streptococcus thermophilus* (Zhou et al. 2017). Enzymes such as NAD-dependent dehydrogenases, which form D-lactate and L-lactate, are involved in the formation of lactic acids (Garvie 1980). The increase in the production of these acids can be achieved by adding residues of activated sludge rich in carbohydrates, as it favors hydrolysis enzymes and improves AGV yield (Li et al. 2015).

The fermentation of propionate type [Fig. 3.2 (D pathway)] during the acidogenic metabolic pathway is performed by anaerobic microorganisms that ferment glucose, generating propionate as main product (Zhu et al. 2009) as well as hydrogen and any valeric acid without significant presence of CO_2 (Kandylis et al. 2016). The genus *Propionibacterium*, a bacterium with substrate based on glycerol and continuous extractive fermentation, stands out as the most popular organism for this type of fermentation (Ahmadi et al. 2017). The higher propionate yields occur between pH 4.0 and 4.5 (Wang et al. 2014) and higher yields of propionic acid with *Propionibacterium acidipropionici* ATCC 4965 using glycerol and mesophilic conditions were demonstrated by Coral et al. (2008). The pathway for the production of propionate comprises the reduction of pyruvate to lactate with catalysis of the enzyme lactate dehydrogenase and then reduction of lactate to propionate by propionate dehydrogenase (Lee et al. 2008).

According to Ren et al. (1997), the pH and the ratio of NADH/NADP coordinate the type of fermentation that will prevail for each process described above. The acetic and propionic acid production will be main at pH between 5 and 6 and NADH/NADP ratio in normal physiological pattern. The fermentation of butyric type occurs at a pH greater than 6 and less than 5 and is considered unstable because it can be converted into a fermentation of the propionic type. Finally, ethanol fermentation occurs at pH 4.5, preserving a balance in the NADH/NADP ratio, which makes the process more stable.

Clostridium species are the main microbial agents present in any anaerobic process involving organic residues, being able to ferment various carbohydrates such as glucose, sucrose, lactose, starch and cellulose and produce mainly acetic, butyric, propionic, lactic acids and H_2 (Svensson et al. 1992). An outstanding member of this class is *Clostridium kluyveri* because it uses ethanol and acetate as sole energy sources and converts these substrates to butyrate and H_2 (Seedorf et al. 2008). *Clostridium disporicum* and *Clostridium quinii* produce acetate, butyrate and hydrogen at pH around 7.4 and mesophilic temperature conditions (Svensson et al. 1992). These conditions favor the more expressive production of acetate and butyrate yielding (more points) (Seon et al. 2014). Besides that, *Clostridium thermocellum* and *C. butyricum* have been intensely reported for producing hydrogen from biomasses such as starch and cellulose (Wang and Wan 2009). The abundance of the *Firmicutes* filo in sludge samples also extended the fermentation

process of fatty acids, producing more hydrogen as a by-product promoting a greater growth of methanogenic compounds that use hydrogen as substrate favoring the production of biogas rates (Lim et al. 2018).

Hydrogen is an important intermediate in the anaerobic degradation of organic matter (Liu et al. 2006). The acidogenic phase is the stage that brings possibilities of obtaining a high yield of hydrogen and consequently a gas rich in H_2 (Silva et al. 2018). The presence of *Clostridium perfringens* in the bioreactor brings remarkable opportunities for H_2 production since the activity of hydrogenase enzymes that regenerate ferredoxin reduced by pyruvate-ferredoxin oxirreductase and NADH-ferredoxin contribute to the vital process to maintain the redox balance during fermentation (Kaji et al. 1999). However, during fermentation, only 10–20% of the energy of the substrate is converted to H_2 and CO_2, since the remainder remains in the liquid phase as soluble metabolic products, among them, volatile fatty acids and ethanol (Cooney et al. 2007).

3.2.3 Acetogenesis

In acetogenesis step, the microorganisms are in charge of converting the intermediates compounds formed in acidogenesis phase to acetate, formate, hydrogen, carbon dioxide and methyl compounds. The principal intermediates compounds biodegraded in this step are propionate, valerate, isovalerate, butyrate, isobutyrate and ethanol and this biotransformation occurs by a process named syntrophic acetogenesis (Speece et al. 2006; Venkiteshwaran et al. 2015; Wang et al. 2018).

This process depends on the relation of hydrogen production and consumption by acetogenic groups microorganisms, generally denominated interspecies H_2 transfer (Batstone et al. 2002; Stams and Plugge 2009; Venkiteshwaran et al. 2015). Some of the characteristics of acetogenic microorganisms are having an optimum pH around 6, being strict anaerobes and requiring long periods for adjust to environmental changes, making their growth slow (Wood and Ljungdahl 1991; Xing et al. 1997; Christy et al. 2014). The syntrophic acetogenesis is responsible for maintaining the anaerobic digestion rapid and stable, since some of the fatty acids, for instance, the propionate, could inhibit methanogenesis at high concentrations and destabilize the entire methane generation process (Mathai et al. 2015; Venkiteshwaran et al. 2015).

Each intermediate compound formed by acidogenesis has bioconversion mechanisms with the purpose of obtaining direct substrates for methane production. These mechanisms, in case of propionate degradation, are developed by syntrophic acetogens from the genera such as *Smithella*, *Syntrophobacter* and *Pelotomaculum*. The oxidation of fatty acids like butyrate happens because of microorganisms from the genera *Syntrophus* and *Syntrophomonas* (Gerardi 2003; Imachi et al. 2007; Jha et al. 2011; Venkiteshwaran et al. 2015; Wang et al. 2018). The processes of intermediate compounds conversion by acetogenic bacteria occur simultaneously, which fatty acids are converted to acetate as well as propionate, but the second one

biotransformation depends on low hydrogen pressure. During acetogenesis, ethanol is converted into molecular hydrogen and acetate through Pelotomaculum. This hypothesis is based in two observations. First the studies by Imachi et al. (2002) and Kosaka et al. (2006) indicating this microorganism capability of growing on ethanol presence and second because of the presence of Pelotomaculum in the propionate degradation pathway, also generating molecular hydrogen and acetate, as previously exposed. This process is shown in Fig. 3.3.

3.2.3.1 Principal Interactions in Propionate and Ethanol Degradation

The process of propionate conversion shown in Fig. 3.3 depends on the action of microorganisms named as syntrophic propionate-oxidizing bacteria. Basically, there are two pathways for obtain the products in this process: through the randomizing methylmalonyl-CoA or the non-randomizing 6-carbon intermediate metabolite (Houwen et al. 1990; Plugge et al. 1993; De Bok et al. 2001; Li 2013). The three principal microorganisms involved in propionate oxidation have the enzyme methylmalonyl-CoA engaged in their metabolism, as can be seen in Fig. 3.3, and this fact induces the idea that all of them follow the randomizing pathway to obtain the products in acetogenesis. However, De Bok et al. (2001) provide evidence that *Smithella propionica* uses the non-randomizing pathway via butyrate. In other words, this microorganism utilizes part of the propionate to be carboxylated to butyrate, and in the next step, it is degraded to acetate by syntrophic β-oxidation. The authors also propose that this alternative pathway via butyrate requires some coenzymes derivatives (Fig. 3.3). The acetyl-CoA, for example, is necessary for the initial activation of propionate, and the crotonase and butyryl-CoA dehydrogenase are needed for the butyrate cleavage (Halpern 1985; De Bok et al. 2001). Since only part of the substrate, in the process described above, is oxidized via butyrate (non-randomizing pathway), it could be hypothesized that *Smithella* genera also use methylmalonyl-CoA enzyme (randomizing pathway) to convert propionate into acetate in synergy with the others microorganisms (Fig. 3.3).

The other two bacteria involved in propionate degradation use the methylmalonyl pathway through different metabolisms and engaging distinct enzymes in the process, as can be seen in Fig. 3.3. Liu et al. (1999) reported the isolation of *Syntrophobacter wolinii* in anaerobic conditions and measured the stoichiometry that this species produces acetate from propionate, obtaining one-mol acetate formed per mol propionate degraded. The authors indicated that this procedure occurs by the dismutation of the substrate to acetate, by methylmalonyl-CoA and butyryl-CoA. Subsequently, happens syntrophic β-oxidation from butyryl-CoA to acetate, indicating the importance of this enzyme for the full conversion. This specie growth can also occur on crotonate, and the speed of growth might be explained by the presence of kinase, an enzyme that also slows down the quantities of butyrate during the action of butyryl-CoA (Liu et al. 1999). Thus, it can be inferred that the action of butyryl-CoA depends on the action of kinase in a way that the second one enzyme causes a change in the metabolism pathway of the

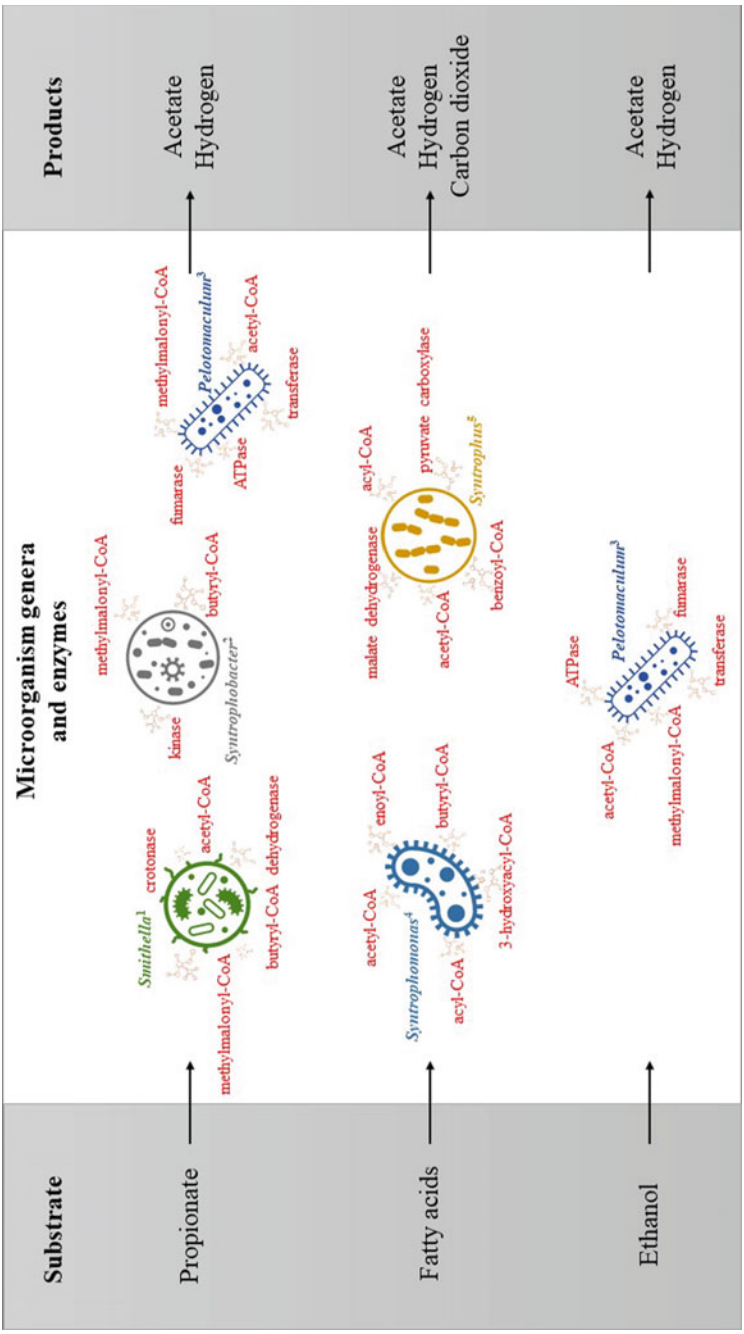

Fig. 3.3 Overview of the principal interactions in acetogenesis between substrates, microorganisms and enzymes (shown in red) and the principal products obtained. *Database of interactions* Imachi et al. (2002, 2007), Gerardi (2003), Kosaka et al. (2006), Jha et al. (2011), Christy et al. (2014), Venkiteshwaran et al. (2015) and Wang et al. (2018). Database of microorganisms metabolism and their enzymes: [1]De Bok et al. (2001), [2]Liu et al. (1999), [3]Kosaka et al. (2006), Imachi et al. (2007) and Li (2013), [4]Sieber et al. (2010), and [5]Jackson et al. (1999), McInerney et al. (2007) and Li (2013)

microorganism. If *S. wolinii* produce large amount of kinase, consequently, the route of converting propionate direct to acetate has to predominate and the bacteria have to produce more methylmalonyl-CoA. The other route could be low amount of kinase, enabling the produce of butyrate and making the microorganism produce acetate through methylmalonyl-CoA and butyryl-CoA.

The last one syntrophic propionate-oxidizing bacteria shown in Fig. 3.3 were reported in two different species: *Pelotomaculum thermopropionicum* by Kosaka et al. (2006) and *Pelotomaculum propionicicum* by Imachi et al. (2007) and Li (2013). Both of them use the same enzymes to convert propionate into acetate (Fig. 3.3), and the authors proposed the randomizing pathway to the process, making the difference between the species basically be the variety of substrates in with they can grow. Other difference observed is that the second one has the characteristic of being obligatory syntrophic life with hydrogenotrophic methanogens, while the first one can grow on fumarate and pyruvate in culture alone (Imachi et al. 2002, 2007; Kosaka et al. 2006). Since both species belong to *Pelotomaculum* genera, it is possible to assume that the pathway for propionate metabolization follow the steps described by Sambrook et al. (1989) and Kosaka et al. (2006), producing five main enzymes (Fig. 3.3). The transferase is present in the first two steps in the process and has the function of catalyze two others enzymes (Propionyl-CoA and Methylmalonyl-CoA), giving to the metabolism of these genera a long lag period characteristic, according to Imachi et al. (2000) and Kosaka et al. (2006). This enzyme can also be used as an intermediate metabolite to the production of acetyl-CoA, which converts propionate to acetate in a short route. The production pathway encompasses several intermediate metabolites and one of the compounds produced during this process is fumarate, which has the possibility of being directly converted into acetate through fumarase, an important enzyme because it offers a direct oxidation to the process and a possibility of being a substrate to the growth of *Pelotomaculum*. Another enzyme engaged with fumarate is ATPase, found in these bacteria in a significant amount and indicating that they can use this enzyme to promote the fumarate respiration and the oxidative phosphorylation (Kosaka et al. 2006).

In studies developed by Imachi et al. (2000) and Kosaka et al. (2006), it was observed that *P. thermopropionicum* could grow in several substrates under anaerobic conditions. One of those substrates is ethanol in cocultures with a hydrogenotrophic methanogen, regarding the assumption that these bacteria and their enzymes, described above, are involved in the process of ethanol degradation in acetogenesis, as shown in Fig. 3.3. Besides that, the database used to describe the main interactions on propionate and ethanol degradation indicates that the microorganisms engaged in this process have a metabolism that needs methanogens microorganisms to grow in synergistic systems. In addition, Fig. 3.3 provides the visualization of some microorganism genera developing similar metabolism and enzymes and living in symbiosis, making possible the supposition that they are a system that have the capability to share or produce together some enzymes. This cooperation makes possible the encouragement of rapid growth in the microbial population through enzymes.

3.2.3.2 Principal Interactions in Fatty Acids Oxidation

The genomic analysis of *Syntrophomonas wolfei* by Sieber et al. (2010) indicates that these bacteria are involved on the reduction of unsaturated fatty acids in syntrophic growth with methanogens, putting these microorganisms in the position of acetogenesis promoters, as can be seen in Fig. 3.3. The study developed by these authors brings some highlights in the metabolism reaction of these genera involving five principal enzymes that work in β-oxidation pathway. The acetyl-CoA is one of these enzymes, which has the function of making ATP and activating butyrate, and after the butyryl-CoA converts butyrate into acetyl-CoA. This conversion has a long route passing through the production of crotonyl-CoA, 3-hydroxybutyryl-CoA and acetoacetyl-CoA, respectively, but after acetyl-CoA is produced, the microorganism can direct obtain acetate (Wofford et al. 1986; McInerney and Wofford 1992; Sieber et al. 2010). As shown in Fig. 3.3, the acyl-CoA and enoyl-CoA enzymes are also present in *Syntrophomonas*'s metabolism, being found nine acyl-CoA dehydrogenase genes and five enoyl-CoA hydratase genes in Sieber et al. (2010) research. The authors discussed their importance under the hypothesis that the microorganism has the possibility of alternate pathways to maintain its metabolism and deal with changes.

As indicated in Fig. 3.3, acyl-CoA and acetyl-CoA were also found in *Syntrophus* genera, making them a common enzyme in the fatty acid oxidation process. In Jackson et al. (1999) and McInerney et al. (2007) studies, it was isolated *Syntrophus aciditrophicus*, a strictly anaerobic bacteria involved in benzoate and fatty acids degradation when associated with syntrophic or hydrogen/formate-using microorganisms. Each of the substrates mentioned before has a pathway to obtain acetate, but in certain moment, the routes have to find each other and follow the same steps. Basically, benzoate degradation first step is to produce benzoyl-CoA and, to fatty acids, it is produce acyl-CoA. Then, in certain point of the pathway, both of them have to use acetyl-CoA to be convert into ATP and acetate. McInerney et al. (2007) also found several intermediate metabolite to the production of acetyl-CoA in the pathways mentioned, such as malate dehydrogenase and pyruvate carboxylase, shown in Fig. 3.3. The combined activity of these enzymes is responsible to synthesize NADPH, an important compound for the microorganism be able to complete the route of generating acetate (Sauer and Eikmanns 2005; McInerney et al. 2007). According to what was exposed above, it is possible to inference that the metabolism of microorganisms found in fatty acids oxidation process is slower than in propionate and ethanol degradation. The explanation of this fact could be the substrate complexity, making the bacteria produce larger varieties of enzymes to conclude the whole process. Since the products in both substrates conversion are essentially the same and some enzymes are produced for more than one microorganism (Fig. 3.3), it can be inferred that the interactions in acetogenesis occur beyond the limits of bacterial metabolism and their pathways known until this moment. Furthermore, it can be observed the existence of a strong relationship between enzymes produced and routes chosen by different microorganism genera, reinforcing the idea of a syntrophic lifestyle.

3.2.4 Methane Production

The last stage of the biogas production process consists of methanogenesis, where methane (CH_4) is ultimately produced from methanogenic microorganisms, *Archaea*, which are strictly anaerobic and produce energy from the biosynthesis of methane (Sarmiento et al. 2011). This stage is considered the most critical of the anaerobic digestion process, being the slowest in biochemical reactions, in addition abrupt changes in pH, increase in salt concentration or even organic matter overload cause system failure (Al Seadi et al. 2008; Vrieze et al. 2012).

Methanogenic archaea are physiologically specialized microorganisms in the conversion of simple substrates, being limited to three main substrates: carbon dioxide (CO_2), acetate and compounds containing methylated groups, transforming into methane, so archaea are dependent on other microorganisms capable of performing the breaking of complex molecules into substrate supplies (Zinder 1993; Al Seadi et al. 2008; Sarmiento et al. 2011). The methanogenesis process is the only way to obtain energy for archaeal growth, and these are the only known microorganisms capable of producing methane as a metabolic process product (Thauer 1998). Therefore, most of the energy available in organic substances is used by other non-methanogenic organisms (Liu and Whitmann 2008).

The methanogenic microorganisms taxonomically belong to the kingdom of Euryarchaeota, classified phylogenetically in five orders: *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanopyrales* and *Methanosarcinales* (Zinder 1993; Thauer 1998; Al Seadi et al. 2008; Liu and Whitmann 2008). The reaction path most used by these microorganisms for the methane production is the reduction of carbon dioxide using hydrogen as an electron donor, which are called hydrologic archaea or hydrogenotrophs (Zinder 1993; Liu and Whitmann 2008; Sarmiento et al. 2011). As for the reactional route where the acetate is used as an energy source, only the microorganisms of the order *Methanosarcinales*, called acetoclásticos (Thauer 1998). The third, and less common, pathway is the production of methane by reducing methyl groups of methylated compounds (Liu and Whitmann 2008).

The hydrogenotrophic reaction pathway, where the reduction of carbon dioxide to methane production occurs, is mediated by different coenzymes, such as methane sulfur (MFR), coenzyme M (CoM) and coenzyme B (CoB) (Liu and Whitman 2008). This process is dependent on the hydrogen or format, having this as the main electron donor of the reactions of methanogenesis via CO_2 reduction. The hydrogenotrophic process is conducted in stages, starting with the reduction of electrons from carbon dioxide producing formamide derivatives, which bind to the amino group of the coenzyme MFR, forming *N*-formyl-MFR. In the subsequent step, the formyl group attached to the MFR coenzyme is transferred to the tetrahydromethanopterin (H4MPT) coenzyme, and then this coenzyme is cyclized in the methanogenesis process, and following a sequence of F420-dependent enzyme-mediated reducing reactions, it produces methyl-H4MPT. The enzyme F_{420} is involved in the catalysis of reactions as an electron carrier, not involved in later

stages, where CO_2 reduction occurs for formyl-MFR and, later, in the reaction of methyl-coenzyme M for CH_4. Subsequently, CoM involvement results in the transfer of the methyl group to the thiol group of CoM, leaving the coenzyme H4MPT and following the coenzyme methyl-CoM reductase (MCR) cycle. Finally, the catalysis is performed by the MCR using CoB as an electron donor for reaction, and this reaction process will generate final product methane (CH_4) (Thauer 1998; Graham and White 2001; Liu and Whitman 2008; Grochowski and White 2010; Leight 2011; Sarmiento et al. 2011). Hydrogen is considered the main electron donor for methanogenesis, and many hydrogenotrophic methanogenic microorganisms can still use formate, ethanol or some secondary alcohols as electron donors. However, methanogens grow little by using alcohols as electron donors (Liu and Whitman 2008).

The reaction pathway using acetate as a substrate for methane production is called acetoclastic, and only two genera of methanogenic microorganisms are able to use this methane: *Methanosarcine* and *Methanosaeta* (Liu and Whitman 2008). This pathway generates less energy for the metabolism when compared to the hydrogenotrophic pathway (Thauer 1998). The process begins with the reaction of acetate by coenzyme A (CoA), resulting in acetyl-CoA, resulting from the coupling of the enzyme acetate kinase and phosphotransacetylase, or acetate kinase. Later, using acetyl-CoA, resulting from the previous reaction, the enzyme carbon monoxide dehydrogenase catalyzes the reaction of the compound with tetrahydrosarcinapterin (H4SPT) or tetrahydromethanopterin, breaking and releasing CoA and transferring CH_3 to H4SPT, forming *N*5-methyl-tetrahydromethanesarcin. In the next step, the methyl group is transferred to CoM, via coenzyme M methyltransferase, which is an energy-conserving enzyme. In this stage, the process of the acetoclastic path joins the hydrogenotrophic pathway, where through reaction with the CoB will occur the production of methane (Thauer 1998; Fournier and Gogarten 2007; Liu and Whitman 2008; Ferry 2011).

Finally, the metabolic pathway where compounds containing methyl groups, such as methylamines and methanol, are used as substrates for the production of methane by the methanogenic archaea is known as methylotrophic (Liu and Whitman 2008; Vanwonterghem et al. 2016).

In all the metabolic pathways of the process of methanogenesis are involved several reactions catalyzed by enzymes, but a specific enzyme plays an essential role in this conversion process, the enzyme methyl-coenzyme M reductase, which participates in the last step of the methanogenesis, the mcrA gene being a coding unit of the alpha subunit of MRT, and being present exclusively in methanogenic archaea (Aronson et al. 2013).

Methanogenesis is an extremely dependent stage of the previous stages of hydrolysis, acidogenesis and acetogenesis, due to the specificity of the methanogenic microorganisms in the conversion of the substrates to methane. This fact, coupled with enzymatic catalysts involved in the process, results in the quality of the biogas produced, because if the other steps do not occur simultaneously, forming a chain of compounds that are substrates for the following steps, the quality of the generated gas will be strongly influenced.

3.3 Inhibition of Biogas Production and Biodegradability

Inhibition in the production of biogas can be understood as the occurrence of anaerobic digestion failures that occurs due to the presence of toxic substances in the biodigester as substrates components or even by-products metabolised by microorganisms (Yatawara 2015).

Numerous substrates can be used to supplement an anaerobic digestion, often because some type of by-product coming from another process of transformation can be found small portions of metals. Some metals present the characteristic of potentiating the production of biogas (Ni, Co, Mn and Fe) as they stimulate activity of microbial community (Abdel-Shafy and Mansour 2014; Yue et al. 2007). However, the presence of heavy metals (Cu, Pb, Cr and Zn) has negative consequences under the digestion process, acting in an inhibitory way, inactivating enzymes that are metabolized by microorganisms present in the reactor (Abdel-Shafy and Mansour 2014; Selling et al. 2008). The inhibitory level depends on the toxicity of metal and accumulation of intermediate substances, such as organic acids, which are produced from the process inhibition of methanogenic archaea (Abdelsalam et al. 2017; Abdel-Shafy and Mansour 2014).

For the final product of biogas production to present quality, a correct functioning of the whole system is necessary, so the balance between what is consumed and what is produced must be prioritized (Ács et al. 2015). An example of this is Hydrogen, in which its presence in an excessive way inhibits the activity of the community of acetogenic microorganisms (Dong et al. 1994). Microorganisms that remove the hydrogen together help in the formation of CH_4, thus contributing to the maintenance of the fermentative activities of the microbiota and the balance of the system (Ács et al. 2015; Rivera-Salvador et al. 2014).

Although is easy to produce methane, anaerobic digestion is a highly complex process, which makes the system exposed to inhibition effects by the concentration of long-chain fatty acids, volatile fatty acids, ammonia and other inappropriate temperature and pH conditions (Amha et al. 2018; Chen et al. 2014).

In high concentrations of long-chain fatty acids, volatile fat acids, hydrogen and humic acids, hydrolytic bacteria acting in the hydrolysis phase are inhibited due to loss of hydrolases activity, which can occur reversibly, when the inhibitors formed during the process are linked to active site of the enzyme, or irreversible ones, that refer to modifications in the structure of the enzyme (Amba et al. 2018; Azman et al. 2015, 2017; Cazier et al. 2015).

The effect of inhibitors is directly correlated with the operating temperature of the digester, and the stability of microbial community, however, is very variable and this is related to the different substrates used (Baserba et al. 2012; Silva et al. 2014; Silvestre et al. 2011).

When agro-industrial waste is used as a substrate for anaerobic digestion, it is possible to find some contaminants such as antibiotics, disinfectants, NH_4, heavy metals, herbicides, among others. These chemicals can also act as inhibitors of the biogas production process (Al Seadi et al. 2008).

The hydrolysis step is considered limiting for the production of methane, because depending on the complexity of raw material used the hydrolytic enzymes are not effective to degrade the compounds present in the substrate, and this fact can cause the inhibition of the subsequent step. In this case, a pretreatment would be an interesting strategy to reduce possible inhibitions during the process (Brémond et al. 2018; Christy et al. 2014).

In addition to the hydrolysis, methanogenesis is also a limiting step, because during the methane formation process, ammonia formation occurs from degradation reactions of nitrogen compounds. The higher the ammonium concentration in the reaction medium, the lower the methane yield, since the methanogenic bacteria are inactivated (Chen et al. 2008, 2016; Kanai et al. 2010). Ammonia has the ability to penetrate the cell membrane which ultimately affects the osmotic balance within the cell (Chen et al. 2016), usually one of the enzymes involved in this process is acetyl-CoA, mainly responsible for nitrogen fixation (Ruiz-Sánchez et al. 2018).

As with ammonia, high dosages of salt present in the substrate can also cause damage to the process, dehydrating the cells, causing stress on the cellular activity of microorganisms and inactivating enzymes responsible for a series of biochemical reactions (Chen et al. 2008; Dereli et al. 2012; Fotidis et al. 2014; Ruiz-Sánchez et al. 2018).

The inhibition process is possibly the result of the action on the cell surface of the microorganisms; the inhibitory substances limit the mass transfer and the access of the microorganisms and enzymes to the corresponding substrate (Amba et al. 2018; Ma et al. 2015). According to some researchers, inhibition may occur in different ways, but among the most viable mechanisms can be mentioned: negatively affect the performance of enzymes in the electron transport chain, oxidative phosphorylation, energy production and decrease in cellular permeability (Amba et al. 2018; Desbois and Smith 2010; Ma et al. 2015; Pereira et al. 2005).

Unfavorable conditions for anaerobic digestion, such as the formation of inhibitory intermediates, cause damage to the DNA replication of microbial cells, which can lead to cell death and process inefficiency (Amba et al. 2018).

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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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Chapter 5
Co-digestion of Animal Manure and Carcasses to Increase Biogas Generation

Deisi Cristina Tápparo, André Cestonaro do Amaral, Ricardo Luis Radis Steinmetz and Airton Kunz

Abstract Livestock productions are changing with scale production increasing and concentration in some geographical areas. As a consequence, the activity environmental sustainability is under concern especially for manure and carcass management, disposal, or treatment. The livestock production system has its own particularities for each rearing process, resulting in residues with different characteristics. News technologies for pre-treatment and treatment for these residues have been established. Anaerobic digestion is an alternative for treatment due to this process combines the waste stabilization producing renewable energy and biofertilizer. The different components of manure excreted by livestock could be influenced on the biodegradation and biogas production. Previous studies are corroborated in this chapter and highlighted the importance of process control and digestate application when the carcass and manure are digested. For the evaluation of the efficiency of treatment processes, reduce environmental risks, and sanitary aspects, the choice of biomarkers is imperative. This chapter presents an approach and review to legislation about the conditions and criteria for the use of manure and carcasses in biodigesters and subsequently biofertilizer.

Keywords Combined process · Process control · Environmental risks

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5.1 Livestock Production

The world food economy is increasing the demand by livestock products and consequently the global livestock production. Livestock is an important economic activity around the world, due to high-value products (Herrero et al. 2013). Livestock products (milk, beef, pork, and poultry meat) are supplied by four animal food systems (beef cattle, dairy cattle, pigs, and broilers) (Weindl et al. 2017). Swine, poultry, and cattle chains have representativeness importance in the global production with approximately 110, 71 and 61 million ton of meat in 2013, respectively, in addition, milk production is around 508 million ton (Gerber et al. 2013).

Livestock operations providing social benefits, mostly in the developing ones, however, are a major impact on the environmental quality through effluent production, large uses of water, and emission of greenhouse gases (GHGs) Sakadevan and Nguyen (2017). GHGs' emissions from cattle represent about 65% of total, while swine and poultry contribute with 9 and 8%, respectively. Table 5.1 is described the emission intensity of each chain.

Manure management practices that ensure the recovery and recycling of nutrients and energy contained in manure along chains can contribute to mitigation of GHG. In many parts of the world, where occurs the increasing of specialized livestock farms, without sufficient land for use these residues for crop production, increase the necessity of treatment alternatives (Petersen et al. 2007).

5.1.1 Cattle

USA is the major producer of bovine meat with 11.9 million ton in 2017, followed to Brazil (9.5), European Union (7.9), China (7.3), and India (4.3), and these countries represent 66% of the world production USDA (2018). For milk production, the leadership continues with USA (87 million ton), followed by India (50 million ton), China (36 million ton), Russia (31 million ton), Brazil (31 million ton), and Germany (29 million ton), and these countries represent approximately 50% of the world's total production (IFCN 2016).

Dairy systems (meat and milk production) are constantly changing due to the market demand and land occupation. The dairy systems are characterized by the following phases (Fig. 5.1) (FAO 2016a):

- Gestation: refers to the pregnancy period after mating, when the calf fetus develops prior to birth;

Table 5.1 Global production and emission intensity for livestock chains

Herd	Production (million tons in 2013)	Emission intensity ($kgCO_2$-eq kg^{-1} product)
Cattle	61.4	67.6
Chicken	71.6	5.4
Swine	110.2	6.1

Source Gerber et al. (2013)

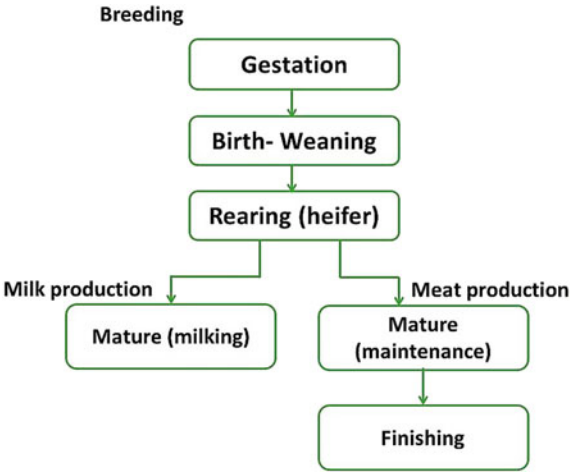

Fig. 5.1 Differences between systems of dairy milk and meat production. *Source* Adapted from FAO (2016a)

- Birth—Weaning: is the period after birth up until the calf is weaned from either its mother's milk or a milk replacement substitute. This stage may have different durations depending on the production system;
- Rearing (heifer): refers to the stage where the female animal (heifer) gains weight postweaning, reaching approximately 65–80% of the adult weight;the heifer may be mated or may be transferred to the beef system for fattening or immediate slaughter. This stage defined that animal is used to milk or meat production;
- Mature (milking): refers to the stage where adult postpartum cows are milked;
- Mature (maintenance): the former refers to the stage where animals are at their minimum mature body weight or may be used for other purposes;
- Finishing: the stage when the body weight is deliberately increased for slaughter.

Mortality on production units can depend on health status and management level, being considered a routine mortality until 5% of herd annually (McConnel et al. 2015; FAO 2016a).

5.1.2 Poultry

Statistics from poultry industry demonstrated that USA is the major producer of meat with 18.7 million ton in 2017, followed to Brazil (13.1 million ton) European Union (11.8 million ton), and China (11.6 million ton), representing 60% the world production (Embrapa 2018; USDA 2018).

The poultry sector is structurally diverse; there are differences in the scale and types of housing, feeding systems, and animal genetics. In a modern system production, the broilers are raised in large, open, or fully enclosed houses. The floors of the houses are covered with litter consisting of wood chips, rice hulks, or peanut shells. Barns are frequently equipped with automatic systems to deliver feed and

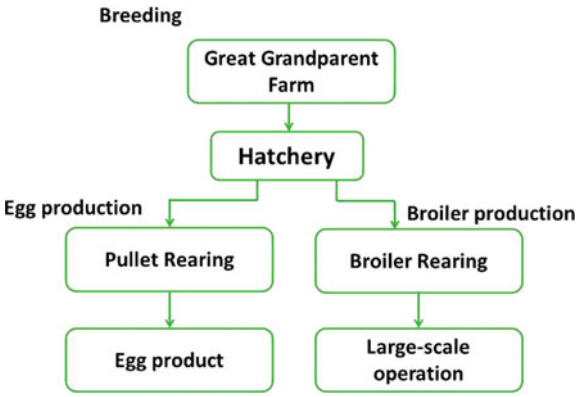

Fig. 5.2 Differences between systems of meat and egg in poultry chain. *Source* Adapted from FAO (2016b)

water (FAO 2016b). Figure 5.2 demonstrates the systems of meat and egg production. In this chain, the routine mortality was between 5 and 9% per year (CAST 2008).

5.1.3 Swine

China is the major producer of swine meat in the world with 53.4 million of ton in 2017, followed by European Union (23.6 million ton), USA (11.6 million ton), and Brazil (3.7 million ton), representing approximately 83% of total global production USDA (2018).

Swine production systems present a high variability ranging from very low (subsistence) to large-scale, in response to a factors socio-economic, markets and consumption. Globally, there is a wide variety of swine production systems, can be characterized by the following phases (FAO 2016c):

- Gestation: breeding females during gestation period;
- Breeding or farrowing: piglets until weighing 7–15 kg between 21 and 28 days of age;
- Nursery or Weaner: pigs, weighing 7–15 kg, reared to 25–35 kg at age 56–84 days;
- Growing to finishing: feeder pigs, weighing 25–35 kg, grown to market weight;

Swine production segregation is organized according to the countries characteristics (FAO 2016c). One example of segregation is demonstrated in Fig. 5.3, at where: farrow-to-feeder (gestation and breeding/farrowing), wean-to-finish (nursery/weaner and growing to finishing), Feeder-to-finishing (growing to finishing), farrow-to-wean (gestation, breeding/farrowing and nursery/weaner) fully integrated systems (gestation, breeding/farrowing, nursery/weaner, and growing to finishing).

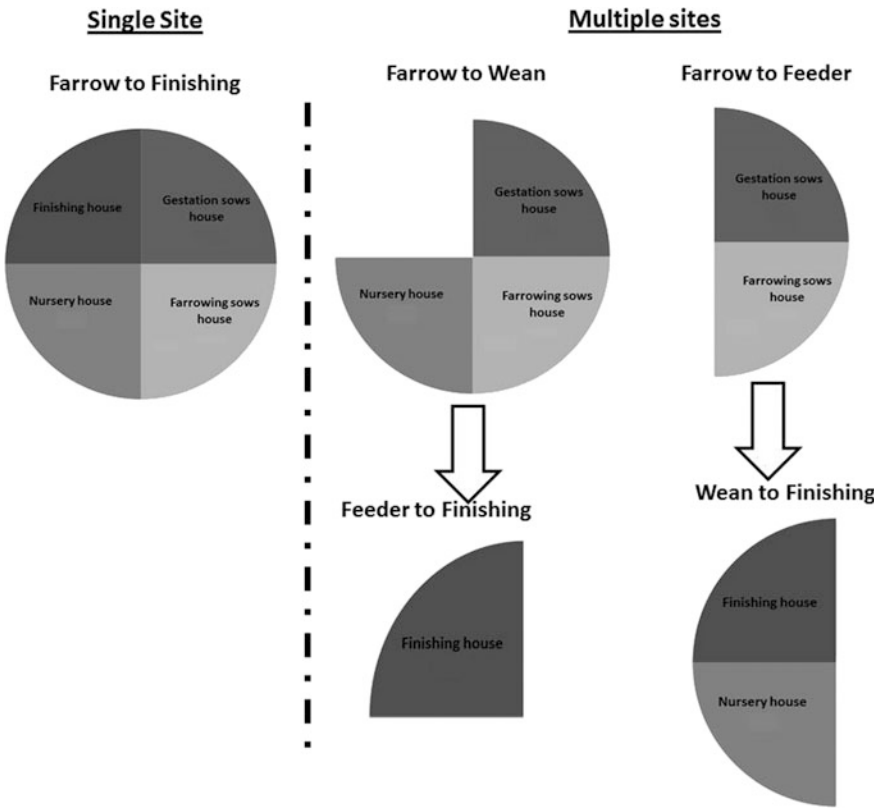

Fig. 5.3 Swine production systems and animal phases. *Source* Cestonaro do Amaral et al. (2016)

Mortality on production units can change depending on health status and management level, generally is between 3 and 9%. Likewise, the number of piglets stillbirths per litter can vary significantly, depending on litter size and sanitary status (FAO 2016c).

5.2 Management and Treatment of Animal Carcasses

There are different types of residues generated in livestock and poultry production that can be separated in: farm and industry levels. At farm level is generated mainly two residues, manure and dead animals, meanwhile, at industry level we have hatchery wastes, residues of meat, fat, feathers, blood, condemned carcasses, and others.

As the livestock industry grows, intensified for global food demands, the necessity of disposal alternatives that effectively manage carcasses and manure are increased. Simple and inexpensive methods such as burial are used for mortalities

disposal on small farms, but they can lead to water and air pollution, and neither is practical for routine, large-scale use (Gooding and Meeker 2016). Responsible and safe animal carcass disposal is an important issue whole the world (Won et al. 2016), and need includes protection of environment, animal, and public health, due to animal carcass may contain pathogens, many of zoonotic importance (Berge et al. 2009; Zhong et al. 2017).

The methods used for carcass disposal include incineration, burial, rendering, composting, and anaerobic digestion.

- Incineration: is thermal-treatment method where animal carcasses or by-products are burnt at high temperatures (>850 °C), during this process is expected to destroy all infective pathogens (NABC 2004) (Fig. 5.4). The principal health concerns with the incineration of carcasses related to gaseous emissions and release of dioxins and furans from flue gas and fly ash, from incomplete combustion can settle in areas around carcass incinerators (Gwyther et al. 2011; Hseu and Chen 2017). Pollution control, it is necessary for the incineration installation, can reduce the risk of noxious emissions.
- From an environmental point, animal carcass incineration has a high energy demand that uses very high temperature (Gwyther et al. 2011). Furthermore, must be taken into consideration about biosecurity risks when transporting animal carcasses off-site (farms) in order to incineration facilities Stanford and Sexton (2006).
- Burial: To be applied this method should be considered, land topography, water table, and soil type of the available land will determine if burial is a valid, although has degradation need time and while production of noxious odors will continue during the degradation Stanford and Sexton (2006). In order to reduce the risk of transmission of bovine spongiform encephalopathy (BSE), the

Fig. 5.4 Animal carcass incinerator equipment. *Source* Lucas S. Cardoso

Commission Regulation (EC No. 1774/2009) prohibited in EU on-farm burning and burial for all fallen stock, irrespective of species susceptibility to prion diseases (except in specific situations, or in areas where access is practically impossible). In USA, burial/permitted landfilling is an accepted practice for animal carcass disposal in emergency management of animal mortalities (USDA 2012).

- Rendering: in this process entails crushing animal carcasses and by-products into smaller particles, heating and separate fat and protein, transforming in meat and bone meal and tallow (Kalbasi-Ashtari et al. 2008). However, after problems with BSE, the feeding of meat and bone meal is currently prohibited in developed countries, owing to rendering plants do not play as significant a role in the disposal of animal wastes, to avoid the dispersion of pathogens (Franke-Whittle and Insam 2013). Tallow from rendering can be used in among other applications as soaps, washing powders, as lipids in the chemical industry and cosmetics (Kalbasi-Ashtari et al. 2008). Rendering, as for incineration, has a high energy demand but if tallow is recovered for subsequent energy production then the net GHG emissions are likely to be low. The main environmental concerns associated with rendering are related to gas and odor emissions (Gwyther et al. 2011). Figure 5.5 demonstrates a flour of animal carcass.
- Composting: is a simple technique that can be undertaken on-farm, typically the process involves the layering of carcasses between strata of carbon-rich substrate such as straw, sawdust, or rice hulks with a final covering of carbon-rich substrate over the entire pile (NABC 2004) (Fig. 5.6); it is a relatively inexpensive technology and the final product can be transformed in fertilizer (Wang et al. 2016). Composting of dead animals requires the addition of a carbon source to ensure proper *C/N* ratios, odor and leachate control and equipment requirements differ the composting process (Kalbasi et al. 2005). The time for composting is a concern due to characteristics of the organic material and pathogens reduction (Glanville et al. 2016), because the organic matter instability, recontamination by pathogenic organisms and ammonia emission (Lasekan et al. 2013).

Fig. 5.5 Flour of animal carcass. *Source* Monalisa Pereira

Fig. 5.6 Schematic of conventional composting system for dead animals

- Anaerobic digestion (AD): is a promising technology that combines a method for carcass disposal with renewable energy production, and other end products including liquid and solid fertilizers (digestate) (Zhang and Ji 2015). Anaerobic digestion of dead livestock is not permitted within current EU legislation without prior treatment of the carcass (sterilization) (EC No. 1069/2009). Figure 5.7 demonstrates an anaerobic co-digestion system that used animal carcass after pre-treatment.

Decision-makers should consider factors that compose each disposal technology (Table 5.2), including the principles of operation, costs, environmental considerations, advantages, and disadvantages of each technology (Baba et al. 2017).

Fig. 5.7 Anaerobic co-digestion of swine carcass and manure. *Source* Monalisa Pereira

Table 5.2 Advantages and disadvantages of methods for livestock carcasses disposal

Disposal methods	Advantages	Disadvantages
Incineration	Superior disease control high volume of waste reduction	Expensive, equipment, and fuel required; ash requires disposal; gas emissions;
Burial	Easy and inexpensive	Possible groundwater contamination tracking of sites required low degradation
Rendering	No generation of residues. Hide and tallow recycled	Logistic limitations; odor and gas emissions
Composting	Organic fertilizer production, easy technology, pathogens inactivation	Need control the time of composting due to odor emission and regrowth of pathogens
Anaerobic digestion	Renewable energy and organic fertilizer production	Necessity of pre-treatment of carcasses

5.3 Anaerobic Digestion Process Using Animal by-Products

5.3.1 Biochemical Methane Potential (BMP)

Due to a large production of livestock and poultry products, thousands of tons of organic by-products in the form of carcass, viscera, feet, head, bones, blood, and feathers are generated. Studies have suggested that residues that contain high concentrations of proteins and lipids (such as carcasses and animal products) are attractive substrates for biogas production (Rajagopal et al. 2014; Zhang and Ji 2015). The BMP test can be very helpful to estimate the biogas generation capacities of different substrates (Table 5.3).

The residues have a high methane potential, on the other hand, mono-digestion methods are susceptible to inhibition due to the accumulation of volatile fatty acids and/or unionized ammonia, resulting in toxicity for methanogenic archaea (Béline et al. 2017), reducing the methane production. One alternative to reduce this effect is simultaneous anaerobic co-digestion with others residues (e.g., manure), which may contribute to the dilution of inhibitory compounds originated during decomposition (Rajagopal et al. 2014). Using livestock manure with the substrate for co-digestion has shown to be an alternative treatment option.

Anaerobic co-digestion (AcoD) between manures and C-rich residues overcome these problems by maintaining a stable pH, within the methanogenic range, and reducing the ammonia concentration by dilution while enhancing methane production (Mata-Alvarez et al. 2011, 2014; Zhang et al. 2016). Most part of studies were conducted using livestock manure to establish different residues, with different types of reactors submitted at different operating parameters as temperature, organic loading rate (OLR), and hydraulic retention time (HRT) (Nasir et al. 2012).

Table 5.3 Biochemical methane potential of different residues of animal by-products

Animal	Material	BMP ($L_{N\ CH4}\ kg^{-1}_{VSadd}$)	Refs.
Bovine and swine	Digestive tract content	400	Luste et al. (2009)
	Meat and bone meal	390	Pitk et al. (2012)
	Fat	978	Pitk et al. (2012)
Swine	Meat tissue	976	Borowski and Kubacki (2015)
	Intestinal waste	826	Borowski and Kubacki (2015)
	Meat	575	Hejnfelt and Angelidaki (2009)
	Carcass	600	Tápparo et al. (2018)
	Solid slaughterhouse	580	Rodríguez-Abalde et al. (2011)
	Manure	406–1157 (biogas)	Cestonaro do Amaral et al. (2016)
Bovine	Soft offal	650	Ware and Power (2016)
	Paunch	228	Ware and Power (2016)
	Manure	204	Kafle and Chen (2016)
Poultry	Intestine residues	512	Yoon et al. (2014)
	Blood	250	Yoon et al. (2014)
	Solid slaughterhouse	460	Rodríguez-Abalde et al. (2011)
	Manure and feather	342	Yoon et al. (2014)
	Feather	210	Salminen and Rintala (2002)
	Meat	500	Salminen and Rintala (2002)
	Litter	259	Kafle and Chen (2016)

5.3.2 *Co-digestion of Animal Carcass*

As discussed above, co-digestion is an interesting alternative to reduce inhibitory effects of carcasses degradation under anaerobic conditions. Tápparo et al. (2018) described biochemical methane potential of swine carcass is around 1076 ± 48 $L_{Nbiogas}\ kg^{-1}_{VSadd}$ until five times more than swine manure. During co-digestion, the potential of methane yield is incremented until 6% per each $Kg_{carcass}$ added at m^3 of manure.

Massé et al. (2008) and Rajagopal et al. (2014) investigated psychrophilic AcoD of swine carcasses and swine manure in a sequence batch reactor (SBR) operated at 25 °C. Their results showed an increase in biogas production and no inhibition at rates of 20 and 40 $kg_{carcass}\ m^{-3}_{manure}$ (that represents up to eight times commercial swine farm mortality rates) (Massé et al. 2008). However, at carcass loading

Table 5.4 Operational conditions of animal carcasses anaerobic co-digestion

Material	Organic loading rate	Reactor type	Temperature (°C)	Refs.
Swine carcass and manure	3.2 g COD L^{-1} d^{-1}	SBR	20–25	Massé et al. (2008)
Swine carcass and manure	3.2 g COD L^{-1} d^{-1}	SBR	25	Rajagopal et al. (2014)
Swine carcass and sugar beet pulp	–	Batch scale	35	Kirby et al. (2018)
Beef carcass, algae, and manure	–	Batch scale	40	Pratt et al. (2013)
Swine carcass and vinasse	6.8 ± 0.4 $kg_{vs\ add}$· m^{-3} d^{-1}	Batch scale	35	Dai et al. (2015)

rates > 230 $kg_{carcass}$ m^{-3}_{manure} simulating emergency disease outbreak, the system was resulting in accumulation of volatile fatty acids and biogas inhibition (Rajagopal et al. 2014).

Several studies have tested different operational conditions for livestock and poultry carcass co-digestion with manure and others residues and are summarized in Table 5.4.

5.3.3 *Sanitary Aspects of Animal Carcass Anaerobic Digestion*

The AD process may be a sustainable method for on-farm carcasses management converting into biogas and organic fertilizers, with environmental and socioeconomic benefits (Hidalgo et al. 2018); however, when the reactors are operated in psycrophilic and mesophilic temperatures, the AD process itself is not sufficient to guarantee sanitary safety aspects (Viancelli et al. 2013; Fongaro et al. 2014; Tápparo et al. 2018).

Temperature is considered the main factor that influences the pathogens inactivation during anaerobic digestion (Franke-Whittle and Insam 2013) due to temperature increase can cause denaturation of proteins in the cell membrane, because it is more permeable and allowing diffusion of compounds into the cytoplasm Ziemba and Peccia (2011). Considering sanitary aspects, for animal by-products use in biogas plants, a pre-treatment is necessary to avoid pathogens dissemination in environment.

Regulation (EU) No 142/2011 (2011) determined that the process must be monitored and *E. coli* and Enterococcus counts must not exceed 1000 (3.0 log10) CFU/g, absence of *Salmonella* and *Clostridium perfringens*, reduction of infectivity of thermoresistant viruses and products must be subjected to a reduction in spore-forming bacteria, where they are identified as a relevant hazard (Commission Regulation (EU) No 142/2011 2011).

5.3.4 Compounds that May Cause Inhibition During AcoD Using Animal by-Products

The AcoD can be inhibited by some parameters that can compromise seriously the biogas-generating process. Some of these parameters that need attention during animal by-products anaerobic digestion are described below. Sometimes, these parameters present synergic effect, making difficult to determine the exact cause of decline in process performance (Moestedt et al. 2016).

5.3.4.1 Free Ammonia (FA)

The anaerobic digestion of livestock wastes and materials rich in proteins can increase total ammoniacal nitrogen (TAN) in digestate, that can cause inhibition of methanogenic microorganisms due to shifting of chemical equilibrium to FA resulting in low methane production (Yenigün and Demirel 2013; Kunz and Mukhtar 2016). The mechanism that explains FA inhibition says that it can freely permeate cell membranes resulting in the change in intracellular pH, increasing the cell maintenance energy requirement, and inhibition of specific enzyme reactions (Tao et al. 2017). Bayr et al. (2012) reported that one FA concentration of 635 mg L^{-1} promotion an inhibition of 50% on methane producing during the digestion of slaughterhouse by-products. High levels of FA also lead to an increase on volatile fatty acids concentration (VFA) during AD process, and this situation indicates an imbalance on microbiological community and facilitates foam generation (Kirchmayr et al. 2011; Resch et al. 2011). Previous studies about swine carcass and manure co-digestion in laboratory scale demonstrated an increase around 10 mg L^{-1} of NH_3–N for each $kg_{carcass}$ added per m^{-3}_{manure}, (Table 5.5).

5.3.4.2 Volatile Fatty Acids (VFA)

Residues that contain high lipids concentration are difficult to degrade, such as animal by-products, hydrolysis must be coupled with the growth of hydrolytic bacteria (Vavilin et al. 2008). Lipids can cause flotation and during hydrolysis, by extracellular lipases, VFA are accumulated (Palatsi et al. 2011). Anaerobic

Table 5.5 Ammonia and free ammonia during swine carcass and manure co-digestion

Swine manure and carcass ratio (kg m^{-3})	Digestate	
	NH_3–N (mg L^{-1})	Free ammonia (mg L^{-1})
0	2180	208
35	2220	269
68	2850	320
100	3000	345

Source Authors

digestion process is stable at a VFA-to-alkalinity ratio below 0.4. However, a severe instability can occur when the volatile fat acids/alkalinity (VFA/AL) ratio exceeds 0.6 (Mézes et al. 2011). Due to the possible accumulation of VFA, the co-digestion with substrate with higher alkalinity has a good option for animal by-products as described by (Rajagopal et al. 2014) and (Tápparo et al. 2018).

5.3.4.3 Foaming Generation

Substrate composition (i.e., lipids and proteins higher) has effects on the AD process viscosity, which may contribute to the increase of foaming (Kougias et al. 2014). Lipids have a tendency to form aggregates and foam causing problems (Cuetos et al. 2008). The presence of foaming in a biodigester can represent operational problems with as reactor overflow and fouling of mixing system (Kougias et al. 2015).

Several studies demonstrated a decrease of methane production because foaming problems and accumulation of fats occurred in the reactor during digestion or co-digestion of animal by-products (Cuetos et al. 2008; Pitk et al. 2013; Borowski and Kubacki 2015; Pagés-Díaz et al. 2015).

An ideal ratio between animal by-products and others residues are necessary for the process occurred without declining in biogas production. If one substrate was identified to cause foam, it was kept generally out of the process if possible or at least reduced in the substrate mix until foaming stopped (Lindorfer and Demmig 2016).

5.4 Legislation Applied for Animal by-Products Treatment and Disposal

European Union follows a regulation about the treatment and disposal of animal by-products (ABP). The European regulation (EC No. 1069/2009) defines different residues into categories based on the risk and material origin:

- Category 1: is a high-risk material, includes animals suspected of being infected by a transmissible spongiform encephalopathy (TSE), wild, pet, and zoo animals;
- Category 2: includes manure and digestive tract content, killed or fallen animals, including animals killed to disease control purposes, fetuses and oocytes, embryos, semen which are not destined for breeding purposes;
- Category 3: is low-risk ABP and comprises the following: carcasses and parts of animals slaughtered, blood, placenta, wool, feathers, hair, horns, and hoof that did not show infected disease communicable.

EU regulation describes the anaerobic digestion as an alternative treatment for Category 2 material (after pre-treatment, pressure sterilization), and Category 3 (some materials need used pasteurization like pre-treatment (EC No. 1069/2009).

In Brazil, national legislation describes that animals that died due to mandatory notification diseases, according to IN 50 (MAPA 2013), is a high-risk material (similar to material of Category 1 described in EU regulation), and have a specific treatment according to, respectively, state legislation. However, for routine mortalities (that could be classified as Category 2), alternative treatments could be applied. With the purpose of to evaluate and develop technological solutions of correct disposal of dead animals along poultry, swine, and bovine chains Embrapa (Brazilian Agricultural Research Corporation) and Ministry of Agriculture, Livestock and Supply (MAPA) developed the project "TEC-DAM, Technologies for disposal of dead animal". One of the objectives of this project is to evaluate the conditions for the use of dead animals in the biogas production chain (Nicoloso et al. 2017).

Due to the less development of anaerobic digesters in USA, no specific regulations about utilized animal by-products are found. However, Wang et al. (2018) suggested that USA could follow the European Union regulations for pathogens control during anaerobic digestion.

5.5 Final Remarks

Residues with high lipids and protein content like animal by-products, especially carcass, have an excellent potential of biogas. However, it is necessary a good process control due to a possibility of free ammonia and volatile fat acids accumulation and consequently inhibitions on methane production and foam generation. Besides that health aspects should be considered for digestion, as like European recommendation, the pre-treatment is imperative to ensure the pathogens inactivation.

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Chapter 6
Coupling Syntrophic Acetate Oxidation and Anaerobic Ammonium Oxidation When Treating Nitrogen-Rich Organic Wastes for Energy Recovery and Nitrogen Removal: Overview and Prospects

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Abstract There is high interest in applying anaerobic digestion to organic wastes for the recovery of biogas as a renewable energy source. In the case of protein-rich residues, the performance of anaerobic digesters might be affected by the accumulation of ammonia and volatile fatty acids. High concentrations of these compounds impact negatively on the activity of the acetotrophic methanogenic archaea (AMA). This limitation can be overcome by promoting the enrichment within digesters of syntrophic acetate-oxidizing bacteria (SAOB) in conjunction with certain groups of hydrogenotrophic methanogenic archaea (HMA). These two microbial populations have a relatively high tolerance towards the aforementioned inhibitory compounds. Hence, when the partial pressure of hydrogen is low enough,

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SAOB metabolize acetate to carbon dioxide and hydrogen, which are syntrophically consumed by HMA. Once the organic matter has been biodegraded, the remaining nitrogen can be biologically removed from digester supernatants by the anaerobic ammonium oxidation (anammox). This pathway consists of the simultaneous conversion of ammonium and nitrite to (di)nitrogen gas, and, therefore, a previous partial oxidation of ammonium to nitrite under aerobic conditions is required. Interestingly, the whole process constitutes a completely autotrophic nitrogen removal strategy. This chapter compiles the current knowledge on the syntrophic oxidation of acetate and on the anaerobic oxidation of ammonium, mostly focusing on technological aspects in view of a sequential bioreactor implementation.

Keywords Anaerobic digestion · Syntrophic acetate oxidation · Methanogenesis · Biogas · Autotrophic nitrogen removal · Partial nitritation · Anaerobic ammonium oxidation

6.1 Introduction

The anaerobic digestion (AD) of protein-rich wastes may result in the inhibition of acetotrophic methanogenic archaea (AMA). These microorganisms are particularly sensitive to the accumulation of compounds such as ammonia (NH_3) and volatile fatty acids (VFA). This limitation can be overcome by prompting the combined enrichment in anaerobic digesters of syntrophic acetate-oxidizing bacteria (SAOB) and certain hydrogenotrophic methanogenic archaea (HMA), since both populations are resilient to relatively high concentrations of these compounds. After the AD of the organic matter, nitrogen (N) mainly remains in a reduced mineral form (i.e. ammonium–N), so that it can be removed through the conventional combined process of nitrification–denitrification (NDN). Yet, the high energy demand for aeration during nitrification and the potential requirement of an external organic matter source for denitrification constrain the viability of this well-known process. Alternatively, N treatment can be enhanced significantly by means of autotrophic N-removal (ANR), based on the coupling of partial nitritation (PN) with anaerobic ammonium oxidation (anammox). In this chapter, the state of the art and prospects on the application of syntrophic acetate oxidation (SAO)-based AD and anammox-based ANR to N-rich effluents are outlined and discussed.

6.2 Syntrophic Acetate Oxidation

6.2.1 Process Description and Microbial Populations

The AD process occurs naturally in environments where molecular oxygen (O_2) and other electron acceptors are lacking, such as waterlogged soils, lake and ocean basin sediments, and the gut of humans and other animals, particularly in ruminants. It can also be an unwanted anthropogenic process as it triggers the release of methane (CH_4) to the atmosphere in landfills, rice paddies and intensive livestock farming, thus contributing to the greenhouse effect. Yet, AD can be turned into a helpful biotechnology when it is applied to the treatment of organic materials like agricultural, livestock, industrial or municipal wastes (Abbasi et al. 2012). The economic viability of the AD process depends on the specific production of CH_4 and on the cost-benefit relationship of the global waste management strategy, among other factors (e.g. policy incentives/subsidies, penalties for polluting, funding opportunities, receptivity and innovation capacity).

The AD process consists in a cascade of syntrophic interactions between several microbial groups. It implies the biological breakdown of complex organic molecules in the absence of oxygen and the formation of biogas; a gas mixture rich in CH_4 and carbon dioxide (CO_2) which can be valorized as renewable energy. The multiple involved reactions occur simultaneously and are grouped into four major steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Batstone et al. 2002). The microbial community responsible for AD is complex, formed by facultative and obligate anaerobic microorganisms, members of the prokaryotic domains *Bacteria* and *Archaea*. The *Bacteria* compose the predominant community and take part in all metabolic processes except methanogenesis, which is exclusively performed by the *Archaea* (Conrad 1999). Thus, although present at much lower levels, methanogenic archaea play a key role in AD as the sole responsible for CH_4 production.

The main representatives of the domain *Bacteria* in anaerobic digesters belong to the phyla *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Actinobacteria* and *Proteobacteria* (St-Pierre and Wright 2014). The hydrolysis step is carried out by hydrolytic-fermentative bacteria that solubilize complex substrates. In acidogenesis, the soluble compounds previously formed are converted mainly to VFA by fermentative bacteria and, subsequently, in the acetogenesis step, to acetate and hydrogen (H_2) by acetogenic bacteria. Acetogens are obligate anaerobic bacteria that use the reductive acetyl-CoA or Wood–Ljungdahl (W-L) pathway as the main mechanism for energy conservation, as well as for the synthesis of acetyl-CoA and cell carbon (C) from CO_2 (Müller 2003; Ragsdale and Pierce 2008). Acetogens are sometimes called "homoacetogens" because of their capability to produce acetate as the only fermentation product (Eq. 6.1).

$$2CO_2 + 4H_2 \leftrightarrow CH_3COO^- + H^+ + 2H_2O \quad (6.1)$$

The AD process is completed by methanogens, which consume the products released by the acetogenic and/or acidogenic microorganisms converting them to CH_4 and CO_2 (Conrad 1999; Liu and Whitman 2008). Depending on the substrate used for CH_4 production, methanogens are mainly classified as acetotrophic and hydrogenotrophic methanogenic archaea—AMA and HMA, respectively—as well as the minor group of methylotrophic methanogens, which are able to produce CH_4 from methylated-C1 compounds (methanol, methylamines, etc.). Methanogens have previously been encompassed within the archaeal phylum *Euryarchaeota,* but new phyla have been proposed recently, suggesting that methanogenesis may be more phylogenetically diverse than initially appreciated (Vanwonterghem et al. 2016). AMA belong to the order *Methanosarcinales*, which includes the family *Methanosaetaceae* that only produce CH_4 from acetate, and *Methanosarcinaceae*, capable of producing CH_4 using all three metabolic pathways for methanogenesis: from CO_2, acetate and methylated-C1 compounds (Stams 1994; Liu and Whitman 2008). On the other hand, HMA are widespread since their capability to use H_2 as electron donor for CO_2 reduction evolved quite early in the history of life (Leigh 2002). Thus, this metabolic pathway is found in members of the orders *Methanomicrobiales*, *Methanococcales, Methanocellales*, *Methanobacteriales* or *Methanopyrales*. The methylotrophic methanogenesis is the most exclusive pathway, mainly limited to the order *Methanosarcinales* and other particular taxa such as the genus *Methanosphaera* (Liu and Whitman 2008).

6.2.1.1 Biological Inhibition by Nitrogen

An important compound regarding the AD process is ammonium, which is formed after the degradation of proteins. Organic-N transformations under anaerobic conditions result in mineralized N in the form of free ammonia (FAN; NH_3–N) and its ionized counterpart, ammonium (NH_4^+–N). In aqueous media, these two species are in equilibrium and their fractionation mainly depends on the temperature and pH. Both species are usually measured together as total ammonium–N (TAN). For a given concentration of TAN, FAN increases with temperature and pH (Hansen et al. 1998).

Nitrogen is an essential nutrient for life and, in particular, TAN is a key player in the growth of microorganisms. Generically, an anaerobic cell mass yield coefficient of 0.15–0.20 g VSS/g $COD_{degraded}$ is assumed for the acid stage, and of 0.03–0.04 g VSS/g $COD_{degraded}$ for the methanogenic stage, with a specific N cell content of about 0.12 g N/g VSS (Henze et al. 1995; VSS is volatile suspended solids and COD is chemical oxygen demand). Furthermore, TAN contributes to the buffer capacity of the medium, but concentrations in the digester >0.3 kg FAN/m^3 can lead to the loss of biological activity (Henze et al. 1995). Too much N will result in the inhibition of methanogenesis, and in the accumulation of intermediate

AD products, such as VFA which, in turn, may contribute further to the instability of the digester. Free ammonia easily diffuses across the cellular membrane, and once in the cytoplasm, it can induce disturbances in the pH, affecting some specific enzymatic reactions (Hunik et al. 1990; Calli et al. 2005; Siles et al. 2010).

TAN inhibition depends on several factors such as the substrate nature, digester running conditions (e.g. temperature, pH, and biomass acclimation) and the microbial community structure of the methanogenic biomass. A wide range of inhibitory TAN levels has been reported in the literature. Under mesophilic conditions (30–37 °C and pH 7–8), partial inhibition was found to occur at 1.5–2.5 kg N/m^3 (Koster and Lettinga 1984; Kayhanian 1994), 5–7 kg N/m^3 (Wang et al. 2016b), 7–9 kg N/m^3 (Sun et al. 2016) and 16 kg N/m^3 (Niu et al. 2015). TAN inhibition under thermophilic conditions (55–60 °C and pH about 8) was reported at 6 kg N/m^3 (1.6–2.6 kg FAN/m^3) when digesting swine manure under continuous conditions (Hansen et al. 1998). However, the same authors (Hansen et al. 1999) reported that FAN inhibition was alleviated just by decreasing the process temperature, while keeping constant the other parameters. When the temperature was adjusted to 37 °C, the concentration of NH_3 within the digester decreased down to 0.75 kg FAN/m^3, leading to a threefold increase in the CH_4 yield.

The TAN content within the anaerobic digester, along with the temperature, reactor configuration and operational parameters, have been identified as major factors that determine the composition of the microbial community (Niu et al. 2015; Poirier et al. 2017). The inhibitory impact of TAN on the microbial populations is considered to be more significant for the last biodegradation stages; particularly for the methanogenesis (Sung and Liu 2003; Calli et al. 2005). Yet, not all methanogens are affected equally. AMA, which under common non-inhibitory conditions are responsible for most of the generated CH_4 in anaerobic digesters, have been described as vulnerable to concentrations of 3.3–5 kg TAN/m^3 (Schnürer and Nordberg 2008; Banks et al. 2012). In contrast, the less sensitive HMA have been reported as capable of remaining active at those concentrations (Wang et al. 2015). Furthermore, TAN inhibition of AMA might result in the accumulation of acetate up to toxic levels (>4 kg acetate/m^3; Lv et al. 2014), contributing to a negative feedback mechanism that eventually leads to a complete reactor failure (Zhang et al. 2014; Wang et al. 2015). Since biogas production by AD involves a complex interaction of microbial populations, a reduced methanogenic activity influences the microbial populations lower in the trophic chain (Wang et al. 2015).

6.2.1.2 Syntrophic Interactions

Microbial syntrophy can be defined as any type of cross-feeding of molecules between different microorganisms, but a restricted definition is applied for the anaerobic syntrophic metabolism. In this case, syntrophy is a close mutualistic interaction in a very specific nutritional situation where the level of exchanged intermediates must be kept low for an efficient cooperation. Under these conditions, syntrophic partners combine their metabolic capabilities to catabolize a substrate

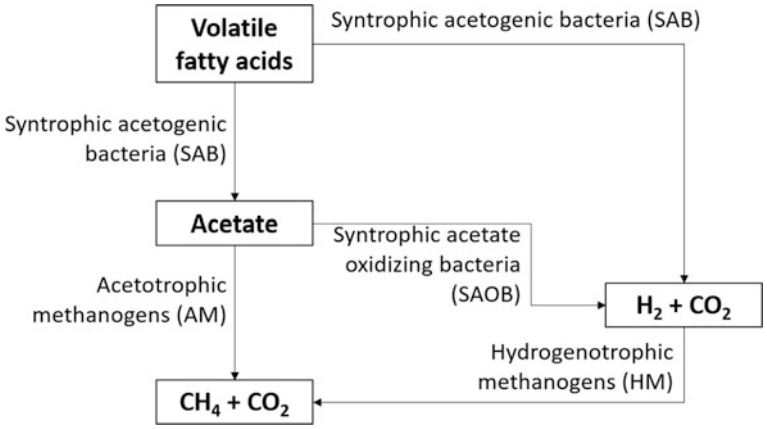

Fig. 6.1 Scheme of the acetogenic and methanogenic steps of the anaerobic digestion process including syntrophic acetate oxidation. Adapted from De Vrieze et al. (2012)

that none of them can process alone (McInerney et al. 2009; Morris et al. 2013). In particular, methanogenic archaea develop syntrophic relations with other microbial communities to biodegrade complex organic compounds (Fotidis et al. 2014).

A group of homoacetogenic bacteria, known as syntrophic acetate-oxidizing bacteria (SAOB), are able to reverse acetogenesis and oxidize acetate to CO_2 and H_2, provided that their products are directly used by H_2-scavenging methanogens (Schnürer et al. 1999). This syntrophic pathway enables the use of acetate as a C and electron source via an alternative pathway to the direct use of acetate by AMA. In the syntrophic acetate oxidation process (SAO; Fig. 6.1), both SAOB and HMA populations are mutually dependent in metabolic terms. The SAO process is thermodynamically feasible only if the syntrophic HMA keep the H_2/formate level low enough, but still suitable, for its biological activity (Stams 1994; Hattori 2008; Schnürer and Nordberg 2008). Besides the homoacetogens, certain methanogens and anammox bacteria, as well as some sulphate reducing bacteria, use the W-L pathway of CO_2 reduction (Ragsdale and Pierce 2008; Müller et al. 2013; Zhao et al. 2018). The syntrophy between SAOB and HMA has been described as beneficial for the AD of N-rich substrates, due to their higher resilience, in relation to AMA, towards the accumulation of FAN and VFA (Schnürer et al. 1999; Schnürer and Nordberg 2008; Hao et al. 2011, 2015; Westerholm et al. 2012; Lü et al. 2013).

The W-L pathway is used by SAOB in a reductive way when growing heterotrophically, and it can be viewed as a series of reactions resulting in the reduction of two molecules of CO_2 to a bound methyl group and a carbonyl group, which finally becomes the acetyl moiety of acetyl-CoA (Ragsdale 2008; Ragsdale and Pierce 2008). The electrons obtained from these cascading reactions must be removed to achieve an appropriate redox balance and regenerate the oxidized electron acceptor. The enzyme *formyltetrahydrofolate synthetase* (FTHFS, EC 6.3.4.3) has been described as crucial for the SAO process (Matsui et al. 2008;

Müller et al. 2013), but the involvement of this enzyme in other metabolic pathways (Ragsdale and Pierce 2008) hinders its potential use as a specific SAO biomarker. Isotope fingerprinting techniques have been used for assessing the predominance of this route[1] through the analysis of the produced biogas, in terms of labelled ^{13}C or ^{14}C (Fotidis et al. 2013, 2014; Gehring et al. 2015, 2016). The fractionation factor is then calculated according to the isotopic composition. In experiments with ^{14}C-methyl labelled acetate, it has been assumed that a $^{14}C–CO_2/^{14}C–CH_4$ ratio <1 corresponds to a predominantly acetotrophic methanogenesis, while a ratio >1 results from a predominantly hydrogenotrophic methanogenesis, which is then associated to SAO (Fotidis et al. 2014). The addition of the radioactive C isotope (^{14}C) allows for a straightforward data interpretation since there is no presence of ^{14}C other than the added tracer. In contrast, the addition of heavy stable C (^{13}C; naturally occurring isotope) leads to a more complex stoichiometry for the combined metabolism of SAOB and HMA (Gehring et al. 2016). Biological systems tend to select for the lighter isotopes over the heavier ones and give specific isotopic ratio signatures that depend on the metabolic processes involved. Hence, as an alternative to isotopic labelling, the analysis of the natural $^{13}C/^{12}C$ isotopic fractionation of biogas components (δCO_2 and δCH_4) has also been used for determining the predominant methanogenic pathway in natural environments (Conrad 2005). The apparent fractionation factor (α_C)[2] obtained from this data indicates the dominance of acetotrophic methanogenesis ($\alpha_C < 1.055$) or hydrogenotrophic methanogenesis ($\alpha_C > 1.065$).

Despite the fact that the W-L pathway has been reported in biogas digesters and in other anaerobic environments, such as lake sediments, oil reservoirs and nutrient-enriched soils (Schnürer and Nordberg 2008; Müller et al. 2013), the information available regarding the occurrence, diversity and role of the SAOB is still limited (Westerholm et al. 2016). So far, the SAOB have mostly been affiliated

[1]The use of methyl or carboxyl labelled acetate isotopes is based on the fact that in the acetotrophic methanogenesis (Equation A), CH_4 originates only from the methyl C in the acetate molecule. Differently, after acetate oxidation, both atoms of C (methyl and carboxyl) are available for the hydrogenotrophic methanogenesis (Equation B) in the form of CO_2. Reactions A and B finally provide the same products (two moles of CH_4 and two moles of CO_2), but only the conversion of labelled ^{13}C-acetate, or ^{14}C-acetate, (identified by the underlined C atom in both Equations) by AMA yields two moles of labelled CH_4 (Reaction A). In contrast, the SAO process (Reaction B) yields a uniform distribution of the labelled C as CH_4 and CO_2 (Gehring et al. 2016).

$$2\underline{C}H_3COOH \rightarrow 2\underline{C}H_4 + 2CO_2 \quad \text{(A)}$$

$$2\underline{C}H_3COOH + 4H_2O \rightarrow 2\underline{C}O_2 + 2CO_2 + 8H_2 \rightarrow \underline{C}H_4 + CH_4 + \underline{C}O_2 + CO_2 + 4H_2O \quad \text{(B)}$$

[2]Carbon isotopic ratio: $\left(\delta^{13}C = \left(\frac{\left(^{13}C/^{12}C\right)_{sample}}{\left(^{13}C/^{12}C\right)_{standard}} - 1\right) \cdot 10^3 \text{ (‰)}\right)$; Fractionation factor: $\left(\alpha_C = \frac{\delta^{13}CO_2 + 10^3}{\delta^{13}CH_4 + 10^3}\right)$

to the phylum *Firmicutes* (Hao et al. 2011; Sun et al. 2014). Some examples of known SAOB are the thermophilic species *Thermacetogenium phaeum* (Hattori et al. 2000) and *Thermotoga lettingae* (Balk et al. 2002), as well as the mesophilic *Clostridium ultunense* (Schnürer et al. 1996), *Syntrophaceticus schinkii* (Westerholm et al. 2010) and *Tepidanaerobacter acetatoxydans* (Westerholm et al. 2011).

Alternatively, it has been suggested that the SAO process could be mediated via the glycine cleavage system (GCS; Nobu et al. 2015). However, this newly proposed pathway for acetate degradation still needs further research. Liang et al. (2016) have recently proposed that, during the AD of long-chain n-alkanes, species belonging to the family *Anaerolineaceae* (phylum *Chloroflexi*) may act as acetogens, SAOB, and formate-producing bacteria. Furthermore, some HMA belonging to the genus *Methanoculleus* could produce CH_4 from H_2/CO_2 and formate. In a recent interdisciplinary metagenomic study, Ruiz-Sánchez et al. (2018) characterized full-scale anaerobic digesters operated in continuous at 6–7 kg TAN/m^3. A shotgun analysis was implemented to explore the presence and function of genome sequences involved in the SAO process, along with the isotopic biogas profiling, and the description of active microbiome. The results suggested that representatives of the phyla *Bacteroidetes* and *Chloroflexi* might be involved in the SAO process because of their potential capability for acetate assimilation/dissimilation through both W-L and GCS pathways (Fig. 6.2).

These authors (Ruiz-Sánchez et al. 2018) also showed the predominance of HMA communities and the hydrogenotrophic pathway upon TAN exposure within the AMA inhibitory range (6–7 kg TAN/m^3), so that SAOB must have played a significant role under such conditions. Similarly, Lv et al. (2014) reported that acetate consumption and CH_4 production via the syntrophic route is especially dominant when the concentration of acetate is above 4 kg/m^3. Most methanogens capable of growing syntrophically with SAOB belong to the genera *Methanothermobacter* (Kato et al. 2014), *Methanosarcina or Methanoculleus* (Mosbæk et al. 2016). Also, recent genomic evidence has pointed out to the relevance of *Methanomassiliicoccus* in this syntrophy due to the metabolic flexibility of this genus, which contributes to both acetotrophy and hydrogenotrophy (Ruiz-Sánchez et al. 2019).

In anaerobic environments, H_2 and formate might act alternatively or simultaneously as electron shuttle molecules between fermentative and methanogenic communities, mechanism that has been described as the most suitable for their syntrophic interaction (Schink et al. 2017). Yet, recent studies have highlighted that some bacterial communities could transfer electrons to methanogenic archaea without shuttle molecules, a phenomenon known as direct interspecies electron transfer (DIET) (Zhao et al. 2016). This electron exchange could be performed by cell-to-cell contact, for instance, via electrically conductive cellular *pili*, allowing biodegradation and CH_4 production in a metabolically more efficient manner. This mechanism has been described for the electron transfer between *Geobacter metallireducens* and *Methanosaeta* spp. in AD reactor biomass and in defined co-cultures (Rotaru et al. 2014). Alternatively, other investigations have shown that

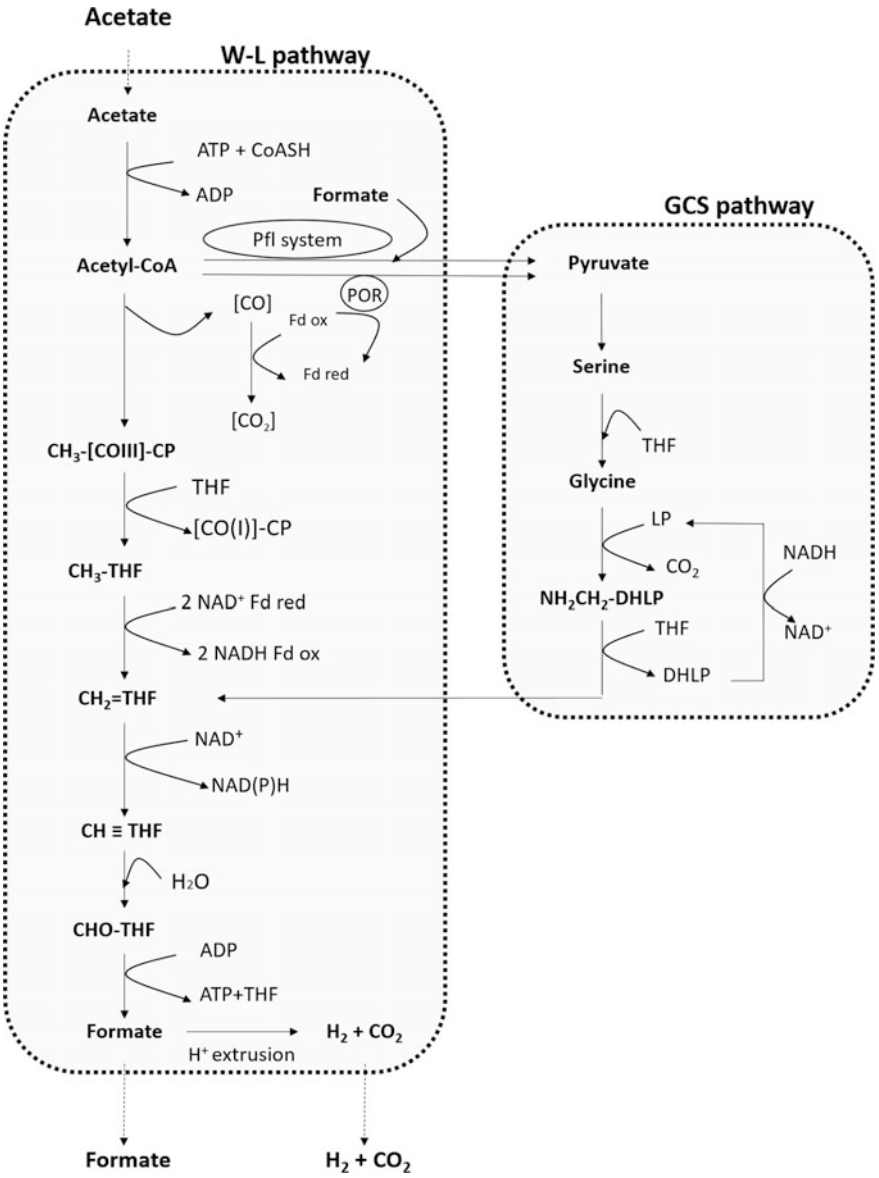

Fig. 6.2 Hypothetic interaction between the W-L and GCS metabolic pathways. Adapted from Nobu et al. (2015) and Ruiz-Sánchez et al. (2018)

the DIET mechanism can be facilitated by adding electrically conductive materials. This strategy improves the efficiency and robustness of reactor performance by introducing new materials in its design and/or operation (Liu et al. 2015; Zhuang et al. 2015; Cuetos et al. 2017). Syntrophic communities might then grow as biofilms attached to these conductive materials, which are used for extracellular

electron transfer. The metabolic energy investment is thus optimized because of the decrease in the need for conductive cellular *pili* (Holmes et al. 2016).

6.2.2 Applied Aspects and Practical Implementation

A biomass acclimation and/or adaptation[3] is usually quoted as the explanation for the wide range of inhibitory TAN levels reported in the scientific literature. Microbial resilience to elevated concentrations of TAN keeps a steady performance of the anaerobic digesters when subjected to perturbations in N load. Some practical experiences on biomass acclimation to high TAN include those of Gao et al. (2015), in a mesophilic continuous stirred-tank reactor (CSTR) for the AD of food waste, with a stepwise load increase from 2 to 4.5 kg N/m^3 over a period of 160 days; Abouelenien et al. (2009) acclimatized mesophilic biomass from 2 to 8 kg N/m^3 over a period of 400 days for the treatment of chicken manure in a sequence of repeated batches. Conversely, Niu et al. (2015) were able to recover the stable operation of a N-inhibited mesophilic CSTR fed with chicken manure as well upon dilution of the influent from 16 to 4 kg N/m^3.

Different strategies have been proposed to enrich or acclimate microbial populations in AD systems with the objective of optimizing the underlying bioprocesses under extreme conditions (Chen et al. 2008; Yenigün and Demirel 2013). The first studies dealing with ammonia acclimation mostly focused on choosing an adequate feed dilution strategy, by applying the co-digestion conditions needed to optimize the C/N ratio in the digester feed or by assessing the effect of discontinuous or pulsed exposure to elevated N concentrations (Demirel and Scherer 2008; Rajagopal et al. 2013; Mata-Alvarez et al. 2014). More recently, other alternatives to prevent inhibition or stimulate microbial activity have also been tested such as ammonia stripping, struvite precipitation and supplementation with additives, either inorganic (micronutrients, ammonia-sequestering agents or biomass support materials) or biological (bioaugmentation with microbial cultures or enzymes) (Walker et al. 2011; Lin et al. 2013; Cruz-Viggi et al. 2014; Sun et al. 2014; Romero-Güiza et al. 2015, 2016; Westerholm et al. 2016). However, these approaches are not always feasible or cost-effective to be applied at full scale.

Acclimation to elevated ammonia levels often implies a shift in the dominant acetate conversion pathways, from the acetotrophic methanogenesis to the more N-resilient SAO/hydrogenotrophic route. This fact has been demonstrated using isotope fingerprinting techniques and by high throughput molecular methods for microbial ecology assessment (Ho et al. 2013; Fotidis et al. 2014; Regueiro et al. 2016; Ruiz-Sánchez et al. 2018). In this regard, isotope fingerprinting has been

[3]Biomass acclimation refers to reversible physiological adjustments of microorganisms in response to rather short-term/limited perturbances in the environment, whereas biomass adaptation usually refers to changes in the microbial community structure and function in response to more intense/persistent environmental changes.

proposed as an early warning tool in stressed anaerobic digesters. Polag et al. (2015) found that these techniques anticipated stress changes affecting the process from five to ten days earlier than other traditionally monitored parameters—e.g. alkalinity ratio or VFA accumulation—in a full-scale mesophilic CSTR running at variable organic loading rates. Other authors, such as Lv et al. (2014) and Gehring et al. (2015), monitored changes in the methanogenic pathways by measuring the α_C in a mesophilic CSTR fed with blends of chicken manure and maize and in a mesophilic upflow anaerobic filter of a two-stage AD system fed with maize silage, respectively.

Microbial shifts towards the dominance of SAOB and HMA are affected by other factors besides FAN, such as the reactor configuration, the hydraulic residence time (HRT) and the solids residence time (SRT) applied during its operation (Demirel and Scherer 2008; Ho et al. 2013; Westerholm et al. 2016). The SAOB are slow-growing microorganisms, with a doubling time of about 28 days in mesophilic conditions (Schnürer et al. 1994; Schnürer and Nordberg 2008). This value is much higher than the approximately 30 min needed for the fast-growing acid-forming bacteria to double its populations, the 1.5–4 days required for the acetogens (Kothari et al. 2014), and even the 2–12 days for the acetotrophic methanogens (Jetten et al. 1992). Thus, in a CSTR without solids retention, elevated N levels (>5 kg TAN/m^3) combined with a prolonged HRT (>45 days) usually lead to the establishment of a SAO-mediated process (Ek et al. 2011). However, there is a wide disparity in the HRT in which SAOB have been detected, i.e. 17–130 days for species taxonomically related to *S. schinkii*, 24–64 days for *C. ultunense*, 24–101 days for *T. acetatoxydans* or 40–60 days for *T. lettingae* (Westerholm et al. 2016).

The SRT can be uncoupled from the HRT by partial recirculation of the digester effluent. The recuperative thickening (RT) belongs to this approach, extending the SRT in CSTR digesters by the recovery and reintroduction of part of the produced and previously thickened sludge. This is a relatively simple and low-cost option to achieve an increased treatment capacity, with relatively little additional requirements of space and capital investment (Yang et al. 2017). The RT increases the concentration of microorganisms (Nagao et al. 2012) and the retention of micronutrients within the digester. Furthermore, the partial reintroduction of solid sludge contributes to optimizing the C/N ratio, due to a decrease in the availability of soluble TAN (Zhang et al. 2013). Another method to promote an increased treatment capacity with no extra energy inputs relies on carefully synchronizing the organic loading rate applied to the digester and the composition of the processed organic substrates. Although this alternative approach may lead to a more stable operation, particularly in the mesophilic range, it appears to be less effective than the RT (Labatut et al. 2014).

Another option to achieve an optimized AD process based on SAO is to promote the growth of the methanogenic biomass as biofilms on carrier materials, like in packed-bed or hybrid reactors. A biofilm is an assemblage of microorganisms that grow attached to a surface thanks to an envelope of extracellular polymeric substance matrix. Hence, biofilm growth extends the SRT over the HRT and also

confers microorganisms with a high resistance towards external perturbations like loading shocks, temperature and pH changes, and the exposure to toxics and inhibitors (Garrett et al. 2008; Flemming et al. 2016). Many different materials have been reported to promote biofilm formation in the AD processes, e.g. clays, alumina-based ceramics, carbon compounds, synthetic polymers, metal or magnetic particles, among others (Muñoz et al. 1997; Lalov et al. 2001; Chauhan and Ogram 2005; Silva et al. 2006; Hellman et al. 2010; Ahammad et al. 2013; Habouzit et al. 2014; Zhao et al. 2015).

The introduction of support materials, biofibres or electrically conductive materials in anaerobic digesters for promoting the formation of biofilms enhances microbial tolerance and process performance under high concentrations of potentially inhibitory compounds, including FAN and VFA (Poirier et al. 2017; Capson-Tojo et al. 2018). The presence of such carriers also favours interesting syntrophic interactions resulting in an enhanced CH_4 production and COD removal. Non-biological conductive materials such as magnetite, graphite, biochar or carbon nanotubes stimulate the methanogenic activity (Cruz-Viggi et al. 2014; Li et al. 2015; Zhao et al. 2015). In these biofilms, bacterial and archaeal cells coexist by forming dense aggregates, which facilitate the DIET mechanism between exoelectrogenic bacteria and electron-utilizing methanogens (Zhao et al. 2016; Baek et al. 2017; Li et al. 2017). The most frequently used digester configuration containing support materials is the packed-bed reactor. The carrier provides a large specific surface for the fast development of biofilms, thus preventing the cell washout of slow-growing microbes and favouring methanogenesis (Qureshi et al. 2005). In principle, the COD removal efficiency and the microbial consortium developed in these digesters are directly related to the characteristics of the support material used for cell immobilization (Chauhan and Ogram 2005). Alternatively, hybrid reactors containing support materials such as bentonite or polyurethane foam, among others, have also been used successfully (Borja et al. 1998; Rajakumar et al. 2012).

6.3 Autotrophic N-Removal Based on Anaerobic Ammonium Oxidation

The characteristics of the effluent from anaerobic digesters, the so-called digestate, are strongly dependent on the processed materials and on the treatment conditions (Makádi et al. 2012). The AD process does not modify the total N content in the material being treated although it favours its mineralization. The use of the digestate in agriculture as organic fertilizer is an interesting option owing to the implicit replacement of mineral fertilizers. However, factors such as transportation costs, water content or presence of heavy metals and pathogens can hinder this option (Nkoa 2014). Thus, further treatment of the digestate may be needed to enhance transportability of valuable components or to protect human health and the quality of agricultural ecosystems, water bodies and the atmosphere. Solid–liquid separation provides two different fractions that need to be handled independently.

The solid fraction can be transported to longer distances for its use as a slow release fertilizer owing to the diminution in the water content, or undergo further treatment, like composting. The liquid fraction—i.e. digestate supernatant—usually contains most of N in the form of ammonium (NH_4^+), and so it can be used for the fertigation of adjacent arable land or post-treated in accordance with its typical low bCOD/N ratio—bCOD is biodegradable COD—for the removal or recovery of N (Magrí et al. 2017). The selection of the treatment method is determined by the concentration of N in the digestate supernatant, among other factors (Magrí et al. 2013). In these circumstances, and because of the low demand for materials and energy inputs, biological ANR based on the anammox process has attracted attention as an interesting technology for the economical treatment of ammonium.

6.3.1 Process Description

The ANR consists in combining both the PN and anammox processes, as shown in Fig. 6.3. In PN, only half of the ammonium is oxidized to nitrite (NO_2^-) under aerobic conditions. The conversion needed for this purpose is at a molar rate of about 57% (Eq. 6.2). This rate fits the typical anammox reaction proposed by Strous et al. (1998) (Eq. 6.3), where the anaerobic conversion ratio is 1.32 mol NO_2^-/mol NH_4^+. Taking into consideration that complete nitrification is performed primarily by two different bacterial groups, i.e. nitritation ($NH_4^+ \rightarrow NO_2^-$) by ammonium-oxidizing bacteria (AOB) and nitratation ($NO_2^- \rightarrow NO_3^-$) by nitrite-oxidizing bacteria (NOB), the activity of the second group should be suppressed to avoid formation of nitrate (NO_3^-). Approximately, two moles of protons are generated per mole of oxidized ammonium (1.14 mol H^+/mol $N_{supplied}$, see Eq. 6.2). Nitritation is thus an acidifying reaction that implies a significant consumption of alkalinity (ALK). Subsequently, the ammonium remaining after PN is oxidized to (di)nitrogen gas, in the absence of oxygen and using nitrite as electron acceptor by the anammox bacteria. A small amount of nitrate is also produced due to the oxidation of nitrite linked to the cellular fixation of inorganic carbon (de Almeida et al. 2011). Hydrazine (N_2H_4) and nitric oxide (NO) are rather toxic intermediate products from the anammox metabolism. Conversely, nitrous oxide (N_2O) is not a product of the anammox reaction, and, thus, the eventual emission of N_2O in ANR is mostly related to the concomitant activity of nitrifying and denitrifying microorganisms (Massara et al. 2017). The maximum activity of anammox bacteria was observed between 35 and 40 °C (Dosta et al. 2008), and exposure to low concentrations of oxygen induces a reversible inhibition (Strous et al. 1997; Seuntjens et al. 2018). Additionally, many other factors can negatively influence the process, such as pH, N-substrates (ammonium and nitrite), organic matter, phosphate, sulphide, salts, antibiotics or heavy metals (Jin et al. 2012). The anammox reaction implies a slight recovery of the alkalinity consumed in PN (0.056 mol H^+/mol $N_{supplied}$). The overall N-removal efficiency attainable in the liquid phase by applying the PN–anammox process is approximately 90%.

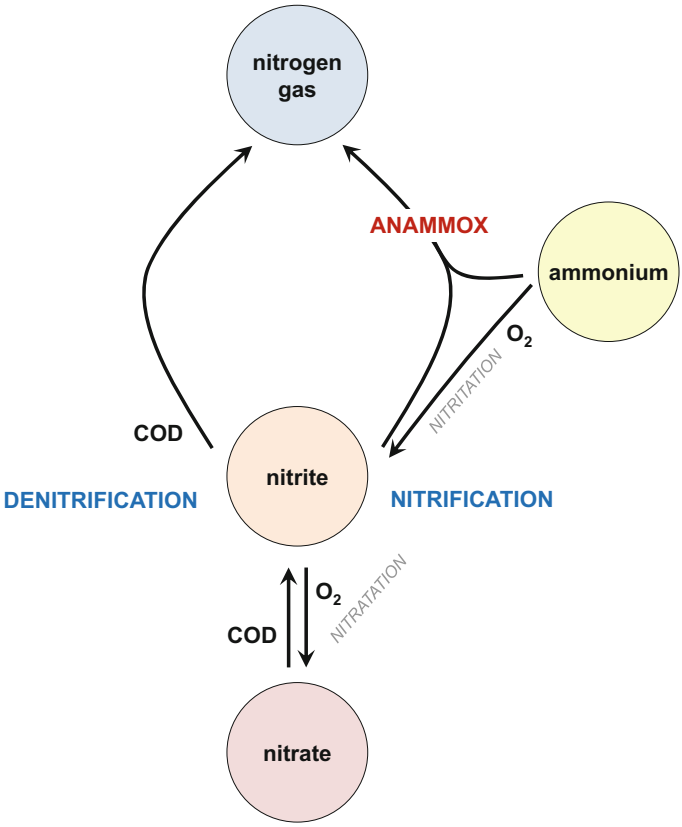

Fig. 6.3 Simplified N cycle in wastewater treatment. Conventional N-removal consists in combining nitrification and denitrification (NDN). Autotrophic N-removal (ANR) consists in combining partial nitritation (PN) and anaerobic ammonium oxidation (anammox)

$$1NH_4^+ + 0.803O_2 + 0.042CO_2 + 0.01HCO_3^- \rightarrow 0.57NO_2^- + 0.42NH_4^+ + 0.01C_5H_7O_2N + 0.56H_2O + 1.14H^+ \quad (6.2)$$

$$1NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (6.3)$$

Compared to conventional NDN, the application of ANR entails significant advantages: a reduction of about 60% in the oxygen uptake during nitrification—i.e. less energy requirements for aeration—no need of organic matter supplementation for denitrification, about 80% reduction in waste sludge production due to the low growth rate of autotrophic biomass, and the achievement of higher N-removal rates, which allows the construction of high rate/small size treatment biosystems (Daigger et al. 2011). As a rule of thumb, energy demand in municipal wastewater

treatment plants (WWTPs) for side-stream processing usually ranges from 0.8 to 2 kWh/kg N (sludge digester supernatants contain about 1 kg NH_4^+–N/m^3). Thus, in comparison to conventional NDN, which has an energy demand of about 4 kWh/kg N, ANR allows energy savings above 50% (Lackner et al. 2014). As of 2014, more than 100 full-scale ANR installations were running worldwide (75% in municipal WWTPs), and the number of new facilities is growing fast (Lackner et al. 2014). In addition, intensive R&D is also being conducted in mainstream wastewater treatment since ANR has been identified by the wastewater industry sector as a priority for innovation (Cao et al. 2017).

6.3.2 Microbial Insights

The most frequently encountered AOB in wastewater treatment systems form a cluster within the β-subclass of the phylum *Proteobacteria* and have been classified within the closely related genera *Nitrosomonas* and *Nitrosospira*. The predominant genus in the treatment facility strongly depends on the operational conditions (Geets et al. 2006; Dytczak et al. 2008). In this regard, *Nitrosomonas* is considered as a *r*-strategist, with a relative high growth rate but low substrate affinity, while *Nitrosospira* is considered as a *K*-strategist that displays a relative low growth rate but high substrate affinity. Other less important microbial groups also responsible for aerobic ammonium oxidation in man-made treatment systems have been found within the phylum γ-*Proteobacteria*—e.g. *the genus Nitrosococcus*—and even in the domain *Archaea* (Ye and Zhang 2011).

Bacteria responsible of the anammox process are related to the phylum *Planctomycetes*. At least five "Candidatus" anammox genera have been found in samples collected from wastewater treatment facilities and natural environments, such as freshwater and marine sediments, i.e. Ca. *Brocadia*, Ca. *Kuenenia*, Ca. *Anammoxoglobus*, Ca. *Jettenia* and Ca. *Scalindua*. These five genera form a deeply branched monophyletic group within the family *Brocadiaceae*. About 20 candidate species have already been identified within these genera (Pereira et al. 2017). The anammox bacteria are slow-growing microorganisms characterized by being strictly anaerobic and chemolithoautotrophic. Measured biomass doubling times range from 2.1 to 11 days at mesophilic conditions (Zhang et al. 2017). In physiological terms, they feature a characteristic membrane-bound intracellular organelle known as anammoxosome, which is surrounded by highly impermeable ladderane lipids and is dedicated to energy metabolism (van Niftrik and Jetten 2012). It has not been possible to isolate anammox bacteria in pure culture, which denotes the importance of symbiotic relationships with other microbial groups (Wang et al. 2016a; Zhao et al. 2018). Anammox enrichment cultures contain about 70–98% of anammox bacteria and are grown either as aggregates or as free cells in bioreactors (Lotti et al. 2014; Connan et al. 2017). However, depending on the applied conditions, anammox cell counts decrease significantly in anammox reactors treating real wastewater (Connan et al. 2018).

6.3.3 Applied Aspects and Practical Implementation

6.3.3.1 Reactor Configuration

The ANR can be engineered according to two main configurations, as PN and anammox running in two separated reactors operated in series, or with both processes running in a single reactor under limited aeration (Van Hulle et al. 2010; Jaroszynski and Oleszkiewicz 2011), as summarized in Table 6.1. In a two-reactor system, both processes, PN and anammox, are operated separately. The individual control of each reactor offers a higher potential for optimization and operational

Table 6.1 Comparison of PN-anammox system configuration as two separate reactors versus one single reactor, in relation to different operational aspects

Operational aspects and parameters	Two reactors	One reactor
System configuration	Complex design, two reactors needing synchronization to run in series	Simple design, only one reactor is considered
System operation	High flexibility, high potential for the individual optimization and intensification of processes leading to higher treatment capacities and reliability, enhanced applicability of recovery strategies after eventual perturbations, easy suppression of nitratation	Flexibility is limited by the coexistence of multiple microbial populations, low risk of anammox bacteria inhibition by nitrite
Online control	Specific optimal conditions must be assured in each of the two reactors	Fine-tuning control is needed for DO and pH
Investment costs	Higher than for one-reactor systems	Lower than for two-reactor systems
bCOD availability in the influent	Organic compounds can be biodegraded in the first stage thus lowering the risk of heterotrophic overgrow in the anammox stage	Availability of bCOD at a low ratio benefits the N-removal efficiency since nitrate formed by anammox is removed by heterotrophic denitrification
N content in the influent	High N levels are expected in the bulk liquid which may help suppressing nitratation in the first stage but increase the risk of inhibition in the second stage	Low N levels are expected in the bulk liquid, lowering the risk of biomass inhibition by N compounds
Dissolved oxygen in the liquid bulk	Absence of oxygen in the anammox stage must be assured by preventing it from entering the reactor	Oxygen can be provided either by continuous or intermittent aeration; an appropriate DO control is of primary importance
N_2O emissions	Emissions are usually lower than in conventional NDN systems	Emissions are usually lower than in conventional NDN and two-reactor ANR systems

flexibility and a more stable process performance than the one-reactor systems. The goal of the first reactor is to produce a good-quality effluent for the anammox process; this is an effluent with a NO_2^-/NH_4^+ molar rate close to 57% (Eq. 6.2). The availability of bCOD in the effluent can be reduced to some extent by favouring heterotrophic degradation in the nitritation reactor, either aerobically (De Prá et al. 2012) or anoxically (Gabarró et al. 2014). Several methods have been suggested to suppress NOB activity in nitrifying bioreactors, including the combination of mesophilic temperature (about 35 °C) and short SRT in the SHARON system—acronym of *single reactor system for high ammonia removal over nitrite*—to promote selective growth conditions (Magrí et al. 2007a), operation under high concentrations of free ammonia (NH_3, FAN) (Magrí et al. 2012b) or free nitrous acid (HNO_2) (Udert et al. 2003) to promote a selective inhibition and operation under low concentration of dissolved oxygen (DO) to take advantage of the lower DO affinity of NOB (Li et al. 2011). N-conversion rates in intensive PN reactors frequently range from 0.35 to 3.1 kg N/(m^3 day) (Fux et al. 2002; Zhang et al. 2011).

The second reactor harbouring the anammox process is particularly sensible to perturbations and inhibitory events due to the low growth rate of the anammox bacteria. Hence, microbial growth as granule and biofilm provide an efficient protective environment in comparison to suspended sludge and help in the retention of anammox cells inside the reactor. N-removal rates as high as 20–75 kg N/(m^3 day) have been reported in several lab-scale studies (Tang et al. 2011). However, such high rates need to be downrated when treating real wastewaters rather than a synthetic substrate. In this regard, anammox reactors treating real effluents have frequently been reported to remove N at rates ranging from 0.2 to 9.0 kg N/(m^3 day) (van der Star et al. 2007; Yamamoto et al. 2008).

One-reactor systems are simpler in configuration, so the investment costs are expected to be significantly lower as well. However, this configuration needs a finer control strategy due to the coexistence of several microbial populations, i.e. aerobic and anaerobic ammonium oxidizers, nitrite oxidizers and heterotrophs (Li et al. 2018). This configuration is the most frequent at full-scale facilities (88%), even though early implementations were based in two-reactor systems (Lackner et al. 2014). Microorganisms will tend to self-organize forming granules or biofilms where conditions are more favourable for their development. According to a possible model for this phenomenon, the AOB would be more active in the outer layers of the biomass structure, producing an appropriate amount of nitrite for the anammox bacteria that would be active in the inner layers. Under these conditions, the mass transfer limitation is usually considered as the bottleneck of the whole process. As long as the concentration of ammonium in the bulk liquid is much higher than the concentration of DO or nitrite, ammonium diffusion into the biomass structure will not limit the process rate. If the nitrite produced in the outer biofilm is consumed in the inner layer, then DO will be the main limiting factor controlling the overall conversion rate. Hence, DO control is of primary importance in one-reactor systems. Aeration can be provided either continuously or intermittently, according to a set point (e.g. 0.3 g O_2/m^3) or within a certain range

(e.g. 0.3–0.9 g O_2/m^3). Overaeration may lead to full nitrification, but also it may just increase the PN rate and the concentration of DO. Higher DO and nitrite concentrations can cause anammox inhibition, leading to a decrease in the N-removal rate and, eventually, to a failure of the system (Jaroszynski and Oleszkiewicz 2011). Different names have been given to ANR-based processes in a single reactor: OLAND for *oxygen-limited autotrophic nitrification–denitrification* (Pynaert et al. 2004), CANON for *completely autotrophic nitrogen removal over nitrite* (Zhang et al. 2012), DEMON for *pH-controlled deammonification* (Wett 2006), SNAP for *single-stage nitrogen removal using anammox and partial nitritation* (Qiao et al. 2012) and SNAD for *simultaneous nitrification, anammox and denitrification* (Giustinianovich et al. 2016). Most of one-reactor systems reported in the literature were operated with N-removal rates ranging from 0.1 to 1.8 kg N/(m^3 day), mainly below 1 kg N/(m^3 day) when treating real effluents (Pynaert et al. 2004; Zhang et al. 2012).

ANR systems can be emitters of harmful gases, such as CH_4, N_2O and NO; the first two are powerful greenhouse agents, and the third is an ozone degrader. The magnitude of these emissions will determine the overall sustainability of the treatment system, and so, they must be minimized. Digestate supernatant contains residual dissolved CH_4 that will be emitted when supplying aeration. In contrast to CH_4, formation of N_2O and NO occurs in situ. The emission of N_2O can be related to the activity of both nitrifying and denitrifying microorganisms and will be influenced by factors such as nitrite accumulation, inefficient control of aeration and the influent bCOD/N ratio, among others (Kampschreur et al. 2009; Massara et al. 2017). One-reactor systems are usually reported as lower N_2O emitters than two-reactor systems with typical emissions, in full-scale installations, below 1.5% of the N load (Vlaeminck et al. 2012; Campos et al. 2016). The emission of NO is normally lower than the emission of N_2O, around a negligible 0.01% of the N load. Yet, NO is easily emitted when formed because of its low solubility in water (Vlaeminck et al. 2012).

6.3.3.2 Effect of Nitrogen Concentration

The aerobic and anaerobic biological N oxidation is inhibited by high concentrations of N-substrates. This is particularly significant in two-reactor systems since their typical operation under high concentrations of N may trigger episodes of instability. Both, total ammonium (TAN: $NH_4^+ + NH_3$) and total nitrite (TNN: $HNO_2 + NO_2^-$), include two different chemical species in equilibrium. The relative concentration of these species mainly depends on total concentration, temperature and pH. The unionized species (NH_3 or HNO_2), rather than the ionized species (NH_4^+ or NO_2^-), are considered as the real inhibitors of the nitrifying microorganisms. Anthonisen et al. (1976) reported threshold concentrations ranging from 8.2 to 123.5 g NH_3–N/m^3 for inhibiting AOB. The same authors also reported values above 0.08–0.82 g NH_3–N/m^3 and 0.06–0.83 g HNO_2–N/m^3 as inhibitory for the NOB. Such wide inhibitory concentration ranges are indicative that the

activity of the nitrifying biomass can vary deeply depending on its acclimation and adaptation to the toxic unionized N-species. The anammox bacteria are generally more sensitive to nitrite than ammonium. There is no clear agreement concerning the nitrite threshold concentration values for the inhibition of the anammox activity (Magrí et al. 2012a; Connan et al. 2016). Lotti et al. (2012) showed that the adverse effect of nitrite is reversible, being NO_2^- rather than HNO_2 the inhibiting compound (with a 50% activity decrease at 400 g NO_2^-–N/m^3). In case of a peak exposure to nitrite, corrective measures should target a diminution in the concentration of nitrite inside the reactor as fast as possible since the contact time has a greater impact than the reached concentration.

6.3.3.3 Effect of Inorganic Carbon and pH

Total inorganic carbon (TIC: $CO_2 + HCO_3^- + CO_3^{2-}$) is generally the main buffering agent in the digestate supernatant against changes in pH, and, therefore, it is the factor that contributes the most to alkalinity. In two-reactor systems, the availability of alkalinity to neutralize protons formed during nitritation determines the accumulation of nitrite in the PN-reactor and, thus, the NO_2^-/NH_4^+ ratio of the effluent. Lack or excess of alkalinity in the supernatant may result in a NO_2^-/NH_4^+ ratio far from the optimal value of 1.32 (Magrí et al. 2007b). If alkalinity is too low, the NO_2^-/NH_4^+ ratio can still be controlled by adding chemicals (Zhang et al. 2011) and adjusting the influent ALK/NH_4^+ ratio to the stoichiometric needs (i.e. 4.1 g $CaCO_3$/g NH_4^+–N supplied according to Eq. 6.2). In contrast, if alkalinity is too high, some corrective actions that may help to adjust the NO_2^-/NH_4^+ ratio are: to fine-tune the N-loading rate applied to the PN-reactor (Magrí et al. 2012b), to bypass part of the anaerobic supernatant and mix it with the effluent exiting the reactor and to promote the heterotrophic denitrification, if there is availability of bCOD in the supernatant, though at the risk of increased emissions of N_2O (Gabarró et al. 2014). If the supernatant to be treated does not contain enough alkalinity, the control of pH in the reactor might be necessary. The pH of the bulk liquid affects chemical equilibria of inorganic C and N, so it may exert an indirect influence on the activity of the biomass. Additionally, the pH may have a direct impact on the anammox activity if it falls out of the appropriate values (Puyol et al. 2014). Of particular interest is the case of the DEMON process, a one-reactor ANR system based on the control of pH (Wett 2006).

6.3.3.4 Effect of Organic Matter

The composition of the digestate is strongly dependent on the processed organic materials and the applied treatment conditions. Usually, the longer the HRT applied in the digester, the lower the remaining bCOD in the digestate. Availability of bCOD in the digestate cannot be overlooked, since its presence during ANR will stimulate the growth of heterotrophic microorganisms (Magrí et al. 2007b;

Kumar and Lin 2010). The effect of the influent bCOD/N ratio on the PN and on the anammox has been assessed when both processes are operated individually (De Prá et al. 2012; Ni et al. 2012), and also when they are combined in a single reactor (Giustinianovich et al. 2016). A low influent bCOD/N ratio (≤ 0.7) does not necessarily have a negative impact on the PN-anammox process, and it may even help to improve the overall N-removal efficiency. This is because the nitrate formed in the anammox reaction can then be removed by heterotrophic denitrifiers (Giustinianovich et al. 2016), but this can be at the cost of increasing the risk of N_2O emission (Jia et al. 2018). If the influent bCOD/N ratio increases (>1), the anammox bacteria are unable to compete with the denitrifiers regarding both spatial organization of communities and electron acceptor uptake (i.e. nitrite), finally resulting in the failure of the reactor performance (Ni et al. 2012; Tang et al. 2013).

6.4 Perspectives on Coupling SAO-Based AD and Anammox-Based ANR

To this date, ANR based on anammox has mainly been applied to the treatment of the supernatant from sludge digesters in municipal WWTPs, which usually contain 0.5–1.5 kg TAN/m^3 (Daigger et al. 2011; Lackner et al. 2014; Connan et al. 2018). However, other sources of digestate supernatant with a similar or higher N load have been reported as well (Table 6.2): livestock manure, agrifood wastes and other complex mixtures of organic materials (Furukawa et al. 2009; Vázquez-Padín et al. 2014; Scaglione et al. 2015). A high availability of bCOD in the digestate supernatant can hinder ANR, so that alternatives might be considered like the aerobic degradation prior to ANR (Lu et al. 2016) or, in the ultimate case, the implementation of an optimized NDN via nitrite strategy (Scaglione et al. 2013). Dilution is an easy way to reduce the concentration of N, and other potential inhibitors in the supernatant fed to the ANR system (Lu et al. 2016). However, high N contents may compromise the interest in ANR-based treatment schemes because of energy requirements. In this regard, a threshold of 2 g TAN/m^3 was suggested by Magrí et al. (2013). The integration of technologies like struvite precipitation, ammonia stripping–absorption and membrane filtration can help in conditioning the supernatant by lowering the N content in view of ANR while producing valuable products (Abma et al. 2010; Pintucci et al. 2017).

The VFA that are commonly present in the supernatant of anaerobic digesters are potential inhibitors of the ANR process (Mosquera-Corral et al 2005; Huang et al 2014). The low contents of VFA required for a good ANR performance can be attained by an appropriate optimization of the AD process using diagnostic tools. Different methods and devices have already been explored in the field of bioprocess monitoring (Feitkenhauer et al. 2002; Ellison et al. 2007; Palacio-Barco et al. 2010; Madsen et al. 2011). Several instruments such as spectral sensors, electronic tongues (e-tongue), electronic noses (e-nose), microwave or acoustic chemometric

Table 6.2 Summary of some case studies dealing with the application of ANR to highly N-loaded digestate supernatants

Digestate supernatant source	Treatment system	Influent characteristics	Influent pretreatment	NLR (kg N/(m^3 day))	Removal and effluent characteristics	Reference
Two-reactor systems						
Sewage sludge	**PN:** SHARON (10 L), 35 °C, HRT = 1 d	**PN:** 1.2 kg NH_4^+–N/m^3	**PN:**–	**PN:** 1.2	**PN:** 1.09 mol NO_2^-/mol NH_4^+ (without pH control)	van Dongen et al. (2001)
	AX: granular SBR (10 L), 30–37 °C, pH = 7–8.5, HRT = 1 d	**AX:** 0.55 kg NH_4^+–N/m^3, 0.60 kg NO_2^-–N/m^3	**AX:** –	**AX:** 1.2	**AX:** NRR = 0.75 kg N/(m^3 day)	
Raw garbage, livestock manure and sewage sludge	**PN:** continuous stirred reactor with gel carriers (8 L), 30 °C, HRT = 0.3–0.9 d	**PN:** 1.5 kg NH_4^+–N/m^3 (79% of TKN), 0.8 g BOD/g NH_4^+–N, pH = 8.5	**PN:** dilution (80–0%)	**PN:** 0.5–3.0	**PN:** 1.0–1.4 mol NO_2^-/mol NH_4^+ (pH controlled at 7–8)	Furukawa et al. (2009)
	AX: continuous stirred reactor with gel carriers (1 L), 30°C, pH = 7.8, HRT = 0.1–0.3 d	**AX:** 0.6 kg NH_4^+–N/m^3, 0.8 kg NO_2^-–N/m^3	**AX:** dilution (70–0%)	**AX:** 5.3	**AX:** NRR >4 kg N/(m^3 day), NRE >80%	
Livestock manure and energy crops	**PN:** SBR (650 L), 30 °C, HRT = 2 d	**PN:** 1.2 kg NH_4^+–N/m^3 (78% of TKN), 5.2 g $CaCO_3$/g NH_4^+–N, 0.4 g sBOD/g NH_4^+–N, pH = 8.1	**PN:** ALK/NH_4^+ ratio adjustment (to 3.6 g $CaCO_3$/g NH_4^+–N with HCl dosage)	**PN:** 0.5–0.6	**PN:** 1.0–1.4 mol NO_2^-/mol NH_4^+, 95% sBOD removed	Scaglione et al. (2015)
	AX: granular SBR (3 L), 34 °C, pH = 7–8, HRT = 2 d	**AX:** 0.56 kg NH_4^+–N/m^3, 0.66 kg NO_2^-–N/m^3	**AX:** dilution (75–0%), NO_2^-/NH_4^+ ratio adjustment (to 1.1–1.3), ALK addition (1 kg $NaHCO_3$/m^3)	**AX:** 0.5–0.6	**AX:** NRR = 0.4–0.6 kg N/(m^3 day), NRE = 91%	

(continued)

Table 6.2 (continued)

Digestate supernatant source	Treatment system	Influent characteristics	Influent pretreatment	NLR (kg N/(m^3 day))	Removal and effluent characteristics	Reference
One-reactor systems						
(Domestic) black water	RBC (2.8 L), 26 °C, pH = 7.7, HRT = 1.3 d	1.2 kg NH_4^+–N/m^3, 0.6 g sCOD/g NH_4^+–N	Gradual replacement of synthetic influent (10–100%)	0.94	NRR = 0.72 kg N/(m^3 day), NRE = 76%	Vlaeminck et al. (2009)
Sewage sludge and food industry wastes	granular SBR (200 L), 18–31 °C, HRT = 0.8–1.5 d	1.1 kg NH_4^+–N/m^3, 0.8 g COD/g NH_4^+–N	–	1.0	NRR = 0.80 kg N/(m^3 day), NRE = 82%	Vázquez-Padín et al. (2014)
Landfill leachate	MBBR (7 L), 30 °C, pH = 7–8	0.4 kg NH_4^+–N/m^3 (previously diluted), 7.5 g COD/g NH_4^+–N	Leachate dilution (by effluent recirculation), aerobic bCOD removal	0.5	NRR = 0.34 kg N/(m^3 day), NRE = 70%	Lu et al. (2016)

ALK alkalinity; *AX* anammox; *bCOD* biodegradable COD; *COD* chemical oxygen demand; *HRT* hydraulic residence time; *MBBR* moving bed biofilm reactor; *N* nitrogen; *NLR* N-loading rate; *NRE* N-removal efficiency; *NRR* N-removal rate; *PN* partial nitritation; *RBC* rotating biological contactor; *(s)BOD* 5-d (soluble) biochemical oxygen demand; *sCOD* soluble COD; *SBR* sequencing batch reactor; *SHARON* single reactor system for high ammonia removal over nitrite; *TKN* total Kjeldahl N

sensors, gas chromatographs and mass spectrometers are now available for an efficient online monitoring of specific AD parameters (Rudnitskaya and Legin 2008; Jimenez et al. 2015). Some promising experiences have been reported recently on the measurement time course evolution of the VFA concentration inside the reactor via e-noses and e-tongues, as well as bioelectrochemical sensors (Costa et al. 2016; Kretzschmar et al. 2017; Schievano et al. 2018).

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Chapter 7
Two-Stage Process to Enhance Bio-hydrogen Production

E. Judith Martínez, Daniel Blanco and Xiomar Gómez

Abstract Bio-hydrogen is generated by renewable feedstocks from biological, chemical, thermochemical and photolytic methods. Biological methods such as dark fermentation have been suggested as a feasible alternative to produce this gas and obtain a sustainable energy source. Bio-hydrogen is not a primary energy source, but it is compatible with electrochemical and combustion processes for energy conversion; this gas can be stored, transported and utilised to fulfil energy needs, and it also contributes to minimise carbon-based emissions reducing environmental pollution and climate change. In the present manuscript, a review is performed about the state of the art of the dark fermentation process and its integration with other processes in an attempt to increase the efficiency of substrate conversion. The two-stage configurations studied involve the bioprocesses for hydrogen production and waste treatment by coupling the dark fermentation process with an alternative biological route such as anaerobic digestion, microbial electrochemical systems or photo-fermentation to promote an efficient stabilisation and use of the organic matter.

Keywords Bio-hydrogen · Dark fermentation · Anaerobic digestion · Microbial electrochemical systems

7.1 Introduction

As global energy consumption continues to rise, the development of a sustainable energy policy is a relevant matter to be seriously considered. The European Union has implemented a comprehensive policy framework to support the development and integration of renewable as a mainstream source of energy (European Commission 2015).

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Expanding the use of renewable energy sources could be an alternative to deal with the important issues in our society, as it is the depletion of fossil fuels, the security of energy supply, the reduction of greenhouse gas (GHG) emissions and continuous increase in energy costs (Cucchiella et al. 2017). Among others renewable energy sources, hydrogen is considered a potential alternative to reduce environmental pollution in substitution to fossil fuels due to its high energy efficiency and cleanness combustion since water is the main product of its oxidation (Meher Kotay and Das 2008; Chu and Huang 2015; Dincer et al. 2016; Argun et al. 2017).

Hydrogen is the lightest element and a valuable gas. It can be directly used as a fuel, it has also industrial applications associated with the prevention of oxidation and corrosion, or it is used as a coolant and can also be used as a feedstock for some other industries, e.g. as a reactant in hydrogenation processes, among others. The consideration of this light gas as a clean energy carrier is based in its high energy content per unit weight, when compared with other gaseous fuels (143 GJ/ton) (Das and Veziroğlu 2001; Argun et al. 2017). However, the low mass content per unit of volume is its main disadvantage converting the storage of this gas in one of the major areas of research in an attempt to increase the efficiency of the storage process and lower the demand of energy necessary to accomplish similar benefits to those of conventional liquid fuels.

Bio-hydrogen, which is obtained from renewable sources, is defined as hydrogen produced via biological means (mostly by those were microorganisms are involved) or via thermal valorisation and conversion of biomass (Manish and Banerjee 2008; Fatih Demirbas 2009; Hallenbeck et al. 2009; Xu et al. 2011; Azadi et al. 2013). It may be considered as a biofuel but also as an energy carrier, since it can be used as a means for transporting of stored energy previously produced by different technologies.

Biological methods of hydrogen production are preferable over common methods such steam reforming, non-catalytic partial oxidation of fossil fuels, coal gasification or auto-thermal reforming, because they offer the possibility of using sunlight, CO_2 and organic wastes as substrates (Gómez et al. 2011). These methods are considered environmentally friendly, because they take place under moderate conditions, mostly operating at lower temperatures and pressures than thermochemical methods, and for this reason, they are expected to be less energy intensive.

The biological methods for generating hydrogen production include direct and indirect biophotolysis and photo-fermentation (light-dependent methods), bioelectrochemical systems (BES) and dark fermentation process (no light-dependent methods).

Dark fermentation is the simplest technology and has higher yields of hydrogen production from carbohydrates than those from photofermentation. In addition, the excellent potential for practical application such as the treatment of organic wastes makes the process an increasingly popular option for hydrogen production (Elsharnouby et al. 2013). The generation of wastes and wastewaters increases with population growth and economic development, and for this reason, the treatment and valorisation of these residues have to be carefully assessed to avoid

environmental problems and to accomplish with the more stringent regulations. The current legislation of wastes in Europe (Directive 2008/98/EC) intends to achieve significant improvements in waste management, increase waste recycling, decrease the landfilling rate and increase the recovery of energy from wastes. Bio-wastes, which is defined, as "biodegradable garden and park waste, food and kitchen waste from households, restaurants, caterers and retail premises, comparable waste from food processing plants and other waste with similar biodegradability properties that is comparable in nature, composition and quantity" hold an enormous potential for recycling and valorisation through the generation of energy (Directive 2008, 2010).

This review includes several descriptions of successful experiences for the production of hydrogen through dark fermentation from bio-wastes and wastewaters. Because the fermentation has a maximum yield of 33% (based on sugar utilisation), this review also discusses the effectiveness to apply a two-stage configuration to increase the hydrogen production from this type of technology and enhance global efficiency.

7.2 Bio-hydrogen Production

Hydrogen can be obtained from a broad number of resources, such as hydrocarbon fuels, water, biomass and chemical elements containing hydrogen (Dincer et al. 2016).

Non-renewable methods for hydrogen generation include processes requiring high temperatures and energy demand, for example steam reforming (most widely used and cheapest production method), non-catalytic partial oxidation of fossil fuels, coal gasification or auto-thermal reforming. Since carbon dioxide is produced as a by-product, this kind of processes can cause environmental impact due to greenhouse gas (GHG) emissions. On the other hand, the electrolytic production of hydrogen involves the use of electricity or thermal energy for splitting water molecules into hydrogen and oxygen (Braga et al. 2017). Electrolysis is the most expensive technology and is usually employed when natural gas is not available or when high-purity hydrogen is required (Ball and Weeda 2015).

In recent years, bio-hydrogen production from renewable and clean energy sources along with the utilisation of waste materials as substrates have received significant attention. These processes are less energy intensive and more eco-friendly compared with conventional methods in addition to facilitate waste recycling (Singh and Wahid 2015; Argun et al. 2017). Taken into account the continuous increase in global energy demand associated with the increase in population and their welfare, the main goal in the near future should be to attain cost-effective production processes. This objective may be attained by the use of renewable sources to fulfil the demand for energy, but it also requires accomplishing the successful integration of the different technologies involved in the production of renewable energies. This is due to the wide diversity of processes and

means for running renewable energy installations, its complex nature and the intrinsic difficulty in having this type of energy available when needed and not just when produced.

The research on bio-hydrogen production methods has focused on bio-photofermentative hydrogen production, bioelectrochemical processes, direct and indirect biophotolysis and dark fermentation (Hallenbeck and Benemann 2002; Lenin Babu et al. 2013; H. Wang et al. 2018; Show et al. 2018; Singh and Das 2018; X. Wang et al. 2018). These methods involve a wide diversity of microorganisms, microbial physiology, metabolisms and, in general, a great variety of overall reactions implicated in the transformation of substrate into hydrogen transformation (Hallenbeck et al. 2009). Table 7.1 indicates the main reactions and classification of microorganism implicated in fermentative hydrogen production.

Generally, in photofermentation, hydrogen is produced through photosynthetic bacteria by a proton-reducing reaction catalysed by nitrogenase under poor nitrogen conditions using light energy and organic substrates (Manish and Banerjee 2008).

The main photofermentative microorganisms are purple non-sulphur bacteria (PNS) such as *Rhodobacter sphaeroides, Rhodobacter capsulatus, Rhodospirillum rubrum and Rhodopseudomonas palustris*. These types of bacteria have the ability to use organic substrates (i.e. volatile fatty acids—VFAs, organic acids such as acetic, butyric, and lactic acids) for hydrogen production under photoheterotrophic conditions (light, anaerobiosis). These photosynthetic bacteria constitute the group of microorganisms which can grow as photoheterotrophs, photoautotrophs or chemoheterotrophs (Basak and Das 2007; Redwood et al. 2008).

Bioelectrochemical systems (BESs) are considered as an emerging and sustainable technology for hydrogen production, but are also capable of attaining the treatment of wastewater, electrosynthesis and desalination (Pant et al. 2012; Mao and Verwoerd 2013; Mohanakrishna et al. 2015). In BES, microorganisms are used to catalyse the reactions of oxidation of the organic matter taking place at the anode, whereas at the cathode electricity can be recovered. This type of configuration allows the treatment of wastewaters or waste streams. When oxygen is excluded from the cathode, the electrons released in the anodic chamber by bacteria reach the cathode and combine with protons to produce hydrogen (Kim et al. 2015; Ghangrekar and Chatterjee 2017).

Hydrogen generation via biophotolysis occurs due to the conversion of solar energy and water by the effect of light on the microbial systems which results in dissociation of water into molecular hydrogen and oxygen. The main microorganisms involved in this processes are microalgae and cyanobacteria (also called blue-green algae). Metabolic pathways can be classified into two different categories: direct biophotolysis and indirect biophotolysis (Show et al. 2018).

Direct biophotolysis has only been reported in green microalgae. During this process, the photosynthetic system captures light and the energy is used for water splitting and for the generation of a low-potential reductant. The electrons' flux from water through the photosystems (PSII-PSI) causes the reduction of ferredoxin, leading also to the reduction of hydrogenase enzyme. This enzyme transfers these electrons to protons and, therefore, is the final responsibility for the release of hydrogen gas (Yu and Takahashi 2007).

Table 7.1 Biological process for hydrogen production: general reactions and microorganisms involved

Process	General reactions	Microorganism involved
Bio-photofermentation	$CH_3COOH + 2H_2O + \text{light} \rightarrow + 4H_2 + 2CO_2$	Photosynthetic bacteria (Purple bacteria as *Rhodobacter sphaeroides*) (Adessi and De Philippis 2012) and Microalgae (Hemschemeier et al. 2009)
Bioelectrochemical process	**Anode** $C_2H_4O_2 + 2H_2O \rightarrow CO_2 + 8e + 8H$ **Cathode** $8e + 8H \rightarrow 4H_2$	Fermentative acidogenic bacteria, non-fermentative bacteria, exoelectrogens (*Geobacter sulfurreducens*) (Hari et al. 2016)
Direct biophotolysis	$12H_2O + \text{light} \rightarrow + 2H_2 + O_2$	Microalgae (Benemann 2000)
Indirect biophotolysis	$12H_2O + \text{light} \rightarrow + 2H_2 + 6O_2$ **Overall reaction**	Microalgae, cyanobacteria (Hallenbeck 2012)
Dark fermentation	$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2$	Fermentative hydrogen-producing bacteria as Clostridium genus and *Enterobacteriaceae* family reported in the mesophilic operation and *Thermoanaerobacteriales* reported in thermophilic conditions (Etchebehere et al. 2016)

Nevertheless, direct biophotolysis presents two major problems; one is the inhibition of the hydrogenase enzyme by the presence of oxygen, and the second is the generation of potentially high-explosive H_2–O_2 mixtures in the reactor. To overcome this weakness, indirect biophotolysis processes involve the separation of H_2 and O_2 into separate stages, along with CO_2 fixation through microorganisms such as microalgae and cyanobacteria. The release of oxygen occurs in the first stage, and hydrogen production reactions take place in the second stage (Huesemann et al. 2010). Further information about biophotolysis in the last decade can be found in several research papers indicating that this process has a promising potential (Smith et al. 1992; Yu and Takahashi 2007; Huesemann et al. 2010; Vijayaraghavan et al. 2010; Gadhamshetty et al. 2011; Jafari et al. 2016; Khetkorn et al. 2017; Nagarajan et al. 2017; Show et al. 2018; Shuba and Kifle 2018).

Dark fermentation is one of the most important bioprocesses for hydrogen generation from a wide variety of substrates (Chong et al. 2009; Balachandar et al. 2013; Rai et al. 2014; Dhar et al. 2015; Rollin et al. 2015). This process involves the conversion of substrates into a mixture of products (H_2, CO_2, acetic acid and butyric acid) in the absence of oxygen and light (Bastidas-Oyanedel et al. 2015).

The preferred carbon sources for hydrogen-producing microorganisms are carbohydrates. The most common products in this type of fermentation are acetate and butyrate (Eqs. 7.1–7.2), with the production of acetate as intermediary giving the highest hydrogen yield (Wang and Yin 2017).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \tag{7.1}$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2CH_2COOH + 2H_2 + 2CO_2 \tag{7.2}$$

Many fermentative bacteria produce hydrogen via dark fermentation including strict anaerobic microorganisms: methanogenic bacteria, archaea, methylotrophs and *Clostridium* (most usual class of microorganisms that produce hydrogen), and facultative anaerobic bacteria such as *Enterobacter*, *Escheriachia coli* and *Citrobacter*. These types of microorganisms have differences in their metabolism and are commonly found in a mixed culture explaining therefore the differences associated with hydrogen yields of reports found in the literature. The predominance on some populations over other ones is usually based on the type of pretreatment applied to the inoculum to eliminate methanogens and obtain a hydrogen-producing culture from mixed microflora and operating conditions of the system (Li and Fang 2007; Zahedi et al. 2014; Dessì et al. 2018).

Dark fermentation offers several advantages over biophotolysis and other bioprocesses. It has a high rate of cell growth, no presenting problems of oxygen limitations, therefore achieving higher hydrogen production rates. The independence from weather conditions (not requiring light energy) makes the process easy to scale up and to keep continuous production during the whole day, not being influenced by seasonal variations. Additionally, the possibility to use a wide variety of substrates including organic wastes (cellulose, food wastes, paper wastes, etc.) and wastewaters offers the advantage of integrating this process into the valorisation

chain of different residues and aids in attaining circular economy objectives (Li and Fang 2007; Meher Kotay and Das 2008; Dhar et al. 2015; Rollin et al. 2015; Elbeshbishy et al. 2017; Prakash et al. 2018).

7.3 Dark Fermentation

Dark fermentation has been widely studied and reviewed for its high capacity of producing hydrogen (Tables 7.2, 7.3 and 7.4). However, it is characterised by low efficiency associated with the low conversion of the substrate into H_2 gas and low stability when the fermentation is compared to the common biological treatment of wastes. These two facts set a great burden on the technical feasibility and hinder its industrial implementation and scale-up (Tapia-Venegas et al. 2015).

Dark fermentation is influenced by process constraints and environmental conditions including the type of feedstock, microbial population, metabolic pathways, pH, temperature, organic loading, the presence of inhibitors and nutrients (Ghimire et al. 2015; Sekoai et al. 2017; Dessì et al. 2018; Vasmara et al. 2018).

The election of an appropriate feedstock is a crucial factor, with the type of the source of organic compounds serving as substrates being a relevant factor affecting fermentations yields and long-term stability of the process. Carbohydrate-rich substrates, especially monosaccharides, such as hexoses including glucose, pentoses and disaccharides, particularly sucrose and lactose are readily biodegradable and extensively evaluated in dark fermentation studies over the last years. Table 7.1 shows several studies with carbohydrates as a main substrate for hydrogen production.

Table 7.2 Common substrates studied for hydrogen production and productivity

Substrates	Seed sludge/ inoculum	H_2 yield	Reference
Glucose	Caloramator celer	2.95 mol H_2/mol glucose	Guo et al. (2010)
Glucose	Agricultural soil	2.2 mol H_2/mol glucose	Show and Su (2011)
Sucrose		3.76 mol H_2/mol $sucrose_{added.}$	Wu et al. (2012)
Glucose		2.8 mol H_2/mol glucose	Elsharnouby et al. (2013)
Lactose	Anaerobic sludge	0.69 mol H_2/mol lactose	Moreno et al. (2015)
Sucrose	Anaerobic sludge	3,5 mol H_2/mol $sucrose_{added.}$	Anzola-Rojas et al. (2015)
Galactose		2.25 mol H_2/mol $galactose_{added.}$	Sivagurunathan et al. (2016)
Glucose	Anaerobic sludge	2.7 mol H_2/mol glucose	Zheng et al. (2016)
Sucrose	Anaerobic sludge	320 ml H_2/g VS_{added}	Salem, Brunstermann, et al. (2018)

Table 7.3 Resuslts reported in literature of lignocellulosic materials studied as a substrate for hydrogen production by dark fermentation

Substrates	Seed sludge/inoculum	Substrate pretreatment	H_2 yield	Reactor type/ Regimen	Reference
Cornstalk wastes	Anaerobic sludge/Heat pretreatment	Enzymatic	141.29 ml/g Cornstalk (optimum conditions)	Batch	Ma et al. (2011)
Paper waste and pulping sludge mixture	Anaerobic sludge/Heat pretreatment	Thermic-chemical (Acid and alkaline pretreatments) + Enzymatic	129.9 ml/g TVS	Batch	Chairattanamanokorn et al. (2012)
Rice straw	Anaerobic sludge/Heat pretreatment	Untreated	25 ml H_2/g TS (initial pH 6.5 and 55 °C)	Batch	Chen et al. (2012)
Grass	Synthetic media: *Clostridium pasteurianum*	Chemical (Acid (HCl) and alkaline (NaOH) pretreatments)	72.21 ml/g dry grass (pretreated with 4% HCl)	Batch	Cui and Shen (2012)
Soybean straw	Synthetic media: *Clostridium butyricum*	Chemical (Acid (HCl) and alkaline (NaOH) hydrolysis)	60.2 ml/g dry soybean (acid pretreatment)	Batch	(Han et al. 2012)
Cornstalk	Synthetic media: T. *thermosaccharolyticum*	Fungal	89.3 ml/g cornstalk (0.75% substrate concentration, enzyme loading of 34 FPU/g cellulose, and initial pH 6.5)	Batch	Zhao et al. (2013)
Rice husk	Synthetic media: *C. beijerinckii* KCTC 1785	Chemical (Acid (H_2SO_4) and alkaline (NaOH) pretreatments) + Enzymatic	2.63 mmol H_2/g cellulose	Batch	Saratale et al. (2014)
Sugarcane bagasse	Cow dung/Heat pretreatment	Enzymatic-Chemical (alkaline (NaOH) hydrolysis)	93.4 ml/g VS_{added}	Batch	Kumari and Das (2015)

(continued)

Table 7.3 (continued)

Substrates	Seed sludge/inoculum	Substrate pretreatment	H_2 yield	Reactor type/ Regimen	Reference
Palm fruit bunch	Anaerobic sludge/Heat pretreatment	Chemical (Acid hydrolysis H_2SO_4)	1.11 mol H_2/mol TS	Batch	Gonzales, Sivagurunathan and Kim (2016)
Rice husk	Anaerobic sludge/Heat pretreatment	Chemical (Acid hydrolysis H_2SO_4)	1.96 mol H_2/mol TS	Batch	Gonzales, Sivagurunathan and Kim (2016)
Waste paper	Anaerobic sludge/Heat pretreatment	Chemical (Acid hydrolysis H_2SO_4)	1.01 mol H_2/mol sugar (initial sugar concentration of 3.84 g/L	Batch	Eker and Sarp (2017)
Sweet sorghum stalks	Synthetic media: C. *thermosaccharolyticum* DSM572	Chemical (Acid hydrolysis H_2SO_4)	2.50 mmol/g substrate (first step fermentation) 5.77 mmol/g substrate (first step + second step fermentation)	Batch	Islam, Guo and Liu (2018)
Cotton stalk	Synthetic media: *Klebsiella sp.*	Chemical (Acid hydrolysis H_2SO_4)	1.44–0.08 mol/mol $sugar_{consumed}$	Batch	Li et al. (2018)

Table 7.4 Different organic wastes and wastewaters studied as a substrate for hydrogen production

Substrates	Seed sludge/ inoculum	Inoculum pretreatment	H_2 yield	Reactor type/ Regimen	Reference
Organic fraction municipal solid wastes and slaughterhouse waste	Anaerobic sludge	Without treatment	52.5–71.3 ml H_2/g SV added	Semi-continuous	Gómez et al. (2006)
Food waste	Anaerobic sludge		205 ml H_2/g VS_{added}	Continuous	Chu et al. (2008a)
Dairy Manure	Dairy Manure	Infrared treatment	31.5 ml H_2/g TVS	Batch	Xing et al. (2010)
Cheese whey powder	Anaerobic sludge	Other	1.03 mol H_2/mol glucose	Batch	Kargi, Eren and Ozmihci (2012)
Vegetable waste (kitchen)	Cow dung	Heat treatment	3.44 ml H_2/g TS	Batch	Bansal, Sreekrishnan and Singh (2013)
Chicken meat	Anaerobic sludge	Heat treatment	6.9 ml H_2/g TS_{added}	Batch	Guo et al. (2014)
Potatoes	Anaerobic sludge	Heat treatment	173.3 ml H_2/g TS_{added}	Batch	Guo et al. (2014)
Brown water			2.52 ml H_2/g VS_{added}	Semi-continuous	Seong, Yoon and Seo (2014)
Food waste	Anaerobic sludge	Heat treatment	2.82 ml H_2/g COD	Batch	Wongthanate, Chinnacotpong

(continued)

Table 7.4 (continued)

Substrates	Seed sludge/ inoculum	Inoculum pretreatment	H_2 yield	Reactor type/ Regimen	Reference
					and Khumpong (2014)
Potato and pumpkin	Anaerobic sludge	Chemical treatment (sodium 2-bromoethanesulfonic acid)	171.1 ml H_2/g VS_{added}	Batch	Ghimire, Frunzo, Pontoni, et al. (2015)
Buffalo manure	Anaerobic sludge	Chemical treatment (sodium 2-bromoethanesulfonic acid)	135.6 ml H_2/g VS_{added}	Batch	Ghimire, Frunzo, Pontoni, et al. (2015)
Olive Pomace	Anaerobic sludge	Chemical treatment (sodium 2-bromoethanesulfonic acid)	54.9 ml H_2/g VS_{added}	Batch	Ghimire, Frunzo, Pontoni, et al. (2015)
Food waste and brown water	Anaerobic sludge	Heat treatment	68.5–99.8 ml H_2/g VS_{added}	Continuous	Paudel et al. (2017)
Chewing gum suspension	Anaerobic sludge	Heat treatment	6.2–2.1 mol H_2/g VS_{added}	Batch	Seifert et al. (2018)
Organic fraction of municipal solid waste with mixed sludge	–	–	33.8 ml H_2/gVS_{added} (maximum value achieved)	Semi-continuous	Angeriz-Campoy et al. (2018)
Potato	Anaerobic sludge	Heat treatment	150 ml H_2/g VS_{added}	Continuous	Salem, Brunstermann, et al. (2018)

(continued)

Table 7.4 (continued)

Substrates	Seed sludge/ inoculum	Inoculum pretreatment	H_2 yield	Reactor type/ Regimen	Reference
Bean	Anaerobic sludge	Heat treatment	80 ml H_2/g VS_{added}	Continuous	Salem, Brunstermann, et al. (2018)
Cattle wastewater	Sewage sludge	Ultrasonic pretreatment (at 100 kHz for 30 min)	319 ml H_2/g COD	Batch	Tang et al. (2008)
Brewery wastewater	Anaerobic sludge	Heat treatment	149.6 ml H_2/g COD	Batch	Shi et al. (2010)
Fructose and molasses wastewaters from Starch and sugarcane	Anaerobic sludge	Heat treatment	166.8 ml H_2/g COD for fructose processing wastewater, and 187.0 ml H_2/g COD for molasses processing wastewater at an initial pH of 6	Batch	Lin, Juan and Hsien (2011)
Snack wastewater	Anaerobic sludge	Heat treatment	0.53 l H_2/l from snack wastewater	Batch	Wongthanate et al. (2014)
Paper mill effluent	Anaerobic sludge	Heat treatment	55.4 ml H_2/g COD	Batch	Vaez et al. (2017)
Sewage sludge	Anaerobic sludge	Heat treatment	355–488 ml H_2/g COD	Batch	Senturk and Buyukgungor (2017)

Wastewaters and lignocellulosic biomass materials including crops, agricultural and forestall residues are the most available resources for hydrogen production (Korres and Norsworthy 2017). Lignocellulosic biomass is considered a suitable substrate despite the limiting steps necessary for an efficient exploitation which comprises the difficult disruption of the complex lignocellulosic structure of the biomass and the subsequent generation of potential fermentative inhibitors associated with the nature of the pretreatment applied. Thus, their biotransformation into hydrogen via dark fermentation represents a great challenge in most cases (Lyberatos 2010; Chu and Huang 2015; Argun et al. 2017). The main components of cell walls of biomass are lignocellulose, and this material creates a highly resistant and recalcitrant structure. The lignocellulose is constituted by approximately 20–45% cellulose, 16–37% hemicellulose and 12–26% of lignin. Consequently, a pretreatment stage (either chemical, physical and enzymatic) would be required to remove the lignin and to hydrolyse complex carbohydrates into their monomers (Sawatdeenarunat et al. 2015). Table 7.3 resumes various examples of hydrogen production conducted with common lignocellulosic materials in various bioprocess designs.

Although extensive studies have been performed regarding the use of lignocellulosic biomass, in order to achieve an effective conversion of this material into hydrogen further investigation is required, particularly, to improve the efficiency of conversion, to obtain strains of bacteria capable of a direct conversion of the lignocellulosic substrate and the employment of and effective pretreatment capable of reduce the presence of inhibitors. In some cases, the pretreatment is complex, energy intensive and can lead to the formation of intermediates, such as furfural, 5-hydroxymethylfurfural, phenolic components or fatty acids which can lead to an inhibition of the process and a depletion of hydrogen production (Łukajtis et al. 2018).

A viable solution for the whole valorisation of lignocellulosic materials may incorporate not only the fermentative hydrogen-producing system but also a second stage, dedicated to enhance the energy and/or materials recovery by increasing hydrogen yields, producing methane or other valuable intermediaries (Islam, Guo and Liu 2018; Łukajtis et al. 2018).

On the other hand, the use of organic wastes as a substrate could also be a promising approach to integrate the hydrogen production into a main valorisation chain, where energy production and waste treatment are the main goals. Hydrogen production from organic wastes has been studied by several authors over the past years, organic fraction of municipal solid wastes, source-separated food wastes, agro-industrial wastes or wastewaters are the most common substrates used (Lay et al. 1999; Gómez et al. 2006; Moreno and Gómez 2012; Balachandar et al. 2013; Fernández et al. 2014; Moreno et al. 2015; Das 2017; Yun et al. 2018). Table 7.4 summarises hydrogen yields and reactor configuration of different dark fermentation studies using common organic wastes as substrates.

Food wastes are characterised by being rich in carbohydrates which makes them an easily hydrolysable substrate with high potential of hydrogen production in comparison with other type of organic wastes (around 3–290 ml H_2/g VS_{added}

reliant to the nature of the food wastes) (Noblecourt et al. 2018; Yun et al. 2018). In addition, this type of substrate has a high content in volatile solids (85–95%) making of this material an ideal candidate for microbial conversion processes where a great fraction of these components are easily transformed into valuable fuels.

Usually, mixed cultures and non-sterilised organic wastes are the preferable inoculum and source of substrates, respectively, for producing hydrogen over the use of pure cultures and aseptic conditions, due to the high feasibility of implementing this technology at large scale (Yokoyama et al. 2007). Compost, soil or anaerobic sludge has been used to provide seeds cultures for hydrogen-producing microflora (Davila-Vazquez et al. 2008; Anzola-Rojas et al. 2015). However, to increase the effectiveness of the process, inoculum pretreatment including mechanical, thermal or chemical methods is applied. Among them, heat pretreatment is the most widely applied method to inhibit methanogenic microflora initially present in the indigenous culture and to favour the proliferation of hydrogen-producing microorganisms, thanks to their capacity of sporulation and survive extreme ambient conditions (Bansal et al. 2013).

A few strategies to improve hydrogen production have been studied by Ghimire et al. (2015b) investigating alternatives for inoculum pretreatment as heat or chemical pretreatment and the use of complex real substrates obtaining good results in the fermentation of fruit, vegetable wastes, buffalo manure and slaughter wastes in terms of hydrogen yields and reporting as best option the chemical pretreatment.

Several pretreatments have been successfully tested for obtaining active hydrogen-producing microflora, and some of these studies are listed in Table 7.4. Nonetheless, when considering large-scale implementation, pretreatments applied either to the inoculum or the substrate to facilitate its assimilation by microorganisms may translate into an increase of operating costs.

Another important factor is the temperature of the fermentation. This operational parameter affects the growth rate and the metabolic activity of microorganisms, hence having direct influence on the rate of hydrogen production. Generally, fermentation reactions can be operated at mesophilic (25–40 °C) and thermophilic (40–65 °C) conditions (Davila-Vazquez et al. 2008). Mesophilic conditions have been widely studied for a broad source of feedstocks, because the process is relatively cheap, robust and simple to operate (van Niel 2016). Nevertheless, thermophilic conditions seem to be more restrict for the contamination by hydrogenotrophic methanogens (Pawar and van Niel 2013) and the stability to the process should be easier to keep under long-term operation.

After the dark fermentation process, an acid stream still remains as a by-product. This is an important limitation, but it can also be considered as a potential for obtaining additional sub-products if the recovery of acid compounds is intended. Other suitable options may be their transformation into methane by a subsequent anaerobic digestion stage, the use of bioelectrochemical systems to produce additional hydrogen or the generation of microbial lipids using oleaginous yeasts (Escapa et al. 2012; Fernández et al. 2015; Liu et al. 2016; Moreno et al. 2018). Different integrating approaches have been evaluated in an attempt to increase the reutilisation of the organic matter initially contained in the feed stream and attain its

transformation into components which can be easily reintroduced into the economy as raw materials. However, some technical approaches are still in need of overcoming some constraints, since the use of certain kind of products which have been previously in close contact with manures during its treatment process, may have difficulties in finding broad public acceptance.

7.4 Dark Fermentation Integrated in Two-Stage Processes

The implementation of the dark fermentation technology is necessarily linked to attaining higher hydrogen yields. The two-stage configuration is a feasible approach and also enhances the efficiency of the global process by increasing the conversion of the organic stream. Two-stage processes can integrate a first dark fermentation stage with a subsequent one involving bio-photofermentation, bioelectrochemical systems or anaerobic digestion.

7.5 Bio-photofermentation

Most of the previous research on hydrogen production from organic wastes are focused mainly on either dark or photofermentative processes (Wu et al. 2010; Pattanamanee et al. 2012; Zhu et al. 2013; Zhang et al. 2017; X. Wang et al. 2018). However, earlier researchers identified some main disadvantages of these processes including low hydrogen yield, high production costs and low energy recovery from the feedstocks. For these reasons, to increase the production of hydrogen and offer a solution to waste treatment, dark fermentation effluents can be used as feedstock in a couple two-stage processes (Stephen et al. 2017).

Equations (7.3–7.5) represent the degradation of glucose by dark fermentation, photofermentation and sequential dark and photofermentation, respectively. In dark fermentation, 4 mol of hydrogen is produced per mole of glucose consumed (Eq. 7.3) (Kim and Zhang 2015). As shown in Eq. 7.4, theoretically 4 mol of hydrogen gas can be produced from 1 mol of acetic acid. The overall maximum theoretical yield in sequential fermentation and photofermentation is 12 mol H_2/mol glucose when acetic acid is the only VFA present (Manish and Banerjee 2008; Argun and Kargi 2011).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \quad \Delta G_0 = -206\,\text{kJ} \tag{7.3}$$

$$CH_3COOH + 2H_2O + \text{light} \rightarrow +4H_2 + 2CO_2 \quad \Delta G_0 = +104\,\text{kJ} \tag{7.4}$$

$$C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO_2 \quad \Delta G_0 = +3.2\,\text{kJ} \tag{7.5}$$

Over the last decade, hydrogen production through the sequential two-stage process has been studied reporting considerable increments in hydrogen yields when compared with the individual dark and photofermentative processes (Redwood et al. 2008; Argun and Kargi 2011; Chandra and Venkata Mohan 2014; Laurinavichene et al. 2014; Zagrodnik and Łaniecki 2017; Ghosh et al. 2018).

Combined dark and photofermentation were studied by Seifert et al. (2018) using chewing gum components as organic substrate and *Rhodobacter sphaeroides* O.U.001 as photoheterotrophic bacteria. The main chewing gum components including xylitol, butyric, acetic, lactic and propionic acids, served as the source of organic carbon for photofermentation. The coupled process under optimised reaction conditions reaches a total amount of hydrogen produced of ~6.7 L H_2/L of non-diluted waste.

As example for wastewater experiences, hydrogen production from palm oil mill (POME) effluent using two-stage sequential dark and photo-fermentation studies developed by Mishra et al. (2016), attained a hydrogen yield of 0.784 H_2/mL POME from dark fermentation and 3.064 mL H_2/mL POME from sequential process dark/photo-fermentation obtaining a considerable enhancement for the overall configuration.

Although some constraints need to be overcome as it is the strict control of environmental conditions, substrate and inoculum concentration and the salinity induced in the liquor of the reactor associated with the control of pH, photobiological hydrogen production as a sequential process with dark fermentation stages can be a feasible option for energy production (Hitit et al. 2017). The favoured choice of the sequential dark/photo-fermentation is due to its dual benefits of higher yield of hydrogen as well as a more complete treatment of the organic waste (Mohan et al. 2013; Ghosh et al. 2018).

7.6 Bioelectrochemical Systems

Usually, bioelectrochemical systems can be classified as microbial fuel cells (MFCs) or microbial electrolysis cells (MECs) depending on whether they operate in galvanic (MFCs) or electrolytic mode (MECs); in other terms, MECs are modified MFCs that produce hydrogen (Rivera et al. 2015; Escapa et al. 2016). MECs transform the organic matter through the oxidation of molecules mediated by microorganisms with the aid of an external circuit containing a power supply (Fig. 7.1). In comparison to the theoretical minimum voltage of 1.23 V required for water electrolysis, MECs have low energy consumption since an applied voltage as low as 0.2 V is considered to be necessary for microbial electrohydrogenesis (Hu et al. 2009; Gómez et al. 2011).

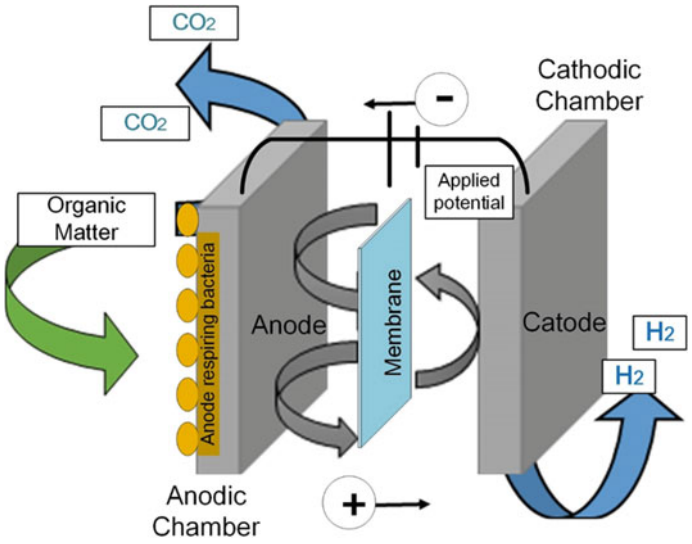

Fig. 7.1 Schematic representation of the working principles of MECs

During the past decade, MECs have been studied as a suitable alternative technology to conventional wastewater treatments, in particular to waste activated sludge systems which are characterised by a high energy demand associated with the need of aeration for maintaining microbial activity. Escapa et al. (2016) analysed the use of MECs for the treatment of real municipal wastewaters and obtained hydrogen at different scales and operating modes (batch, semi-continuous). Different studies revealed a hydrogen production rate from 0.007 to 0.74 L H_2/L day, with an applied voltage ranging from 0.2 to 1.1 V and a percentage of organic matter removal of 33.7–92.0% (Lu et al. 2009; Escapa et al. 2012, 2015; Gil-Carrera et al. 2013; Heidrich et al. 2014; Brown et al. 2015).

Two-stage approach, combining MECs with dark fermentation, might be a feasible route to obtain an increase in hydrogen yields and better efficiencies in the conversion of substrate with the simultaneous reduction of either the chemical oxygen demand (COD) or solid content of the wastes. Acetic acid has been one of the substrates most frequently studied in MEC systems, because it is rapidly assimilated and has been tested in many start-up operations of MECs or for evaluating the performance of the process under different conditions. Based on the successful results obtained when using this substrate, coupling dark fermentation and MECs seems to be the most reasonable option. The dark fermentation may be used for converting the organic material into acid forms and the subsequent MEC may be dedicated to end the transformation by turning the organic acids into hydrogen. The effluents of the dark fermentation process usually contain high concentrations of VFAs including acetate, butyrate and propionate, which can be interesting for their suitable use in other processes (Lenin Babu et al. 2013;

Chandrasekhar et al. 2015). However, major difficulties in the extraction of VFA from the acid stream are associated with their similar boiling points which make difficult the recovery of the single species from the fermentation broth and increase overall costs (Pandit et al. 2014). This makes the conversion of these species a preferable option rather than it direct recovery. The conversion of VFAs has been widely studied obtaining successful results in MECs for hydrogen production.

Table 7.5 shows a compilation of published works that report on the production of hydrogen from organic substrates and wastewaters by coupling dark fermentation and MECs.

In general, the results of the different studies showed that coupling dark fermentation and MECs for organic waste and wastewater treatment constitute a suitable route for increasing the efficiency of the conversion of organic substrates into hydrogen (Lenin Babu et al. 2013; Chookaew et al. 2014; Li et al. 2015; Rivera et al. 2015; Marone et al. 2017). Nevertheless, the optimisation of the bioprocess parameters, like pH, temperature, organic loading rate, hydraulic retention time and effluent recycling ratio, is still necessary. In addition, the large capital costs associated with the installation of MECs are the main barrier to their scale-up and commercialisation.

Table 7.5 Studies of two-stage approach: combining dark fermentation and MECs

Substrate DF	H_2 yield in DF	Effluent DF-Feed MECs	Applied voltage in MECs	H_2 yield in MECs	Reference
Corn stover lignocellulose	1.64 mol H_2/mol glucose	Acetic acid, ethanol, formic, lactic, and succinic acids	0.5 V	750 ± 180 mL H_2/g COD	Lalaurette et al. (2009)
Glucose	1.21 mol/kg $COD_{removed}$	Volatile fatty acids	0.2–1.0 V	Maximum at 0.6 V applied: 2.03 mol/kg $VFA_{removed}$	Lenin Babu et al. (2013)
Crude glycerol (purity 50%)	0.55 mol H_2/mol glycerol	Volatile fatty acids: Acetic acid, propionic acid, isocaproic acid and butyric acid	0.6–1.0 V	Maximum at 1.0 V applied: 106.1 ± 8.5 mL H_2/g COD	Chookaew, Prasertsan and Ren (2014)
Synthetic Media	–	VFA: 25% acetic, 12% propionic and 63% butyric	0.55 V	Hydrogen production rate was 81 mL H_2/L/day (initial COD from 400 to 1200 mg/L)	Rivera et al. (2015)
Ricotta cheese whey	122.27 mL H_2/g COD	Butyrate, succinic acid, acetic acid and ethanol	0.2 V	714.7 mL H_2/g COD	Marone et al. (2017)
Vinasse from spirits production	87.70 mL H_2/g COD	Butyrate, succinic acid, acetic acid and ethanol	0.2 V	1399.57 mL H_2/g COD	Marone et al. (2017)
Cassava starch processing wastewater	At HRT of 24 h: 260 mL H_2/g COD	Acetic acid, butyric acid, propionic acid and valeric acid	0.1–0.8 V	Maximum at 0.6 V applied: 205 mL H_2/g COD	Khongkliang et al. (2017)
Paper mill wastewater	37.37 mL H_2/g COD	Butyrate, succinic acid, acetic acid and ethanol	0.2 V	219.4 mL H_2/g COD	Marone et al. (2017)

7.7 Anaerobic Digestion of Wastes: Coupling Hydrogen and Methane Production

Two-stage processes, coupling hydrogen and methane production by anaerobic digestion of wastes have multiple advantages and could enhance the global efficiency of both processes. These advantages, Besides higher gas-energy recovery, the advantages of coupling hydrogen and methane production by anaerobic digestion, include the stabilisation of the waste streams in a reasonable time, more stable reactor performance of the hydrogen production unit without the need of adding any extra reagent for pH control of the hydrogen reactor since this can be easily attained by the recirculation of the alkalinity present in the digested effluent, and the maintenance of microbial population (hydrogen-producing bacteria) in high concentration.

The two-phase fermentation process to obtain hydrogen in the first phase and methane in the subsequent one may become an attractive solution to reach higher energy yields and at the same time solve the problem of waste treatment. Anaerobic digestion is a complex bioprocess where the degradation of organic compounds is conducted by a wide diversity of microorganisms. An overall scheme of the breakdown pathways of organic matter and biogas production is shown in Fig. 7.2. The process can be summarised in four major steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis which are carried out by a consortium of mutually

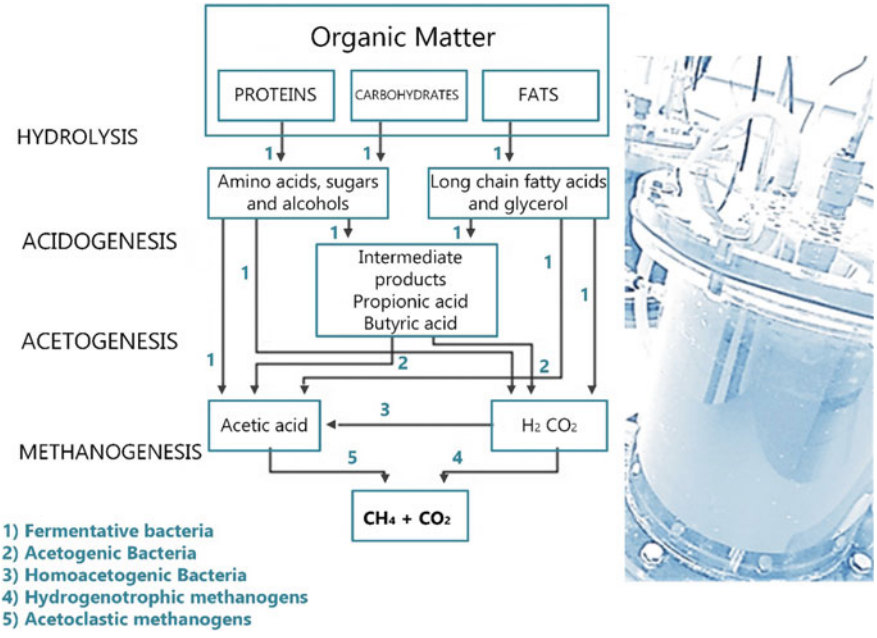

Fig. 7.2 Main stages of anaerobic digestion

dependent microorganisms including hydrolytic-fermentative bacteria, proton-reducing acetogenic bacteria, hydrogenotrophic methanogens and acetoclastic methanogens (Zinder 1984). Hydrogen is produced by fermenting and syntrophic bacteria in the anaerobic degradation of organic matter as an intermediate product and consumed by the hydrogenotrophic methanogenic archaea (Conrad 1999).

The main variables influencing both single- and two-stage process performances are temperature, inoculum, C/N ratio of the substrate, reactor retention time, pH and substrate concentration. The optimisation of these variables are imperative to promote the effective sequence of the two phases and to increase the combined yields of hydrogen and methane (Chu et al. 2008a; Fernández, Villaseñor and Infantes 2011; Lin et al. 2011; Paudel et al. 2017; Wang et al. 2017; Salem, Brunstermann et al. 2018).

The performance of the combined process using anaerobic digestion as a second stage has been studied by several authors (Table 7.6). The data show that higher energy recovery was obtained from the combined approach than from the single process.

Gómez et al. (2006) concluded that the incorporation of a hydrogen recovery unit into the traditional anaerobic process for the treatment of municipal solid wastes and slaughterhouse wastes was appropriate for attaining stable hydrogen production under the hydraulic retention times (HRTs) evaluated (3 and 5 days). The amount of hydrogen obtained from the fermentation process was in the range of 52.5–71.3 L H_2/kg VS.

According to the results of Lavagnolo et al. (2018) for the two-stage treatment of the organic fraction of municipal solid wastes, the digestion of the organic material was improved thanks to the faster rate of methane production and the decrease in the lag phase obtained during batch tests with different initial pH and feed/microorganism ratio.

Cheese whey has also been studied as a promising feedstock for fermentative hydrogen production (Valta et al. 2017). In a thermophilic anaerobic digestion of cheese whey evaluated for two-stage configuration (H_2–CH_4) in a sequencing batch reactor (Fig. 7.3), Fernández et al. (2015) concluded that the digestion of cheese whey was successfully achieved at an HRT of 8.3 days. However, the evaluation of the process under two-stage configuration resulted in a VFAs build-up in the methanogenic reactor as a response to inhibitory conditions.

An instable operation of a continuous stirred-tank reactor (CSTR) fed with raw cheese whey (with an organic loading rate of 30 g COD/L day) was reported by Castelló et al. (2018). Hydrogen production oscillated significantly reaching a maximum value of 0.9 L H_2/L day and decreased after 17 days of operation. In spite of this unsteady performance, hydrogen-producing organisms as *Clostridium* and their hydrogenases genes were detected along the operation, and the process did not improve the hydrogen production or showed better stability.

The co-digestion of two or more substrates offers multiple advantages for the process, and the main is the improvement obtained in biogas production along with the process stability. In some cases, this factor may be considered relevant to

Table 7.6 Hydrogen and methane yields obtained from scientific studies on two-stage process couple DF-AD

Substrate	Reactor	HRT (H_2 phase)	H_2 yield	CH_4 yield	OLR (H_2 phase)	**Reference**
Food waste from a dining hall	CSTR (10 L) + CH_4 reactor with suspended media (40 L). Thermophilic–Mesophilic	0.3 day	0.205 m^3 H_2/kg VS	0.464 m^3 CH_4/kg VS	38.4 kg VS/ m^3 d	Chu et al. (2008a)
Food waste from restaurants	Rotating drum (200 L) + CSTR (800 L). Mesophilic	240–96 h	0.049–0.065 m^3 H_2/kg VS	$\leq$0.546 m^3 CH_4/kg VS	22.7–37.8 kg VS/m^3 d	Wang and Zhao (2009)
Food waste from cafeteria	CSTR (10L) + CH_4 packed reactor (40 L). Thermophilic	3.8–1.28 day	0.056–0.118 m^3 H_2/kg VS	0.451 m^3 CH_4/kg VS	12.4–37.0 kg VS/m^3 d	Lee et al. (2010)
Co-digestion of cow manure and waste milk	1-L batch digester Thermophilic	–	0.035 m^3 H_2/kg VS maximum at 50:50 CM to WM ratios	0.627 m^3 CH_4/kg VS maximum at 30:70 CM to WM ratios	–	Lateef et al. (2014)
Cheese whey from after protein recovery	Automatic plant with mesophilic H_2 reactor (3L) + CH_4 Thermophilic SBR reactor (25 L)	28–13 day	0.007–0.018 m^3 H_2/kg COD	0.248–0.350 m^3 CH_4/kg VS	12.7–25.3 kg VS/m^3 d	Fernández et al. (2015)
Paperboard Mill Wastewater	Multi-phase ABR (30 L). Mesophilic	12 h	0.042 m^3 H_2/kg COD	0.018 m^3 CH_4/kg VS at HRT 36 h	–	Farghaly and Tawfik (2017)
OFMSW from organic waste treating plant	2 × 500 ml glass bottles in batch configuration. Mesophilic	45 day	0.007–0.030 m^3 H_2/kg VS	0.474–0.619 m^3 CH_4/kg VS	–	Lavagnolo et al. (2018)
Potato wastewater,	H_2 Two parallel CSTRs (4 L) + CH_4 UASB (45 L). Mesophilic	13.1–14.6 day	0.059–0.252 m^3 H_2/kg VS	0.199–0.507 m^3 CH_4/kg VS	–	Salem, Mietzel, (et al. 2018)
Bean wastewater	H_2 two parallel CSTRs (4 L) + CH_4 UASB (45 L). Mesophilic	13.1–14.6 day	0.093–0.152 m^3H_2/kg VS	0.127–0.463 m^3 CH_4/kg VS	–	Salem, Mietzel, et al. (2018)

Continuous stirred-tank reactor (CSTR), sequencing batch reactors (SBR), anaerobic baffled reactor (ABR), upflow anaerobic sludge Blanket (UASB)

increase the economic feasibility. Co-digestion of sewage sludge and other organic wastes could enhance biogas production and organic matter degradation due to dilution of inhibitory compounds and a more balanced carbon-to-nitrogen ratio (Martínez et al. 2011, 2012).

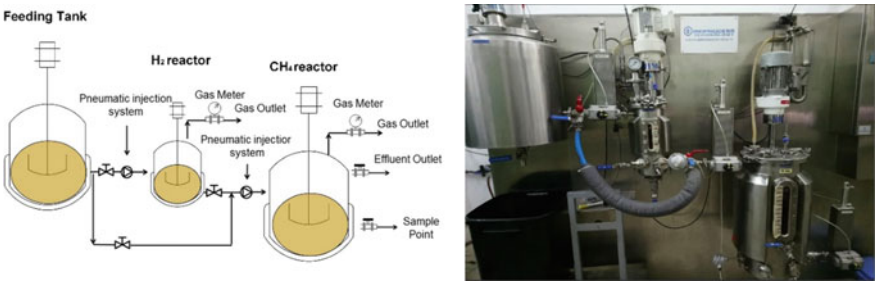

Fig. 7.3 Pilot plant used for anaerobic digestion of cheese whey, coupling H_2 and CH_4 production

Recent studies are particularly related to the sequential production of hydrogen and methane employing an organic waste as a single substrate (Chu et al. 2008b; Wang and Zhao 2009; Lavagnolo et al. 2018; Yun et al. 2018). Other researchers have also demonstrated that hydrogen yields can be enhanced by the co-digestion of organic wastes with sewage sludge (Kim et al. 2004; Kim et al. 2013; Lee et al. 2014; Arain et al. 2018; Maragkaki et al. 2018; Náthia-Neves et al. 2018; Silva et al. 2018).

Hydrogen and methane production from co-digestion of food waste and sewage sludge was evaluated in a two-stage system under mesophilic and batch conditions by Silva et al. (2018). Their results showed higher hydrogen yields compared with the experiments carried out with only one of these substrates. The co-digestion with sewage sludge plus raw glycerol results in an 87% increase in the production of hydrogen if compared with the control experiments (mixture of food waste and sewage sludge), with the maximum hydrogen yield obtained being 179.3 mL H_2/g VS for the mixture of the tree substrates at a glycerol concentration of 3% v/v.

7.8 Conclusion

Environmental friendly bio-hydrogen can be obtained by means of the transformation of organic wastes and wastewaters through dark fermentation. Several factors influence the fermentative hydrogen production process, including operating constraints and environmental conditions, type of feedstocks, microbial population, metabolic pathways, pH, temperature, organic loadings, the presence of inhibitors and nutrients, which have to be optimised.

The most important problems that need to be overcome are the low production rates and hydrogen yields. During the last decade, several efforts have been made to attain economic feasibility. Two-stage processes integrating the production of hydrogen by dark fermentation and a subsequent stage to assimilate the acid stream obtained as by-product from the first one offer a promising alternative to close the

technical gaps of implementing this technology at large scale. This fermentation is able to deal with a wide variety of organic substrates, but lowering operating costs and increasing the global efficiency of the process is still a pending task.

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Chapter 8
Impact of Antibiotics on Biogas Production

Ricardo Luís Radis Steinmetz and Vanessa Gressler

Abstract Besides their use in human treatments, antibiotics have been extensively used to control animal diseases and, in some countries, still used to promote animal growth in livestock industry. To attempt human diet necessities, concentrated animal feeding operations (CAFOs) are necessary, increasing antibiotics consumption and manure production. Once antibiotic active agents and its metabolites are excreted in urine and feces, these substances are present in manure and can reach the environment. Around the world, especially in rural areas, manure is the main substrate for biogas production. This chapter presents a review about fate of antibiotics, with special focus on livestock by-products, and effects during the anaerobic digestion (AD). The antibiotic interaction has two important emphases addressed: (a) inhibition on the biogas and methane production process by the presence and action of these compounds and metabolites in the digester and (b) the ability of AD to degrade the molecules of antibiotics and thereby reduce the adverse effect caused by these compounds on the environment.

Keywords Anaerobic digestion · Veterinary drugs · Inhibition

8.1 Introduction

Antibiotics were discovered in the 1920s but only introduced into medicine in the 1940s (Etebu and Arikekpar 2016) to treat or prevent diseases in human and livestock (CDDEP 2015), changing the pattern of modern way of living. With the increasing prosperity and world population growth, the demand of these substances rises constantly, where more antibiotics are necessary to human necessities as well as to attempt animal husbandry due to the growing demand for animal products (e.g., meat, milk, egg, and their products) for human consumption (Tilman et al. 2002).

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In humans, the consumption of antibiotics between 2000 and 2010 grew by 35%, from approximately 50 to 70 billion standard units, based on data from 71 countries (Van Boeckel et al. 2015). In most countries, about 20% of antibiotics are used in hospitals and other healthcare facilities, and 80% are used in the community (CDDEP 2015). In livestock, global antibiotic consumption was estimated at approximately 63,000 tons in 2010 (Van Boeckel et al. 2015), accounting for nearly two-thirds of the estimated 100,000 tons of antibiotics produced annually worldwide (Bbosa and Mwebaza 2013). At less proportion, antibiotics are also given to pets, used in food industries, as preservatives (Silva and Lidon 2016), in aquaculture for shrimp and fish production, crop growing (CDDEP 2015; O'Neill 2015), in pharmaceutical production plants, and other uses.

Considering this scenario, it is possible to differentiate the substrates that contribute to the occurrence of antibiotic compounds in the biodigester in three possible groups (Fig. 8.1): from animals, from humans, and from industry (Alexy et al. 2004). In industrial effluents, antibiotics occur in high concentration, since they are the processes that generate the drugs. In this case, biological processes for the wastewater treatment are generally ineffective, requiring additional physical and chemical process (e.g., activated carbon or advanced oxidative processes) prior to UASB reactor or anaerobic filter (Yan and Lam 2015). Similarly, it occurs in hospital effluents. On the other hand, domestic effluents have a much lower concentration of antibiotics and in this case the inhibition possibility could be substantial during the digestion of the sludge from wastewater treatment plants. However, considering the three groups of substrates, the major contribution to the interaction with antibiotics occurs in large-scale biogas plants that take advantage of animal manure to feed the anaerobic digesters. For this reason, this chapter will focus on the occurrence and interaction of veterinary drugs found in manure.

To meet the needs of animal-derived food, livestock production has become increasingly dominated by concentrated animal feeding operation (CAFO).

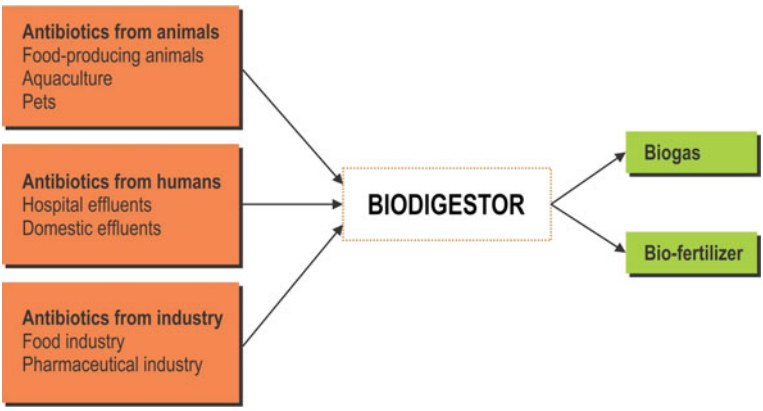

Fig. 8.1 Sources of antibiotics-containing residues which can be used as substrate in anaerobic digestion

The high population density of CAFOs in a relatively small area results in sharing of both commensal flora and pathogens, which can be conducive to rapid dissemination of infectious agents. Also, commensal bacteria found in livestock are frequently present in fresh meat products and may serve as reservoirs for resistant genes that could potentially be transferred to pathogenic organisms in humans. As a result, livestock in these environments commonly requires management strategies, which often include the use of antibiotic therapy to ensure the herd health and optimize production (Landers et al. 2012; Hao et al. 2014; O'Neill 2015).

In CAFOs, different antibiotic utilization manners are applied. Two terms are frequently used to describe the use in livestock: therapeutic and subtherapeutic. The first term, therapeutic, is used when a veterinarian prescribes a drug to treat animal with clinical signs of an illness or a condition like a respiratory infection or a skin infection. To this prescription, high doses of antibiotics for brief periods are administered to a single animal or a large group of them. The second term, subtherapeutic, is used when the antibiotics are administered in animals, which are susceptible to diseases or infection that can kill them quickly, in a preventative manner (lower doses for long periods of time), to prevent an outbreak. This definition fits also the use as growth promoters, where antibiotics are given mixed in feedstuff to improve daily weight gain and feed efficiency (CDDEP 2015; Schlüsener et al. 2003; Shi et al. 2011; Venglovsky et al. 2009; Landers et al. 2012).

The use of antibiotics in food production animals brings up an environmental preoccupation, once it is known that after administration (oral or parenteral) each antibiotic has its particular route and a significant proportion are excreted by urine or feces (17–90%, varying from compound to compound), in the unchanged or as active metabolites of the parent species (Martínez-Carballo et al. 2007; Zhou et al. 2013). Besides the occurrence of antibiotics, in CAFOs a very high manure amount (also with high concentrations of organic matter and nutrients) is produced with a worldwide estimation bigger than 9×10^9 ton annually (He et al. 2016). Thus, livestock wastes cannot be directly discarded in water bodies; therefore, they are a potential environmental contaminant on air, soil, and water resources if its destination is not correctly managed (Kunz et al. 2009). Once in the environment, it is difficult to predict how quickly antimicrobials will degrade, whether they come from animal use, human use, or manufacturing, as they are very diverse chemically. Some degrade easily, while others bind to organic matter and can persist in their active states for long periods of time (O'Neill 2015).

Because manure has been integrated as part of sustainable crop production by direct land application as biofertilizer (He et al. 2016), usually manure production is higher than the necessities for this purpose or not economically practicable by the distance between CAFOs and cropland (Seganfredo and Girotto 2004). As an alternative to manure disposal problem and recycling, multi-step advanced treatment systems were adopted enabling further effluent discharge in water bodies or water reuse. Other alternatives to reduce manure's pollution potential are composting (compost/fertilizer production) and anaerobic digestion (biogas generation). Both generate by-products with agronomic value, but they do not reduce land area needed to recycle the nutrients (Kunz et al. 2009; Scheeren et al. 2011).

It is the notorious importance of the antibiotics in both human and animal health and welfare. However, antibiotic resistance became one of the biggest threats to global health, food security, and development today (World Health Organization 2018), once infections with antibiotic-resistant bacteria may not respond to regular antibiotic treatments, resulting in increased mortality, morbidity, and social and economic costs. There are evidences that the use of antibiotics in animal food production is a source of antibiotic-resistant bacteria which are transmitted to people via occupational exposure on farms, along the food production chain, through food itself and through environmental pathways like contaminated soil and water supplies by animal production wastes (excreted material or manure-treated effluents) containing antibiotic residues (Koch et al. 2017).

Healthy animals mean a safe food supply and in turn healthy people. However, in food production animals, the use of antimicrobial drugs brings benefits and risks. These health products have important benefits such as: prevention, treatment, and control of bacterial and parasitic diseases contributing to good animal welfare and ensuring human food security and avoiding foodborne illness; protection of human against zoonosis preventing human hospitalizations and deaths; enhancement of animal production by the improvement of feed conversion ratio, animal growth, and reproductive performance; and improvement of environment by reducing manure disposal amounts and consequently the emissions of greenhouse gases. On the other hand, animal antibiotic use impacts on the development of drug-resistant pathogens, residues in food products occurrence, and also may influence on biological treatment methods of waste products (Hao et al. 2014; Sneeringer et al. 2015).

8.2 World Antibiotic Consumption in Food-Producing Animals

Antibiotics have been used to treat infections in animals for as long as they have been widely available (CDDEP 2015); however, the incorporation in animal husbandry practices became more frequent in the twentieth century. Agricultural activities represent a large proportion of the usage of antibiotics in worldwide antibiotic consumption (O'Neill 2015; Gonzalez Ronquillo and Angeles Hernandez 2017). For example, in the USA, of the antibiotics defined as medically important for humans by the US Food and Drug Administration (FDA), over 70% (and over 50% in most countries in the world) are sold for use in animals (O'Neill 2015). There is a lack of reliable information on global use of antibiotics in livestock; however, it is estimated that in 2010, more than 63,000 tons of antibiotics were used in food animals worldwide and probably will reach more than 105,000 tons by 2030. The five countries with the largest shares of global of antimicrobials used in livestock in 2010 were China, USA, Brazil, Germany, and India with approximately 15,000 ton (23%), 8500 ton (13%), 5500 ton (9%), 2000 ton (3%), and 2000 ton (3%), respectively, and the five countries with the greatest projected percentage of antimicrobial consumption by 2030 are China, USA, Brazil, India,

and Mexico with approximately 33,000 ton (31%), 12,000 ton (11%), 8500 ton (8%), 4500 ton (4%), and 2500 ton (2%), respectively (Van Boeckel et al. 2015).

Looking at antimicrobial use between countries, heterogenic geographic distribution can be seen. The reasons often are: particular preferences (handling, market, etc.), national custom, practice and legislation, level of industrialization of animal production, and availability of number of authorized veterinary medicines (Jarvis et al. 2011; Gonzalez Ronquillo and Angeles Hernandez 2017). Changes in disease pattern (outbreaks) and changes in climatic condition per year also influence antibiotic consumption variations (De Briyne et al. 2014; Federation of Veterinarians of Europe—FVE 2016). An overview of classes and amounts of

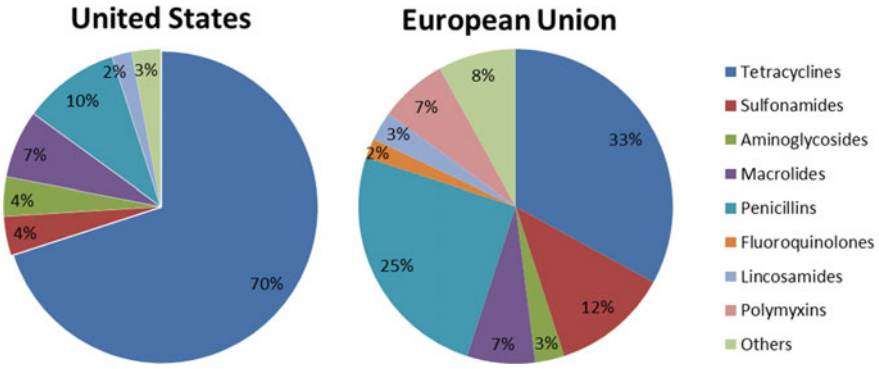

Fig. 8.2 Percentage of antimicrobial sales (by class) for food-producing animals in the USA in 2016 and European Union in 2015. *Source* ESVAC (2017); FDA (2017)

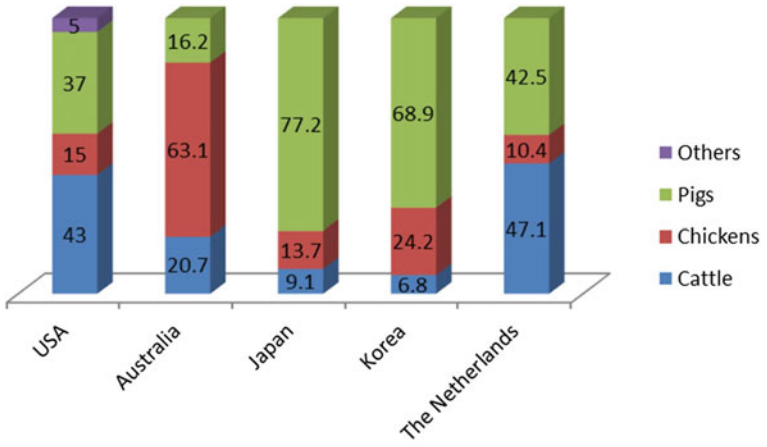

Fig. 8.3 Estimative of the intention use (percentual based on domestic sales) of medically important antimicrobials in food-producing animals. *Source* FDA (2017); Van Boeckel et al. (2015)

antimicrobials used in food-producing animals in USA and in European Union (EU) is shown in Fig. 8.2. Figure 8.3 presents an estimative of veterinary antimicrobial consumption by livestock species in food animals of five countries.

Certain antimicrobial drugs used in food-producing animals are considered "not medically important" like ionophores, polypeptides, orthosomycins, pleuromutilins, aminocoumarins, glycolipids, and quinoxalines. Ionophores, for example, are only used in veterinary medicine; thus, public health risks are much lower than medically important antimicrobials. Despite being not antimicrobial resistance target compounds, not medically important antimicrobials are widely used; for example, in USA, in 2016, tetracycline accounted for 5,866,588 kg and ionophores for 4,602,971 kg of the domestic sales (FDA 2017).

8.3 Antibiotic Used in Food-Producing Animals

Antibiotics, by definition, are chemical substances produced by microorganisms or by synthetic ways which acts by disrupting various molecular targets within bacteria and cell surface preventing growth (bacteriostatic) or initiating killing (bactericidal) (Etebu and Arikekpar 2016). There are several kinds of antibiotics, and they can be classified by different forms; however, the most common are based on their chemical structure or mechanism of action. Assuming that antimicrobial consumption in cattle, pigs, and chickens represents the majority of antimicrobial consumption in food-producing animals, Table 8.1 contains the most common groups which may be used at different times in the life cycle of these animals.

The frequently occurring diseases in food-producing animals that are likely to be treated with antibiotics are mastitis, uterine infections (metritis), joint infections, foot rot, digital dermatitis, and salmonellosis in dairy cows; enteritis, septicemia, umbilical infections and polyserositis, pneumonia, diphtheria, and foot rot in calves; erysipelas, joint infections, foot rot, mastitis, and uterine infections (metritis) in breeding sows; enteritis, septicemia, meningitis, umbilical infections, and skin infections in weaned piglets; enteritis, pneumonia, and tail bite infections in fattening pigs; enteritis, respiratory infections, septicemia, and yolk sac infection in chickens (De Briyne et al. 2014; ESVAC 2017).

8.4 Antibiotic Occurrence in Manure

Animal operations may vary widely in the administration of medicine. The occurrence in manure is dependent from the quantity administered (dosage) and the capability of excretion by the animal. Some of the factors that can influence on the manure antibiotic amount are: difference breeding performance among farms, prescription pattern for different animal types or stage of production (e.g., piglets, growing-finishing pigs, swine sows, dairy cow, and calf), susceptibility of diseases

Table 8.1 Most common group of antimicrobials used food-producing animals

Antimicrobial class	Mechanism of action[a,b,c]	Examples[d,e]
Tetracyclines	Inhibits protein synthesis	Tetracycline, chlortetracycline, oxytetracycline
Sulfonamides	Inhibits nucleic acid synthesis (folic acid metabolism)	Sulfadimethoxine, sulfamethazine
Aminoglycosides	Inhibits protein synthesis	Dihydrostreptomycin, gentamycin, Hygromycin B, Neomycin, spectinomycin
Macrolides	Inhibits protein synthesis	Erythromycin, gamithromycin, tildipirosin, tilmicosin, tulathromycin, tylosin, Tylvalosin
β-lactams		
Penicillin	Inhibits steps in bacteria cell wall synthesis	Amoxicillin, benzylpenicillin (penicillin G), ampicillin, cloxacillin
Cephalosporins		Ceftiofour, cephapirin
Fluoroquinolones	Inhibits DNA replication and transcription (DNA gyrase)	Danofloxacin, enrofloxacin
Lincosamides	Inhibits protein synthesis	Lincomycin, pirlimycin
Polymyxins	Destroys cell membrane structure or function	Polymyxin B
Streptogramins	Inhibits protein synthesis	Virginamycin
Amphenicols	Inhibits protein synthesis	Florfenicol
Diaminopyrimidines	Inhibits nucleic acid synthesis (folic acid metabolism)	Ormetoprim, trimethoprim
Polypeptides	Destroys cell membrane structure or function	Bacitracin
Orthosomycins	Inhibits protein synthesis	Avilamycin
Pleuromutilins	Inhibits protein synthesis	Tiamulin
Aminocoumarins	Inhibits DNA replication and transcription (DNA gyrase)	Novobiocin
Glycolipids	Inhibits steps in bacteria cell wall synthesis	Bambermycins
Quinoxalines	Unknown	Carbadox
Ionophores (polyether)	Modifies the permeability of cellular membranes	Lasalocid, monensin, salinomicyn, narasin, laidlomycin

[a]Etebu and Arikekpar 2016; [b]Lambert 2012; [c]Sperelakis 2012; [d]FDA 2017; [e]ESVAC 2017

according to seasons, breeding scale (in heads), relation between antibiotic price and farm income, type of animal diet, and difference between animal races (Haller et al. 2002; Jacobsen and Halling-Sørensen 2006; Pan et al. 2011; Chen et al. 2012a, b; Tong et al. 2012; Zhou et al. 2013).

For example, much higher concentration of sulfonamides and tetracyclines compounds was found in piglet manures when compared with fattening pig or sow

manures (Pan et al. 2011). Some studies found higher antibiotic residues in swine manure in winter time (Chen et al. 2012b). The seasonality could be explained according to two possibilities: In winter, the animals are more susceptible to respiratory illness and need more antibiotics. On the other hand, in summer the high temperatures favor the bacteria activity that generally improve the use of drugs to combat diarrhea causes (e.g., anticoccidial) in preference to antibiotics to combat respiratory diseases. In addition, higher temperatures accelerate biodegradation and probably reduce antibiotic residues in manure at summer time. Table 8.2 shows a resume of antibiotics found in manure and livestock by-products around the world.

Table 8.2 Examples of antibiotics levels reported in livestock waste samples

Antimicrobial	Animal type	Sample	Levels	Reference
Tetracyclines				
Tetracycline	Swine	Sludge	130.6–3617.2 μg/kg	Pan et al. (2011)
	Swine	Manure	98.2 mg/kg	Chen et al. (2012a)
	Swine	Liquid manure	0.36–23 mg/kg	Martínez-Carballo et al. (2007)
Chlortetracycline	Swine	Manure	139.4 mg/kg	Chen et al. (2012a)
	Swine	Liquid manure	0.1–46 mg/kg	Martínez-Carballo et al. (2007)
	Dairy cattle	Manure	1450 μg/kg	Zhou et al. (2013)
Sulfonamides				
Sulfamethazine	Swine	Manure	3.3–24.8 mg/kg	Chen et al. (2012a)
Sulfathiazole	Swine	Manure	0.10–12.4 mg/kg	Haller et al. (2002)
Sulfamonomethoxine	Dairy cattle	Effluent	4.03 ng/L	Zhou et al. (2013)
Macrolides				
Tiamulin	Swine	Manure	0.1 mg/kg	Pan et al. (2011)
Tylosin	Swine	Wastewater	0.56–42 μg/L	Tagiri-Endo et al. (2009)
Lincosamides				
Lincomycin	Swine	Feces	0.16–17.0 mg/kg	Zhou et al. (2013)
	Dairy cattle	Effluent	700–6600 ng/L	Brown et al. (2006)
Quinolones				
Enrofloxacin	Swine	Dung	0.48–33.26 mg/kg	Zhao et al. (2010)
	Poultry	Manure	1420 mg/kg	Zhao et al. (2010)
	Poultry	Litter	30.97 mg/kg	Leal et al. (2012)

(continued)

Table 8.2 (continued)

Antimicrobial	Animal type	Sample	Levels	Reference
Norfloxacin	Swine	Dung	0.56–5.50 mg/kg	Zhao et al. (2010)
	Poultry	Manure	225 mg/kg	Zhao et al. (2010)
	Poultry	Litter	4.55 mg/kg	Leal et al. (2012)

Different analytical strategies have been employed to determine the antibiotic occurrence in wastewater and manure. Manure and wastewater samples are very complex to be analyzed, mainly because of the particulate matter, organic matter content, and various inorganic species which could cause serious matrix interferences. To eliminate or reduce interferences, usually are applied sequential liquid–liquid extraction and/or cleanup with solid phase extraction (SPE) on the sample preparation step (Hu et al. 2008). For estimation of antibiotic quantity, some authors report the use of very simple methods based on ELISA or radioimmunoassay (Aga et al. 2003). These methods, although cheap, are nonspecific and very inaccurate generally used as screening tool. For better specificity and accuracy, analysis is necessary to apply chromatographic methods like HPLC-UV, LC-MS, or LC-MS/MS (Schlüsener et al. 2003; Jacobsen and Halling-Sørensen 2006; Hu et al. 2008; Chenxi et al. 2008; Zhou et al. 2012).

8.5 Antibiotic Interaction in AD

Various antibiotics used in humans or animals have been studied asanaerobic digestion interactions agents. Studies have been focused to degrade substrates such as sewage, manure (cow and pig), and pure substances (e.g., glucose and organic acids standards) (Massé et al. 2000; Gartiser et al. 2007; Shimada et al. 2011). Interference reported in the anaerobic digestion is diverse, such as excessive foaming in the reactor, decline in biogas productivity, accumulation of organic acids, and imbalances in microbiology community (Fischer et al. 1981; Sanz et al. 1996; Shimada et al. 2011).

8.5.1 Methods for Evaluation of Antibiotic Effect in AD

Different methods are used to evaluate the antibiotic effect during anaerobic digestion, varying according to the objective of the study proposed by each author. Usually, the methods are based on a batch test, similar to a biochemical methane potential (BMP) assay, to establish comparisons at the biogas production and

kinetics parameters between tests with different antibiotic concentrations. The baseline for the experimental setup is found at the standard protocol ISO 13641, Water quality—Determination of inhibition of gas production, also called anaerobic toxicity assay (ATA). This test permits to evaluate the acute inhibition effect in biogas production (International Organization for Standardization 2003).

The ISO protocol consists of incubation of the anaerobic inoculum together with a standard substrate (yeast extract and glucose) and spiked with different concentrations of the inhibitory agent to be evaluated. Cumulative biogas volume produced is measured after 3 days of incubation at 35 °C and compared to the test without addition of the inhibitor. For each test, the percent of inhibition is obtained applying Eq. 8.1. The ISO experimental setup consists in 0.1–1 L of pressure-resistant gastight closed glass test bottles, coupled at precision pressure meter for measuring total biogas production. Original equation is based on the pressure variation measured by manometric systems. Equation 8.1 presents an adaptation by equivalence of volume variation:

$$I(\%) = \left(1 - \frac{V_t}{V_c}\right) \times 100 \tag{8.1}$$

where I is the inhibition percentual, V_t is the cumulative volume produced with test material (with antibiotic), and V_c is the cumulative biogas volume produced in the control at the same time. After, as illustrated in Fig. 8.4, it is possible to plot I against the logarithm of the concentrations of test material. The inhibitory concentration (IC or EC) value could be assessed visually or by regression analysis. Alternatively, it is possible to express a correlative inhibition based on inoculum mass used in each assay (International Organization for Standardization 2003).

Usually, the IC_{10} (concentration that produces 10% of inhibitory effect on biogas production) represents the minimum quantified level of inhibition or the method limit of detection. The IC_{50} (concentration that produces 50% of inhibitory effect on biogas production) is the standard parameter to compare toxicity between different antibiotic compounds.

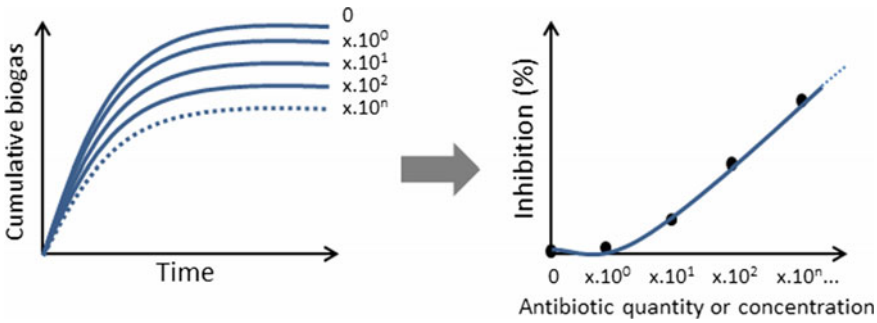

Fig. 8.4 Graphical demonstration of biogas data processing to obtain the inhibition response in ATA

Table 8.3 Suggested substrates for activity evaluation of a different trophic group anaerobic digestion inoculum

Population	Substrate
Hydrolytic	Cellulose
Acidogenic	Glucose
Proteolytic	Casein
Acetogenic	Propionic acid + n-butyric acid
Acetoclastic	Acetic acid
Hydrogenotrophic	$H_2 + CO_2$

Adapted from Angelidaki et al. (2009)

By the way, this methodology can be adapted according to the study purposed. Alternative substrates could be used to evaluate the activity or inhibition effect on the group of target microorganisms, as presented in Table 8.3 (Angelidaki et al. 2009). For example, Cetecioglu et al. (2012) used acetate to evaluate sulfamethoxazole, erythromycin, and tetracycline effect in acetoclastic activity after 5 days of digestion, using domestic anaerobic sludge as anaerobic inoculum. Other example is microcrystalline cellulose as the standard substrate applied by Steinmetz et al. (2016) to simulate a condition of co-digestion of manure and agricultural wastes. According to Angelidaki et al. (2009), each kind of substrates corresponds to stimulate the activity of different trophic groups of microorganisms from the anaerobic inoculum (Table 8.3). This means that during the ATA procedure it is possible to adapt the test conditions according to the type of substrate desired and generate results to specific conditions.

Similarly, other change in the ISO methodology could be proposed. Instead of evaluating only biogas production, it is also possible to use kinetic parameters as response variables to estimate the ATA of a substance. In this case, it is possible to evaluate if the toxic compound acts to inhibit on the adaptive phase (e.g., hydrolysis), on the biogas rate, or on the BMP.

Chronic (long-term) toxicity evaluation is also possible, but the methods are not standardized. Usually, for this case, a laboratory-scale reactor is continuous (or semi-continuous) feeding with a fixed organic loading rate and with progressive small increment of antibiotic loading (Bressan et al. 2013). The increment of antibiotic should be done by biogas productiveness stability or defined by fixed time (e.g., respecting 3 times of HRT).

8.5.2 Review of Inhibition Effect in Biogas Production Process

Inhibition during anaerobic digestion, when it occurs, has a negative impact on the generation of the products derived from the process. In addition to the reduction of

the volume of biogas produced it can also reduce the contents of methane and consequently the accumulation of intermediates (e.g. organic acids). These effects in the digester are generally related to unbalance of microcosmos. The literature reports about antibiotic inhibition show variations in the toxicity level. Such variations depend on the type of chemical substance used as antibiotic, on the conditions of the ATA test, and also on the possible conditions of acclimatization of the anaerobic sludge (which could be related to the development of antibiotic resistance mechanisms from the microorganisms).

Even though ATA tests are performed using direct spike of the antibiotic before the inoculation test, there are reports confirming the inhibition of biogas production using manure from medicated animal. Turker et al. (2013) evaluated the anaerobic digestion manure from bovine medicated with oxytetracycline (concentrations of 0.8–3.4 mg/L). After mesophilic anaerobic digestion (37 °C), they found inhibition effect of 17–24% in biogas yield from manure with 0.8–3.4 mg/L of oxytetracycline, respectively. The authors also identified the reduction of *Methanomicrobiales*, *Methanobacteriales,* and *Methanosarcinaceae* and suggested as the main cause for the biogas reduction.

Sanz et al. (1996) found 20% of inhibition in methane production in digestion with 35 mg/L of gentamicin. Authors used synthetic media, a mixture of organic acids as standard substrate, and spiked different concentrations of gentamicin (5–250 mg/L). The inhibition effect was evaluated after 2 weeks of incubation and described accumulation of propionic and butyric acid in order of 10–50% based on the acid added and compared to the free antibiotic control test. In another study, Gartiser et al. (2007) found IC_{10} for biogas in gentamicin concentration range of 0.4–7.2 and IC_{50} in range of 57.2–231.8 mg/L. At this case was applied the ISO test scheme for ATA using inoculum source from a municipal sewage treatment plant and measured biogas inhibition after 7 days in mesophilic condition (35 °C).

Fischer et al. (1981) related a stress condition of swine manure anaerobic digestion process. They observed a drop of 75% on the gas production, high propionic acid content (>3000 mg/L) in the digestate and operational problems by foam in the digester headspace. The reactor stress condition was related to the possible presence of antibiotic lincomycin. Ji et al. (2013) used a luminescent fast method to evaluate the methanogens biological activity and related to the acute inhibitory effect of lincomycin in anaerobic digestion. The authors found IC_{50} level of 3.5–5.7 g/L and related synergic toxicity effect when the lincomycin was mixed with other antibiotics (amoxicillin, kanamycin, and ciprofloxacin). The authors report a strong synergism in the toxicity effect of lincomycin in the presence of metabolites from anaerobic digestion process (ethanol, acetate, propionate, and butyrate). This indicates a possibility of lincomycin present in the digestate to decrease the digester efficiency in reactors operating at stressed conditions (e.g., overload or feedstock nutrient unbalance) or any reason to promote accumulation of VFA inside the reactor. At this case, the process could be more susceptible to suffer inhibition when receiving lincomycin.

Already for the macrolide tylosin, Poels et al. (1984) did not see disturbance effect in swine waste anaerobic digestion in the presence of 1.7–16.7 mg/L.

The experiment was conducted in a 1.5 L CSTR operated at 30–33 °C. However, Sanz et al. (1996) found 20% inhibition of methane production in batch assays, using tylosin at 15 mg/L. Besides that, as a consequence they found accumulation of propionic and butyric acids in order of 10–90% added.

Shimada et al. (2008) evaluated addition of small quantities (1.67 mg/L) of tylosin in glucose-fed ASBR. They observed decrease in the rates of propionate uptake and methane production, without effects on COD removal efficiency. But, at higher concentration (167 mg/L) of tylosin added in the digester, the authors observed high disturbance in the reactor performance: decrease in the glucose uptake rate, accumulation of acetate and propionate, and drop in the COD removal efficiency. Subsequently, the authors identified that there was an imbalance between the population of fermentative bacteria and methanogens archaea, with great impact on acetoclastic methanogens. Gram-positive glucose-fermenting bacteria maintained activity with tylosin, and propionate-oxidizing syntrophic bacteria were detected less frequently after tylosin introduction. The authors postulate relation between the inhibition in propionate uptake rate and occurrence of *Syntrophobacter* (consuming propionate bacteria) sensitive to the antibiotic. Finally, the methane reduction efficiency was explained for the combination of tylosin resistance in glucose-fermenting bacteria and inhibition of propionate-oxidizing bacteria resulted in accumulation of VFA.

Studies about methane emission of swine manure in anaerobic lagoons reported methane emission tended to plateau rapidly between 20 (after 72 h) and 45% (336 h) with addition of antibiotic lincomycin and tylosin in dosage between 1 and 25 mg/L (Loftin et al. 2005). The same test was done with an inoculum sludge collected from another lagoon with less antibiotic exposure, however it was observed quicker methane emission reduction. This could be related to a higher inhibition effect and could be a consequence of a better microorganisms' adaptation in the lagoons that were in greater contact with the antibiotics.

Bressan et al. (2013) evaluated a long-term exposure (450 days) of colistin sulfate (polymyxin E) in a UASB bench-scale reactor. The UASB was inoculated using swine manure and feed with acetate as substrate. The concentration of colistin was varied from 0.1 to 100 mg/L. Authors report that methanogenesis activity showed high tolerance to colistin, not showing relevant inhibition in methane production, even at the highest concentrations tested. The highest concentration tested is much higher than the one expected in swine wastewater (generally below 1 mg/L).

In other chronic toxicity experiment, Shimada et al. (2011) developed a long-term study of the digestion of swine manure in the presence of macrolide antibiotics. They observed accumulation of acids, especially propionic acid, and verified that antibiotics directly affected the action of propionate-oxidizing syntrophic bacteria, especially of the genus *Syntrophobacter*, and indirectly inhibited *Methanosaeta*.

8.5.3 *Persistence in Digestate: Factors of Fate Residual Antibiotic*

It is also important to identify the risk mitigation potential for the degradation of the antibiotic compounds in the digester. Besides the necessity to elucidate the effects of the antibiotic compounds during the anaerobic digestion process, it is relevant to understand the persistence and mechanism of degradation during the anaerobic digestion. The persistence of antibiotic after digester could release contaminant on soil by fertilizer pathway or represent inhibition effect to sequential biological process. Several studies have been demonstrating an opportunity to use the anaerobic route to the removal of antibiotic residues in wastewater or animal manure.

Recently, Steinmetz et al. (2016) observed a significant reduction of tetracycline compounds in a batch test after 35 days at mesophilic digestion (37 °C). The authors were evaluating the persistence of spiked (1.3–809 mg/L) tetracycline, chlortetracycline, metacycline, and oxytetracycline, using LC-MS/MS analysis. The most part of assays show reduction of 76–98% at antibiotic concentration. Only higher concentration of tetracycline (508 mg/L) and metacycline (104 mg/L) had efficiency removal decrease. For these assays were observed 46 and 57% of antibiotic reduction, respectively.

The reduction levels of tetracycline were similar to those described by Tong et al. (2012). The author found 88.6–91.6% of tetracycline reduction and 97.7–98.2% of chlortetracycline reduction after 45 days of swine manure anaerobic digestion at mesophilic conditions (35 °C). Turker et al. (2013) reported 55–70% of oxytetracycline reduction after 30 days of anaerobic digestion, at 37 °C, in manure feed with the antibiotic.

Generally, the antibiotic persistence is dependent of synergic effect of temperature and microbiological activity. Schlüsener et al. (2006) defined a high persistence (half-life > 200 days) of the macrolide tiamulin during the swine manure storage under anaerobic condition and 20 °C. In another study, Li et al. (2018) compared antibiotic persistence in manure samples stored at 15 and 35 °C. Antibiotic reduction was more notable when digestate was stored under mesophilic conditions. Regardless of storage conditions, in cases when organic matter was further biodegraded, the residuals of antibiotics in digestate were lower. In general, more biological activity results in less antibiotic persistence after AD.

8.6 Conclusion

The presence of antibiotic substances promotes adverse reactions in biogas production. However, the toxicity degree is dependent on a broad of factors: type of antibiotic, residual concentration, temperature of the digester, and whether the

microorganisms from the sludge underwent any process of acclimatization and enrichment for resistance development.

This reinforces the importance of knowing possible contaminants present in the substrate and thus predicts process changes. In some cases, the effect from antibiotic could promote synergic effect (e.g., FVA accumulation) and the anaerobic process could be more susceptible to suffer inhibition when the reactor was operated at stressed conditions. In addition, the biological effects of the combination of some drugs are still unknown.

Nevertheless, the anaerobic digester still is an important tool to treat the residual antibiotic content present in manure, for example. In this way, anaerobic digestion of livestock wastes represents an opportunity to risk mitigation potential related to intensification use of veterinary drugs in SPACs.

Despite increasing efforts to increase the rational and prudent use of antibiotics in all contexts to prevent the development of resistant bacteria, the presence of antibiotics in urban, industrial, and agricultural effluents will continue to exist.

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Chapter 9
Effect of Short-Chain Fatty Acid Production on Biogas Generation

Marina Celant De Prá, Andréia Anschau, Cleverson Busso, Naiana Gabiatti and Marcelo Bortoli

Abstract The short-chain fatty acids (SCFAs) are generated in the acidogenesis step of the anaerobic digestion, and their production is very important for the global process and efficient biogas production. SCFA production takes place inside the cells of fermentative microorganisms, which are the first ones to start the complex soluble carbon degradation. The SCFA reactions are the most energetic among the anaerobic digestion steps, which means that this step will hardly be limiting for biogas production in normal conditions—except if the previous hydrolysis is rough or impaired. The SCFAs produced are subsequently converted to acetic acid, which is effectively converted to methane by methanogenic acetoclastic archeas. Nevertheless, acetic acid production from SCFA releases a large amount of hydrogen in the medium, and in some situations, it will reduce the process pH to inhibitory levels for methanogenic archeas and consequently suppress biogas production. This chapter will focus on these events, approaching SCFA formation, the functional microorganisms involved, and their importance for the global process.

Keywords Biogas production · Short chain fatty acids (SCFAs) · Acidogenesis

9.1 Introduction

As discussed in previous chapters, anaerobic digestion involves four processes: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. The individual degradation steps are carried out by different consortia of microorganisms. Through

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these steps, and depending on the balance among them, complex polymers are bioconverted to biogas. To study and detail the effects of short-chain fatty acids (SCFAs) on biogas production, this chapter will focus mostly on the first two steps, hydrolysis and acidogenesis processes, since they are the most important and responsible for SCFA formation.

The biogas quality is directly related to the final methane concentration formed by the anaerobic digestion process. On the other hand, methane formation is dependent on the presence of SCFAs, because they are the methanogenesis substrates. Several studies have already observed that in addition to dependence, methane production is also improved with higher concentrations of SCFA. This is mainly because methanogenic microorganisms become more active when there are increases in the SCFA concentration (Buyukkamaci and Filibeli 2004).

The straight SCFA amount influence on methane production has been widely studied, and most of these studies correlate methane production with the total concentration of SCFAs, which is also called as total volatile acidity (Anderson and Yang 1992).

In general, the influence of SCFAs on biogas production is not limited only to the amount that is present in the same medium during anaerobic digestion. Several studies show that there is a correlation among the quantity of SCFAs and other process variables associated with methane production, such as organic loading rate (OLR) (Rincón et al. 2008; Nagao et al. 2012; Jiang et al. 2013; Ferguson et al. 2016), pH (Zhang et al. 2009; Endres and Barberg 2007; Dai et al. 2013; Dong et al. 2016), and hydraulic retention time (HRT) (Dong et al. 2016; Koch et al. 2015, 2016). The temperature associated with SCFA concentration also influences methane production, in an indirect way, though. The decrease in temperature causes a decrease in microbiological activity in general, leading to an inhibition in all activities of anaerobic digestion steps and consequently in the methane production and biogas generation (Bowen et al. 2014).

Despite its relevance, the amount of SCFA may be inhibitory for anaerobic digestion, especially in imbalanced or low alkalinity systems. When there is an abrupt decrease in the methanogenic microorganism activity or SCFA production is somewhat uncontrolled, SCFA accumulation may occur in the medium, causing a reduction in the systems' pH. This pH reduction can reach critical acidity values and cause inhibition of the anaerobic digestion process. Irreversible inhibition levels could be reached, causing a system collapse (Chen et al. 2008; Intanoo et al. 2016).

Therefore, it is important to understand that the operational conditions, the key control factors, and its direct relationship with the SCFA concentration are determinant for the efficient biogas production and success of anaerobic digestion. SCFAs are substrate for biogas generation and concomitantly can cause imbalances in the buffer system medium until process inhibition, which justifies a better understanding of the SCFA mechanism formation, interferences, and functional microorganisms responsible for biochemical reactions, as we will see in the following topics.

9.2 Mechanism and Principle of SCFA Formation

The SCFA production process is performed by different microorganisms and goes through several transformations until its formation. The SCFA generation reaction can be described as below (Zygmunt and Banel 2009).

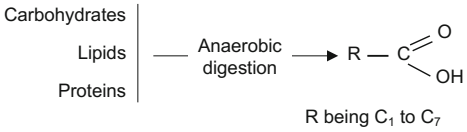

Figure 9.1 shows a graphical representation of the different steps of anaerobic digestion and its metabolic pathways in a simplified way. In the case of SCFA production process, the limiting step is represented by the hydrolysis, in which the majority of the complex and polymerized organic compounds must be degraded

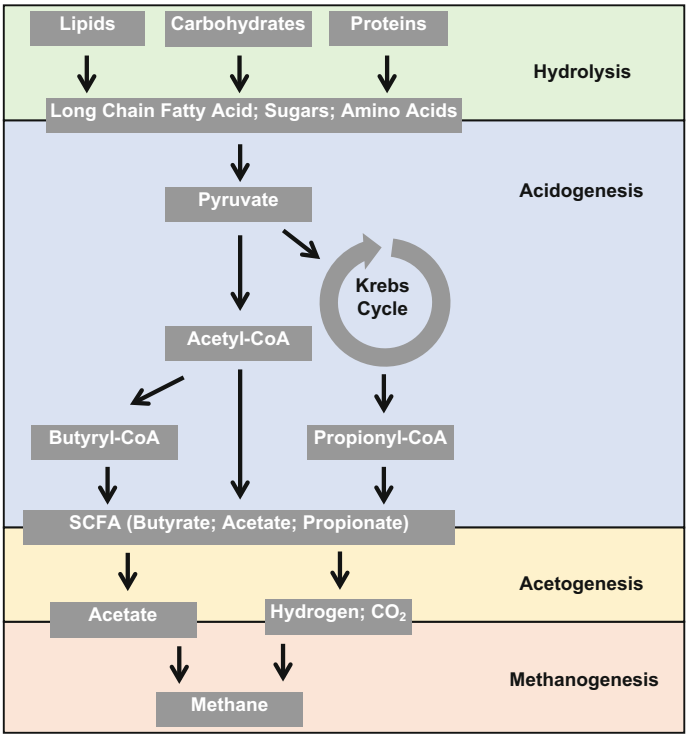

Fig. 9.1 Schematic pathway of anaerobic fermentation for SCFA production from metabolic pathway intermediates. In the diagram are indicated the four stages for the biogas formation (hydrolysis, acidogenesis, acetogenesis, and methanogenesis)

into simpler products. During hydrolysis, the hydrolytic bacteria, using extracellular enzymes, cleave organic compounds, such as cellulose, hemicellulose, starch, pectin, proteins, and lipids, to smaller compounds such as amino acids and volatile fatty acids (VFAs) also known as long-chain fatty acids, LCFA (Evans and Furlong 2011).

Subsequently, in the acidogenesis step (Fig. 9.1), these simplest organic compounds are transformed again. During acidogenesis process, sugars and the other compounds generated in the previous step, including long-chain fatty acids, and other low-molecular weight molecules are bioconverted to SCFA, CO_2, H_2, NH_3, SO_4^{-2}, and alcohols by the acidogenic bacteria activity. SCFAs are the major nutrients produced by acidogenic microorganisms in charge of solubilized hydrolysis product metabolization. The bacteria involved in this step proliferate very quickly, about 30–40 times faster than methanogens, and can survive in extreme conditions such as low pH, high temperatures, and high organic loads (Amani et al. 2010). SCFAs produced during acidogenesis include formic acid (CH_2O_2), acetic acid ($C_2H_4O_2$), propionic acid ($C_3H_6O_2$), butyric and iso-butyric acid ($C_4H_8O_2$), iso-valeric and valeric acid ($C_5H_{10}O_2$), caproic and iso-caproic acid ($C_6H_{12}O_2$), and heptanoic acid ($C_7H_{14}O_2$) (Tchobanoglous et al. 2013). Figure 9.1 shows the steps of biochemical conversion of the acidogenesis products into other compounds for the biogas production (acetogenesis and methanogenesis).

Despite the acidogens' adaptation ability to such harsh circumstances, it is extremely important to control and monitor the operational conditions to ensure the proper process progress. Acidogenesis is influenced by several factors such as substrate characteristics, environmental conditions, and other parameters such as temperature, pH, medium agitation, retention reactor time, and the organic volumetric load applied (Gavala et al. 2003; Jiang et al. 2013). Furthermore, an unbalanced environment is not favorable to methanogenesis, thus avoiding the consumption of the produced SCFA for methane formation (Zhang et al. 2009).

The phenomenon that interrelates the biochemical and microbial activities of acidogenic and methanogenic community, controlling the global function of the reaction, is known as syntrophy (McInerney et al. 2009). Considering a stable reactor operated under optimal conditions for microbial growth, in the absence of stress factors, acidogenic, acetogenic, and methanogenic steps should be synchronized, so that there will be an equilibrium in the intermediate compounds' production and consumption rates.

On the other hand, the accumulation of acetate and hydrogen in the dissolved phase could lead to thermodynamic inhibition of important metabolic conversions. The medium acidification resulted from SCFA accumulation contributes to alkalinity consumption, and pH decreases intensification (Yuan et al. 2015). This explains why volatile fatty acids, pH, and alkalinity are important indicators of anaerobic reactors stability.

Knowing the mechanisms involved in SCFA production and consumption as well as operation control strategies is the key for maintaining the system stability and process efficiency. These are topics that will be covered in the following topics.

9.3 Key Control Factors

Particular features, such as substrate and product composition, as well as bioreactor types generally drive the operation conditions for SCFA production. Regarding the bioreactors and the rate of biomass decomposition, Lee et al. (2014) recommended that the batch or semi-continuous operation mode is better than the continuous system for anaerobic sludge bed reactor (UASB), packed bed biofilm reactor (PBBR), and anaerobic fluidized bed reactor (AFBR).

Besides the operation mode, the ideal operating conditions such as pH, temperature, organic loading rate (OLR), and retention time vary largely according to substrate conditions and different types of bioreactor systems. Some specific actions, such as hydraulic flushing and sludge pre-treatment, can help in the bioreactor acidification process and hence maximizing the SCFA production through anaerobic digestion.

9.3.1 Substrate Influence on SCFA Generation

The rapid-to-moderate biodegradable matter content of matter, such as carbohydrates, proteins, and lipids, is related to the microorganisms' development and, therefore, affects qualitatively and quantitatively the intermediates and biogas production (Maciel and Jucá 2011). According to Zhang et al. (2012), the substrate volatile solids concentration (SV) also refers to its biodegradable organic matter. Therefore, solids characterization can be a reliable low-cost alternative parameter to evaluate a substrate SCFA and biogas potential generation.

Substrates rich in organic matter like waste-activated sludge (WAS) and primary sludge (PS) obtained from municipal wastewater treatment plant have been considered as potential sources for SCFA production due to the great volumes generated from biological wastewater treatment (Jiang et al. 2007a, b). Regardless the substrate organic matter concentration, an important reference to ensure the system balance is the ratio of SCFA $\times$ alkalinity in the medium which, according to Sánchez et al. (2005), should be around 0.1 and 0.5.

Table 9.1 shows the different SCFA compositions after the acidogenesis step according to the substrate type and characteristic. Wastes rich in protein, also meaning high nitrogen content, present a high organic matter, high biological oxygen demand (BOD), and low C/N ratio (Esposito et al. 2012). In general, acetic acid is the dominant SCFA. Authors show that the SCFA composition varies from 20 to 75% acetic acid, followed by propionic acid with concentrations varying between 3 and 35%, butyric acid between 8 and 35%, and other SCFAs to a lesser extent (Q. Yuan et al. 2011; Ucisik and Henze 2008).

Table 9.1 SCFA proportions in function of different substrate characteristics

	Acetic (%)	Propionic (%)	Butyric (%)	Other (%)	
Food waste	66.9	3.7	29.4	–	Jiang et al. (2013)
Food waste	37.6	20	32.1	10.3	Yin et al. (2014)
Waste-activated sludge	26–31	43–49	14–18	–	Ucisik and Henze, (2008)
Waste-activated sludge	24.7	14.3	24.6	36.4	Xiong et al. (2012)
Waste-activated sludge	41–69	8–29	9–21	11–15	Yuan et al. (2011)
Swine manure	56.7	19.7	11.8	11.8	Bortoli et al. (2013)
Dairy cattle manure	72.8	16.8	10.4	–	Patni and Jui (1985)
Sewage sludge	47.0	47.6	3.7	1.8	Buyukkamaci and Filibeli (2004)

9.3.2 Temperature

Temperature plays an important role on SCFA accumulation by anaerobic digestion. Q. Yuan et al. (2011) reported SCFA accumulation by waste-activated sludge (WAS) at distinct temperatures (4, 14, and 24.6 °C). The highest SCFA production (2154 mg/L) was achieved at 24.6 °C. Lower temperatures resulted in 782 mg/L (4 °C) and 2149 mg/L (14 °C) of SCFA. The yield of SCFA was also improved at psychrophilic and mesophilic temperature ranges (Q. Yuan et al. 2011; Zhuo et al. 2012).

The results obtained by these authors could be explained by the higher carbohydrates and protein solubility at high temperatures. The hydrolysis rate also increased at higher temperatures (Liu et al. 2012).

Some authors observed that temperatures variation during production process had no straight influence on the produced SCFA type. Q. Yuan et al. (2011) also verified that the composition of the SCFA produced at 4, 14, and 24.6 °C presented no significant modifications. However, the authors reported that mild temperature increases leaded to a reduction in the acetate production, while butyrate and propionate had its production improved in the same condition.

Zhuo et al. (2012) investigated the influence of temperature on ultrasonic pre-treated WAS cultivation at alkaline pH. The results indicated no significant variation in the SCFA is produced. In another study, the temperature increase up to 70 °C did not result in positive effect on SCFA production (Yu et al. 2013). On the other hand, Zhuo et al. (2012) reported a reduction of 40 °C in total SCFA production when the temperature decreased from 40 to 37 °C.

It should be highlighted that microorganisms in different waste materials will present significant differences in growth rate at diverse temperatures. In addition, the changes identification in distinct microorganisms' growth rates could be a promising investigation study in the temperature influence in SCFA production.

9.3.3 pH

The primary factor that directly affects the content of SCFA produced is the amount of organic matter being hydrolyzed. Just like substrate composition, pH has a significant function in the yield and production rate of SCFA in anaerobic digestion.

When different pH values were compared to test SCFAs accumulation in excess sludge, alkaline conditions (pH = 10) were able to maximize the SCFA content (Jie et al. 2014). This result was supported by another study (Wu et al. 2010) in which primary sludge was used in alkaline fermentation for SCFAs formation.

Acidogenic microorganisms are inhibited in low pH (below 3) or high pH (above 12) (Liu et al. 2012). Although it was mentioned above that the optimal pH for sludge hydrolysis was around 10, the waste source used may require a diverse pH value, which can vary from 5.25 to 11 (Lee et al. 2014). Anaerobic digestion of kitchen waste resulted in optimum pH of 7.0 (Tang et al. 2016) whereas for wastewater treatment the optimum pH was around 6.0 (Bengtsson et al. 2008).

Acidic or alkaline conditions can affect the acidogenic (Zhang et al. 2012) and methanogenic (Ghosh et al. 2000) microorganisms activity in SCFA production from WAS anaerobic fermentation. Yuan et al. (2006) showed that the accumulation of SCFA from WAS was greatly enhanced in alkaline systems (such as pH 10). Nevertheless, in the full-scale sludge treatment plant, the pH control during the whole process is still challenging. Kang et al. (2011) studied the effect of initial pH adjustment on sludge hydrolysis and acidification by ultrasonic pre-treatment.

The highest SCFA concentration in anaerobic digestion by the excess of sludge can also be determined by fermentation with inoculum and the reactor hydraulic retention time (HRT), resulting in changes at the optimum pH of the process. For example, Wang et al. (2014) investigated the influence of pH on different inoculum types, in eight different batch bioreactors, over 20 days of fermentation. The results indicated the maximum yield (918 mg/g VSS removal) and concentration (51.3 g-COD/L) for SCFA at pH of 6.0.

For SCFA generation, the SCFA to soluble chemical oxygen demand (SCOD) ratio is referred to the number of soluble components converted into SCFAs (Jiang et al. 2013). Investigations showed that applying a pH range from 5.0 to 6.0 resulted in the highest ratio value of SCFA to SCOD (75%), regardless of the type of inoculum used while producing SCFA from food waste. Nevertheless, this study did not include the results concerning an extreme alkaline level (pH > 10) (Wang et al. 2014).

Zhao et al. (2015) showed that an initial pH of 11 was the optimum condition for SCFA accumulation. Furthermore, the authors verified that the activity of specific enzymes for SCFA generation at alkaline conditions was higher than in acidic or neutral pH.

Although the composition of SCFA produced primarily depends on the substrate composition, changes in pH levels can also affect the SCFA produced at acidogenic fermentation (Lee et al. 2014). Prior to the selective production of any specific SCFA, the optimum pH value needs to be determined.

9.3.4 Retention Time

In anaerobic digestion using waste materials, the microorganisms and the retention time in the reactor are considered as key control factors of the process. Retention time refers to solid retention time (SRT) and HRT to the allocated time for selected prevalent microorganisms and the reactor volume, respectively. SCFA production is affected more by the HRT than to the temperature during the fermentation (Kim et al. 2013).

High HRT provides enough time for the acidogenic bacteria to convert the waste into a soluble component, and consequently, it favors the SCFA yield (Bengtsson et al. 2008). In terms of system, the HRT depends on the composition and type of substrate. In anaerobic LBR digesting a substrate with a high solid matter, an HRT of 1.5 days was used for SCFA accumulation (Cysneiros et al. 2012). In other studies, an HRT of 1.9 days was the best performance for the acidogenic step of the organic fraction of municipal solid waste (OFMSW) (Romero Aguilar et al. 2013).

Prolonged HRT reached to SCFA accumulation. An investigation produced SCFA by acidogenic food fermentation (Lim et al. 2008). The results showed that the SCFA production was higher as the HRT increased up to 192 h, but no further increment in SCFA content was observed until 288 h.

It has been also verified that the methanogenic microorganism's growth rate is slower when compared to the acidogens' growth rate. Low SRT does not let sufficient time for the methanogenic microorganisms to consume SCFA producing carbon dioxide (CO_2) and methane (CH_4) (Lee et al. 2014). The acidogenic microorganisms also require a minimum SRT to hydrolyze the substrate. A long SRT provides enough time to the methanogenic microorganisms activity and consequently improve the biogas production. Wastewater treatment system using submerged anaerobic membrane bioreactors (SAnMBRs) has an SRT going from one to three months (Huang et al. 2013; Khan et al. 2016).

9.3.5 Organic Loading Rate

The OLR is affected by the substrate type and composition of as long as the bioreactor arrangement. Yet, no direct relation has been reported concerning the production rate or yield of SCFA by changing the OLR. Meanwhile, the bias of SCFA production could be foreseen by changes in the OLR (Khan et al. 2016).

Lactic acid fermentation shows that the amount of lactic acid increased raising the OLR. The lactic acid content reached 37.6 g/L at the same time as the OLR rose up to 18 g-TS/L/day). In contrast, the increment of the OLR until 22 g-TS/L/day resulted in a decrease of acid production to 22 g-TS/L/day. These outcomes could be related to the reduction of the hydrolysis rate if the OLR exceeds the optimum level (Tang et al. 2016).

A fermentation research is tested an olive oil mill solid waste (OMW) and different OLRs. In this study, at OLR of 12.9 g-COD/L/day the production of SCFA was maximized (Rincón et al. 2008). A similar trend was observed using food waste as substrate. An increment in the SCFA generation was obtained at an OLR up to 13 g/L/day; however, above this rate the bioreactor became inconstant. Summarizing, the SCFA production was enhanced by the initial increment in OLR and the production rate decreased when OLR was raised later in the process, regardless of the substrate and its composition (Lim et al. 2008). Nevertheless, additional studies are required to characterize the optimum range in OLR together with substrate used and the type of bioreactor.

9.3.6 Bioreactors for SCFA Production

The mechanisms for SCFA production most used are suspended and attached growth (Tchobanoglous et al. 2013). Both of these types of growth technologies have been used in several bioreactor models. In packed bed bioreactor (PBR), cell mass is attached to packing material but can implicate in clogging. The fluidized bed bioreactor (FBR) is an efficient alternative to prevent the clogging issue, and the cell mass grows linked to a small solid-like sand keeping in suspension by the upstairs fluid movement (Grady et al. 2011). Moreover, the continuous stirred tank reactor (CSTR) is the ideal reactor for completely mixing waste and microorganism cells.

In order to produce SCFAs, reactors can be projected to produce SCFA as primary products (Wang et al. 2014) or as by-products (Peces et al. 2016). Several bioreactors have been designed and afforded promisor SCFA production such as sequencing batch reactor (SBR) (Frison et al. 2013), anaerobic leach bed reactor (LBR) (Cysneiros et al. 2012), packed bed biofilm reactor (PBBR) (Scoma et al. 2016), continuous flow reactor (J. Luo et al. 2014), CSTR (Bengtsson et al. 2008), expanded granular sludge bed (EGSB) (D. Zhang et al. 2011), and two-stage thermophilic anaerobic membrane bioreactor (TS-TAnMBR) (Wijekoon et al. 2011).

Anaerobic fermentation using membrane bioreactor (MBR) for SCFA accumulation has attracted a lot of attention, aiming to produce value-added products and reduce the volume of waste simultaneously (Zhao et al. 2015). In 2017, the global MBR market was around USD 1.54 billion, and by 2026, it is expected to come to USD 5.59 billion (Credence Research Inc 2018).

9.3.7 Other Parameters

Besides the process optimization parameters, other measures can improve the SCFA production rate and yield. Some studies indicated that the hydraulic flush rises the SCFA production by 32% in buffered LBRs where digested substrate was used with high solids matter (Cysneiros et al. 2012).

Feng et al. (2009) reported that the content and composition of SCFA are affected by the carbon to nitrogen ratio (C/N) of the substrate. Moreover, chemical additives can increase significantly the SCFA production. The addition of biosurfactant, for instance, can significantly increase the SCFA content. Huang et al. (2015) reported that the use of surfactin or rhamnolipid can significantly improve the SCFA concentration alkyl polyglycoside (APG seems to be a promissory for SCFA production from WAS). Zhao et al. (2015) related a production of SCFA up to 280 mg of chemical oxygen demand (COD) per gram of volatile suspended solids (VSS) using the biosurfactant APG in a concentration of 0.2 g/g dry sludge (DS) in a MBR sludge anaerobic fermentation. Furthermore, APG can also reduce the fermentation time.

Ji et al. (2010) observed a hydrolysis increase when the surfactant sodium dodecylbenzene sulfonate (SDBS) was added to the mixture of WAS and PS. The increment of the sludge hydrolysis reached higher SCFA accumulation. The addition of SCFA also affects its composition, but as this occurs it is not yet elucidated.

Sodium dodecyl sulfate (SDS) is an anionic surfactant effectively used to speed up the solubilization of WAS and increase the concentrations of proteins and carbohydrates in the aqueous phase. Jiang et al. (2007a, b) related that the SCFA accumulation increased significantly using SDS. Using 0.1 g/g of SDS, a total SCFA concentration of 2243.04 mg COD/L was obtained. Luo et al. (2011) studied the combined effect of enzymes (neuter protease and a-amylase) and SDS on the acidification and hydrolysis of WAS. The authors observed that the composition of the SCFAs produced in SDS plus enzyme systems suggested that acetic acid, propionic acid, and iso-valeric acid were the main SCFAs in WAS hydrolysate.

9.4 Functional Microorganisms Involved

The accumulation of short-chain fatty acids (SCFAs) in sewage sludge systems can be increased when the anaerobic digestion process occurs under alkaline conditions. However, it is observed that temperature variations under different pH conditions result in different levels of SCFA, as well as the constitution of the local microbiota. Analysis with fluorescence in situ hybridization (FISH) and PCR-based 16S rRNA gene clone library have identified that the ratio of bacteria to archaea as well as bacteria producing SCFA change in the following ratio: 20:1 (ambient temperature without pH control); 68:1 (ambient temperature at pH 10); 101:1 (mesophilic at pH 9) and 177:1 (thermophilic at pH 8) (Zhang et al. 2010).

Mostly, anaerobic bacteria producing SCFA are members of the Clostridia class, which belong to Firmicutes phylum. In digesters under thermophilic and mesophilic conditions supplied with corn silage, cattle, or pig manure, it was possible to isolate acetic, propionic, and butyric acid forming bacteria. The great majority of isolated bacteria were related to the genus *Clostridium*, e.g., *C. thermoamylovorans, C. sporosphaeroides, C. aminovalericum,* and *C. cochlearium/C. tetani* (Cibis et al. 2016). Acetic acid is produced anaerobically by these microorganisms via the glycolytic pathway and pyruvate intermediates. It is usually a co-product of bacteria such as *Clostridium* and *Propionibacterium* genus that synthesize propionic and butyric acid as major compounds (Baumann and Westermann 2016).

Propionibacterium species utilize the glycolytic pathway and pyruvate to synthesize butyric acid and also succinate intermediates to produce propionic acid (Fig. 9.2) (Wang et al. 2013; Rogers 2006; Dahiya et al. 2018).

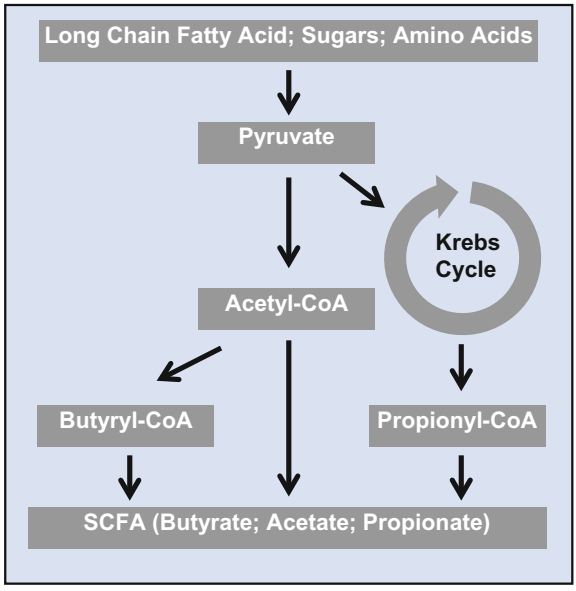

Fig. 9.2 Schematic pathway of short-chain fatty acids production from metabolic pathway intermediates

As previously mentioned, although it is possible to increase the SCFA concentration—especially acetic, propionic, and butyric acid—the increase and accumulation of these substances can result in a reduction of pH, damaging the whole system. An alternative would be blocking the supply of new substrates, which is often not the best option, since optimization for continuous system operation is more advantageous. The separation of acidogenesis and methanogenesis may be an alternative to control the acidification process and consequently prevent the reduction of the population of methanogenic microorganisms during the methane generation stage (Fu et al. 2017).

A study from India demonstrated to be possible to obtain an enriched inoculant from a wastewater bioreactor residue. The use of this inoculant in an anaerobic digestion system allowed an increase in the production of acetic acid and SCFA from food residues adjusted to pH 10. Selective enrichment of the inoculant microbiome was obtained by means of acidic shocks and the operation of the system at alkaline pH. This pre-treatment induced the prevalence of acidic Firmicutes spores and fatty acid-forming Bacteroidetes which together with saccharolytic (*Soehngenia saccharolytica*) and proteolytic bacteria (*Bacillus cellulosilyticus*) induced the effective digestion of complex carbohydrates. Alkaline biodigestion seems to benefit the phosphoroclastic pathway by enhancing the production of acetate and H_2 by the microorganisms, since the pre-treatment of the inoculant agent promotes the acidogenic pathway with parallel inhibition of the methanogenic bacteria without affecting the H_2-producing bacteria (Sarkar et al. 2016).

Proteomic and sequencing analyses of the 16S-rDNA region in a biodigestor containing grass identified a high activity of microorganisms using sugars and producing SCFA in the acidogenic phase. The study was conducted in a two-stage production system under mesophilic conditions (37 °C) and thermophilic conditions (55 °C). In the samples of the mesophilic biodigestor, the prevalence of microorganisms of Bacteroidetes and Firmicutes phyla was observed, producing glycolytic proteins associated with degradation and catabolism of sugars. Among the 1700 proteins quantified at this stage, approximately 120 were expressed at different levels when compared to the 5 thermophilic proteins. However, thermophilic proteins have a high degree of representativity of chaperones, which makes them more stable and suggests their fundamental role in stability on stressful conditions of biodigestion (Abendroth et al. 2017).

Although biodigestion for biogas production can be carried out with different types of plant biomasses, a predominance of Firmicutes during the acidogenesis phase can be observed in several studies. This bacterial phylum has a great enzymatic versatility, mainly related to the decomposition of xylans, among them xylanases, xylosidases, and cellulases (Hassa et al. 2018). However, what is observed in a biomass degradation system is a microbial consortium acting together, some secreting free enzymes with different substrate specificities and others, such as the bacterium *Clostridium thermocellum*, constitute a group that secretes subunits of cellulases and xylanases in large multienzymatic complexes known as cellulosomes (Herpoël-Gimbert et al. 2008; Raman et al. 2009).

Metagenomic studies in a microbial consortium called EMSD5 of corn straw identified a population composed mainly of members of the phylum Proteobacteria, Bacteroidetes, and Firmicutes. In the last group, synergistic population activity was attributed to the degradation of polysaccharides in plants, mainly by the expression of xylan-degrading enzymes, including xylanases, β-xylosidases, α-L-arabinofuranosidases, α-glucuronidases, and acetyl xylan esterases. The synthesis of these enzymes is associated with the generation of most SCFA. The study of the microbial composition in a substrate and the association of the enzymes synthesized by these microorganisms are extremely important for the understanding of regulating and optimizing mechanisms in the production of SCFA in acidogenesis.

Although bacteria and archaea are the main constituents of the microbiome in a biodigestor, there are studies suggesting that fungi, although in a smaller amount, have a relevant role in the biodigestion process. Due to its excellent adaptability and versatility in the production of different enzymes, fungi can contribute to the initial hydrolysis process in a biogas reactor. *Saccharomyces cerevisiae* is known to increase the number of viable total cells of cellulolytic bacteria in the cow rumen (Lila et al. 2004). It is believed that this effect can occur in a biodigestor, where the yeast could contribute to increase the number of cellulolytic bacteria optimizing the cellulose degradation process from the plant biomass. This fungus has already been found in a bioreactor containing substrates with more than one year of activity (Bengelsdorf et al. 2012).

Fungi can also be used as pre-treatments in processes to accelerate the decomposition of lignocellulose. Aerobic digestion may be employed in this process, since this constituent of the plant biomass is composed of recalcitrant material, such as cellulose (40–50%), hemicellulose (20–40%), and lignin (5–30%). The use of fungi in pre-treatments of biomass is an alternative to existing physical treatments that present high costs, and can also be a substitute for chemical treatments, which are known to generate toxic products in the digested material inhibiting the microbial activity in the steps of hydrolysis and fermentation.

The use of the fungus *Trichoderma viridae* as aerobic pre-treatment in a municipal waste substrate (plant material, paper, coffee beans, tree debris) resulted in a threefold increase in methane production using a laboratory scale. The availability of nutrients promoted by the cellulolytic enzymatic action of the fungus favors the continuity of the anaerobic digestion process, allowing a greater hydrolytic activity promoted by a bacterial consortium. However, the presence of high moisture content for cellulases is essential in lignocellulosic substrates. The low moisture content inhibits cellulase production due to the lower solubility index of the substrates, preventing swelling and promoting high water stress (Kalogeris et al. 2003; Ghanem et al. 2000).

Acidogenesis is a fundamental step of anaerobic digestion; it is at this stage that the greatest amplitude of microbial diversity occurs, since there is the joint work with the microorganisms involved with the hydrolysis. Factors such as the origin and product resulting from the hydrolysis, pH, temperature, and composition/capacity of the enzymatic activity produced by the microbial consortium end up limiting the subsequent step influencing the quality and quantity of biogas.

After all the information presented in this chapter, it is expected that the effect of SCFA formation and its interference on biogas production has been clarified. This is a small and important part of a larger anaerobic digestion process, which needs more advanced research and still has unresolved challenges. The search for new alternative energy sources and the vision of future development should be a scientific priority in the next decades. About SCFA, controlling their production with the aforementioned alternatives, it is possible to overcome the technological challenges and to improve biogas production.

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Chapter 10
Positive Impact of Biogas Chain on GHG Reduction

María Cruz García-González, David Hernández, Beatriz Molinuevo-Salces and Berta Riaño

Abstract Nowadays, it is a well-accepted fact that greenhouse gases (GHG) contribute to the global warming of the planet and that they are a very real and very serious threat to the whole world. It is estimated that 10% of total GHG emitted is from sources in the agricultural sector and over 3% from waste management. Most countries agreed to reduce GHG emissions through the mitigation of GHG sources and application of technologies to stop global warming; however, there is much work to do as GHG are increasing every year. Among these technologies, anaerobic digestion appears as a well-established technology in most countries that can contribute to mitigate GHG emissions from organic wastes. Capture of these gases from uncontrolled organic wastes processes from municipal solid wastes, human excreta, wastewaters, tanneries, distilleries and other industries discharged in public swears is necessary to reduce these emissions and to profit methane from this biogas; otherwise, they are a source of fugitive GHG contributing to the global warming. Anaerobic digestion has the potential for global warming savings, due to the potential substitution of fossil fuel by biogas, also from carbon storage in soil and inorganic fertilizer substitution through use of the digestate as a fertilizer.

Keywords Global warming · Sustainability · Anaerobic digestion · Greenhouse gases · Organic wastes

10.1 Introduction

The United Nations Framework Convention on Climate Change (UNFCCC) is the main legal instrument for international response to the challenge of climate change that seeks to stabilize the concentrations of greenhouse gases (GHG) in the atmosphere to prevent dangerous anthropogenic disturbances in the climate system. To ensure the continuity of the efforts made with the Kyoto Protocol after 2020,

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the 195 member countries of the UNFCCC approved on December 2015 the Paris Agreement, which establishes measures to reduce GHG emissions through the mitigation, adaptation and resilience of ecosystems for the purpose of global warming.

According to the European Environment Agency, in 2015 total GHG emissions (excluding land use, land use change and forestry, in the EU-28 plus Iceland), amounted to 4317 million tonnes CO_2 equivalent (including indirect CO_2 emissions). Over 54% of the total was emitted from fuel combustion and fugitive emissions from fuels, over 24% from the transport sector, 8% from industrial processes and product use, 10% from sources in the agricultural sector (fuels and biomass burning, organic matter decomposition, soil tillage, etc.), and over 3% from waste management (Fig. 10.1) (Eurostat 2018). In the case of the energy sector, the most important energy-related gas is CO_2 that makes up 75%, followed by CH_4 that is responsible for 2% and N_2O for 1% of the total GHG emissions. Regarding the agricultural sector, contributions from CH_4, N_2O, and CO_2 of 242, 185 and 10.3 Mt CO_2-eq, respectively, represented 5.6, 4.3 and 0.24% of the GHG emissions, respectively. And finally, in the industrial processes and product use sector the most important GHGs are CO_2 (6% of total GHG emissions), HFCs (3%) and N_2O (0.3%) (EEA 2017). As indicated, the main GHG related to the agricultural sector are carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O). The effect of each on climate change depends on three main factors: concentration or abundance, residence time in the atmosphere and the impact strength in the atmosphere. For each GHG, a global warming potential (GWP) has been calculated to reflect how long it remains in the atmosphere and how strongly it absorbs energy. Gases with a higher GWP absorb more energy and thus contribute more to warming Earth. In spite of presenting the lowest GWP of 1, carbon dioxide has the highest direct warming impact because its concentration and the emitted quantities are much higher than that of the other gases. Methane is the second most important greenhouse gas, with a GWP of 23. Once emitted, methane remains in the atmosphere for approximately 9–15 years. Nitrous oxide is present in the atmosphere in extremely small amounts; however, its GWP is of 296 and has a very long atmospheric lifetime (114 years) (Steinfeld et al. 2006).

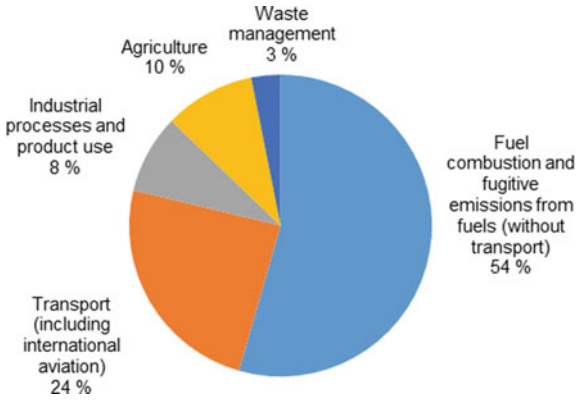

Fig. 10.1 Main contributors to GHG emissions in the EU-28. *Source* Eurostat (2018)

Livestock activities that produce large amounts of animal manure and slurries, as well as anthropogenic activities that produce wet and dry organic waste streams, emit considerable amounts of these three gases, and therefore, representing a constant pollution risk with a negative impact on the environment, human and animal health and food safety. To prevent emissions of GHG and leaching of nutrients and organic matter to the natural environment, it is necessary to close the loops from production to utilization by optimal recycling measures (Holm-Nielsen et al. 2009). One of these strategic measures is the application of the anaerobic digestion (AD) process to convert organic residues into energy and fertilizers, and therefore, to prevent GHG emissions.

According to different authors, AD contributes to GHG emissions, mainly from use of fossil energy at the facility, emissions from the bioreactor and combustion of biogas, and emissions from the digestate when applied to soil (Fig. 10.2) (Møller et al. 2009). However, AD also has the potential for global warming savings, especially from substitution of fossil fuel by biogas, also from carbon storage in soil and inorganic fertilizer substitution through use of the digestate as a fertilizer (Møller et al. 2009), and eliminating uncontrolled fugitive CH_4 emissions from stored wastes, as manure (Riaño and García-González 2015) or landfill (Yoshida et al. 2012).

In this chapter, the positive impact of the AD process on GHG emissions mitigation is described. The chapter is mainly focused on the treatment of organic wastes, which include animal manure and slurries, as well as wastewater and organic waste from municipal wastes. It describes the contribution of AD to emissions mitigation through renewable energy production, as well as different sources of fugitive GHG emissions related to organic waste degradation, how AD contributes to GHG emissions reduction compared with other technologies, some strategies to increase GHG mitigation during AD and, finally, the role of digestate management on GHG reduction.

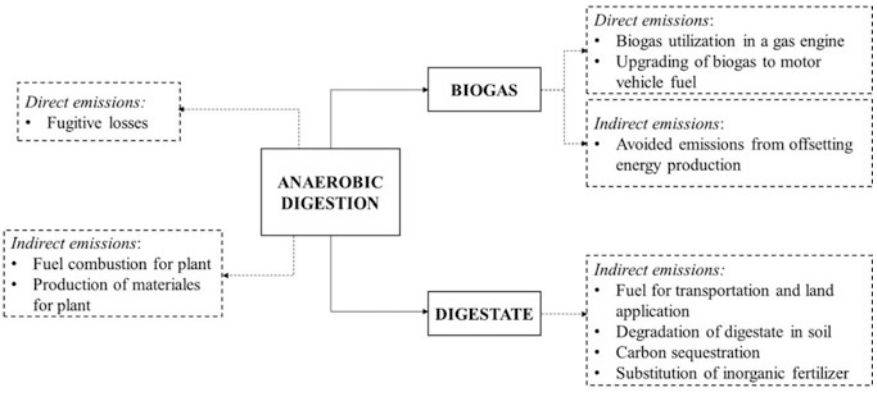

Fig. 10.2 Direct and indirect emissions from a biogas plant. Adapted from Møller et al. (2009)

10.2 Emission Mitigation Potential Through Renewable Energy Production

The need of GHG emissions mitigation is not only to create a sustainable environment but also to build a sustainable economy through renewable energy resources (IPCC 2014). As fuel combustion and fugitive emissions from fuels, as well as transport, play an important role as main contributors to GHG emissions, energetic alternatives must be developed and applied. In this sense, as transportation of people and goods play the most important role in CO_2 emissions, the EU has proposed a decrease in CO_2 emissions from vehicles of 30% by 2030 (European Commission 2017) by accelerating the uptake of zero and low emission vehicles (less than 50 g CO_2/km). The implication of governments and regional politics is essential to develop fossil-independent fuels like biogas. A successful example of the implementation of biogas technology for public transportation (mainly bus) has been performed in many cities, allowing the maturation of this technology (Ammenberg et al. 2018; IPCC 2014).

As mentioned, biogas from AD can be used for vehicles specially adapted to the use of methane, but a biogas upgrading step must be performed to separate methane from hydrogen sulphide, water and CO_2. Currently, biomethane is widely used for buses and heavy vehicles and different projects worldwide are performed to upgrade biogas and transform it in the main transportation biofuel (Holm-Nielsen et al. 2009; Ammenberg et al. 2018). From a theoretical point of view, biogas production may be tenfold higher than current production if food waste, agriculture, energy crops and industrial residues were utilized to produce biogas (IPCC 2014).

Also, hybrid vehicles equipped with both conventional (internal combustion) and electric engines may be an interesting alternative to decrease GHG emissions. However, most of the hybrid vehicles are based on petrol-fuels; hence, the use of a biogas-hybrid vehicle may result in an environmentally friendly alternative. On the other hand, electric vehicles do not emit GHG to the atmosphere and may be an interesting alternative to conventional cars, but currently an important amount of this electric energy is produced from fuel-based power plants. In this sense, a combination of non-fuel-based power plants and electric vehicles may be a suitable option to get zero-emission cars. Hence, there is a big potential for decreasing CO_2 emissions in the transport sector (European Commission 2017).

Biogas may be also used to produce electricity. In the EU, electricity from biogas increased from 3 GW in 2005 to 10 GW in 2015 (Scarlat et al. 2015). The main electricity producers from biogas are Germany, Italy and Czech Republic with 53, 13 and 4%, respectively (International Energy Agency 2016). Differences between them are remarkable, since Germany has favoured electricity production and biogas installation through positive policies, while supports in other EU countries are lower. In this manner, the main bottleneck for the electricity

production from biogas in the EU is political issues (Scarlat et al. 2015). The reduction in support for biogas and the increase in costs to inject electricity into power grids have led to biogas producers to use the heat and electricity in their exploitations undersizing their biogas installations in order to avoid electricity surplus. However, despite the lack of tax incentives or bonuses, significant progress in installed electric capacity from biogas plants is expected as it is shown in Fig. 10.3 (Scarlat et al. 2015).

The worldwide share of biogas used as vehicular fuel is still very low (<1%) (Sahota et al. 2018), which is basically due to high operation costs and high energy consumption of the upgrading processes. In this sense, there is a need of establishing new biogas upgrading technologies and optimization of the older ones to reduce operation costs and consume less energy in the biogas creation (Sahota et al. 2018). Although in Europe, more than 90% of biogas produced is used for electricity generation, there is a tendency in biogas plants to upgrade their biogas instead of finding local sources for biogas consumption (Skovsgaard and Jensen 2018). The upgraded biomethane can be used as a substitute for natural gas, if the final composition meets the natural gas quality standards (Sahota et al. 2018). Therefore, the most promising future of biogas is to replace natural gas in its multiple uses.

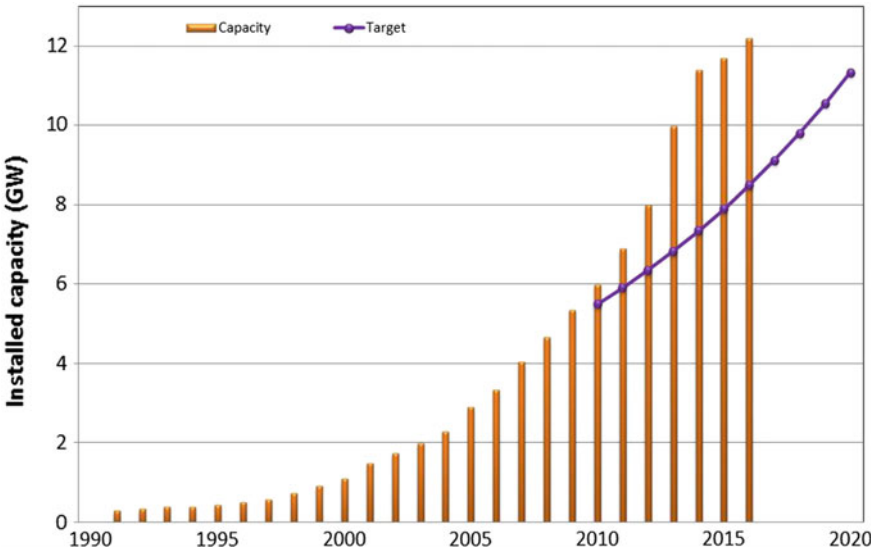

Fig. 10.3 Evolution of installed electricity from biogas plants (blue colour) and targets in the European Union (red colour). Adapted from Scarlat et al. (2018)

10.3 Fugitive GHG from Non-controlled Organic Waste Degradation

The main human-related sources of non-controlled GHG emissions are coal mining, production of natural gas and oil, livestock enteric fermentation, landfills, manure management, wastewater treatment and agriculture. Among these activities, only landfills, manure management and wastewater treatment will provide opportunities to reduce fugitive biogas emissions and to capture much of the generated biogas for its use as energy source (Abbasi et al. 2012), as the AD technology is already well-established in most countries. In this section, an overview of the fugitive GHG from landfills and manure management will be presented, as wastewater treatment is developed in Sects. 10.4 and 10.5.

10.3.1 Landfill

Landfilling is an important component of municipal waste management, a very common practice in many cities around the world. Methane and CO_2 are generated in landfills and open dumps as they are by-products of anaerobic decomposition of organic waste. The main components in landfilling are CH_4 (55–60%), carbon dioxide (40–45%) and N_2O (<5%) (Scheutz et al. 2009). Several factors influence methane generation in landfills. They include composition of the waste and availability of readily biodegradable organic matter, the age of the waste, moisture content, pH and temperature (Machado et al. 2009), as well as the design and management practices at the site (Abbasi et al. 2012). The processes that lead to the formation of landfill gas are bacterial decomposition, volatilization and chemical reactions.

Waste management is one of the main social and economic challenges, since it is estimated that production of municipal solid waste (MSW) in the world will double in next years, increasing from 1.32 billion tonnes per year in 2010 to 2.2 billion tonnes per year in 2025 (Pawlowska 2014). In 2014, 47.4% of the residues from EU-28 countries were deposited in landfills, being most of them open to the atmosphere, while 36.2% of wastes were recycled (Eurostat 2014). In the case of the USA, 54% of total municipal waste generated was discarded to landfill in the year 2012 (USEPA 2015). It is estimated that methane from the MSW landfills represents over 12% of total global CH_4 emissions (USEPA 2006). It amounts to over 730 million metric tonnes of CO_2 equivalent ($MtCO_2eq$). Countries like USA, Africa, Eastern Europe and China together account for 42% of the world's CH_4 emissions, and it is expected to reduce these emissions in next years due to specific regulations; however, in countries as India and Eastern Europe a steady growth in landfill CH_4 emissions is expected (Abbasi et al. 2012). This will be caused by the increase in municipal waste disposal, but also by the increasing proportion of biodegradable fraction of these wastes.

To control landfill emissions, three main strategies may be carried out: (1) To decrease the total amount of degradable waste left on the landfill; (2) to accelerate the AD process under controlled conditions (inoculation of anaerobic bacteria, temperature optimization, watering the landfill waste and diminishing oxygen concentration); (3) to collect and to burn the biogas produced. After biogas burning, emissions may be minimized by appropriate barriers. These practices will minimize the landfill impact on the atmosphere (Pawlowska 2014).

Collecting the biogas produced for its further use is the most spread strategy, avoiding its release from the landfills (Fig. 10.4). For that, perforated plastic pipes of about 15 cm in diameter are installed in the landfill. They are packed in gravel, and the pipe and the gravel are further enclosed in larger pipes. This is done to prevent refuse from plugging the perforations. A network of such extraction wells is installed across the landfill. Gas extraction can also be done by drilling boreholes in the landfill and installing extraction pipes. The individual gas wells are connected by a series of pipes leading to larger pipes that deliver the gas to the processing and conversion stations. The entire piping system is under a partial vacuum created by blowers or fans at the processing station, causing landfill gas to migrate towards the wells (Abbasi et al. 2012).

Although landfills are fugitive sources of GHG emissions, they still remain a good alternative for municipal waste disposal because of their simplicity and versatility, and as mentioned, there are strategies and technologies to control these emissions. More information about the control of GHG emissions from landfill sites can be found in Sect. 10.5. Recycling of non-renewable raw materials will prevent its disposal in landfills, as well as applying technologies to recover organic matter and nutrients from organic wastes.

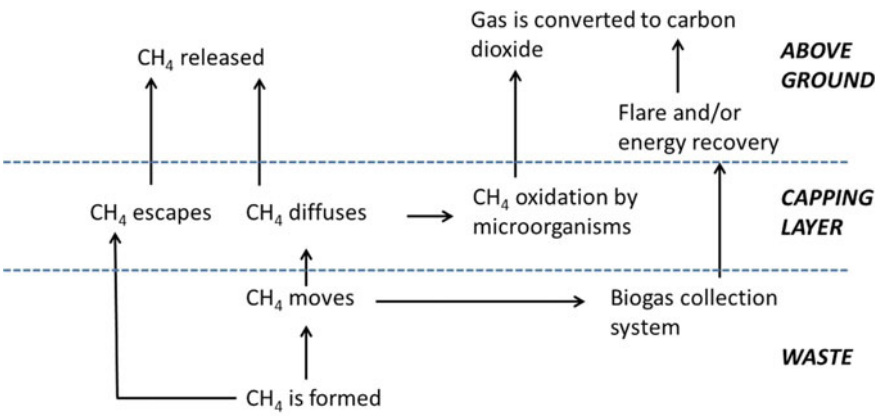

Fig. 10.4 Biogas release pathways in landfills. Adapted from Abbasi et al. (2012)

10.3.2 Manure Storage and Management

The handling and use of manure on livestock farms contribute to emissions of the GHG CH_4 and N_2O, especially with liquid manure management (Petersen 2018). GHG emissions from livestock vary by animal type and growth stage due to different diets, feed conversion mechanisms, while GHG emissions from storage and treatment of manure depend on the type of storage, duration of storage, ambient temperature and manure management practices (Chadwick et al. 2000; Borhan et al. 2012).

Most of the manure is collected in storage/treatment structures or left to decompose in the open, which poses a significant environmental hazard (Borhan et al. 2012), being therefore a fugitive emissions source. GHG emissions from animal houses are influenced by house ventilation, ambient temperature, floor type and existence and type of bedding material. In pig houses with natural ventilation, CH_4 emissions are significantly lower than in houses with forced ventilation due to lower temperature in the first ones. Concerning floor type, emissions from deep litter in pig houses are lower than those from pig houses with slats and slurry tanks (Sommer et al. 2013). Regarding N_2O, as manure remains in an anaerobic ambient there is little opportunity for the ammonia to be nitrified, and thus N_2O may theoretically be produced at the air–liquid interface of stored slurry or on slats and solid floors where urine and faeces are deposited. Emissions of N_2O may be affected by TAN concentration and pH, since high NH_3(aq) concentrations inhibit nitrification (Sommer et al. 2013). However, in houses with deep litter systems N_2O emissions will vary depending on air exchange in the deep litter.

In outside slurry stores, CH_4 emissions vary over the year due to temperature variations and management practices (Sommer et al. 2009). In those countries where slurry stores are emptied in spring, only small amounts of slurry are exposed to high temperatures during summer, whereas in countries where slurry is stored in lagoons for years, emissions may be higher. Emissions may also be higher from lagoons that are not stirred, because dry matter settles out to the bottom and is seldom removed from the lagoons (Sommer et al. 2013). Usually, manure stored outside is under anaerobic conditions, thus emissions of N_2O via nitrification and denitrification without a floating cover are insignificant (Sommer et al. 2000). However, a natural or artificial surface crust on top of the stored manure can create anaerobic and aerobic conditions, thus creating an environment where N_2O can be produced (VanderZaag et al. 2009).

Depending on the management system employed to process manure, GHG emissions will differ significantly. Therefore, strategies for mitigating net GHG emissions should be aimed to manipulate manure properties or the conditions under which CH_4 and N_2O are produced and utilized during manure storage and treatment. However, GHG mitigation options are critical and depend on several factors, which are economic, technical and material resources, climatic conditions, existing manure management practices, bioenergy sources, and a source of high-quality fertilizer and soil amendments (Borhan et al. 2012).

According to Misselbrook et al. (2016), GHG mitigation techniques for slurry storage include promotion and capture of CH_4 in purpose-built anaerobic digestion plants, slurry crusting or covering the slurry surface with a floating material and slurry acidification. Methane produced by bacteria under stored conditions is transferred to the air above the slurry surface through ebullition. Therefore, surface crusts and coverings may provide the opportunity for CH_4 oxidation to CO_2, thereby reducing emissions (Husted 1994; Petersen et al. 2005; Qi et al. 2015; Sommer et al. 2000); as methanotrophs have been identified in slurry surface crusts (Duan et al. 2014). However, CH_4 can escape from the slurry through cracks or breaks in the covers, minimizing the oxidation of CH_4 (Petersen et al. 2013).

Manure management includes land application. In this case, emissions of CH_4 occur immediately after manure application to land and are usually short-lived, as oxygen diffusion into the manure inhibits CH_4 formation. Methane emitted immediately after application of manure to land is CH_4 trapped within the manure, having been generated during its storage (Sommer et al. 2013). After application of manure to soil, organic matter is mineralized forming ammonia that may be subjected to nitrification forming NO_3^-. Besides, O_2 demand increases and O_2 supply reduces in the soil, which affects the potential for N_2O emissions because the production is determined by the balance between O_2 demand and O_2 supply, rather than by O_2 supply alone. The effect of slurry will depend on soil conditions at the time of application. For example, in a dry soil increase in N_2O can be expected after slurry application, because slurry with a high content of degradable volatile solids increases O_2 demand and much more N_2O is produced. In this case, reducing degradable volatile solids content of manure through AD will reduce N_2O emissions (Sommer et al. 2013). More information on this subject is provided in Sects. 10.5 and 10.6.

10.4 Reduction of GHG During Anaerobic Digestion Compared with Other Technologies

Recent works about GHG emissions show that industrial and domestic wastewater treatment plants (WWTP) are anthropogenic GHG potential sources. Wastewater and organic wastes treatment can contribute to greenhouse gases through production of CH_4, CO_2 or N_2O from treatment processes as well as CO_2 produced from the energy required for treatment. Therefore, they contribute to the climate change and air pollution. The increasing interest towards climate change has led to the development of new tools for wastewater and organic wastes treatment plants design and management. Anaerobic digestion treatment plants are among these tools, which according to several studies have the potential for global warming savings.

In the last years, several studies have been conducted to compare GHG emissions from traditional wastewater treatment processes with those produced during

anaerobic digestion. This technology results in a good alternative for the reduction of GHG produced during industrial and municipal waste and wastewater treatment. Although AD contributes to GHG emissions (see Sect. 10.5), this process also has a great potential for global warming savings. This section is devoted to briefly compile the reduction of GHG emissions during anaerobic digestion compared with other technologies or management practices applied to municipal, industrial and livestock wastes and wastewaters.

10.4.1 Agricultural Wastes

The constant growth of intensive pig farming has led to increased livestock waste in small and located areas worldwide. Within these areas, local use of manure as an organic fertilizer leads to nutrient over-application (N and P mainly) in agriculture, resulting in water and soil pollution (Bernet and Béline 2009). The most common management practice for liquid manure is to store it in uncovered anaerobic tanks for between four and six months, prior to exportation for landspreading (Burton and Turner 2003). Storing swine manure in uncovered anaerobic tanks entails a number of significant environmental impact issues, including GHG emissions (Riaño and García-González 2014; Vanotti et al. 2008). In fact, GHG released from livestock attributed to manure management account for 30% (Bernet and Béline 2009; Steinfeld et al. 2006). In this context, alternative technologies for manure treatment have been developed and implemented in order to achieve enhanced environmental protection, including the reduction of GHG emissions. These technologies include physical–chemical, aerobic and anaerobic processes.

Few comparative studies have explored the GHG emissions of the various manure management systems. Among those, some works compare GHG emissions of aerobic and anaerobic processes with the baseline scenario (i.e. conventional manure storage and further land application). For example, annual GHG emissions were cut by 62% through the installation of a swine manure treatment plant based on solid–liquid separation and nitrification–denitrification of the liquid phase compared with the baseline scenario (Riaño and García-González 2015). This reduction was in the range (53–75%) of estimated reduction for the implementation of anaerobic digestion for manure treatment (García-González et al. 2016). These authors calculated the GHG emission reduction for several full-scale anaerobic digestion plants that used manure as the main substrate, comparing with the baseline scenario. Most of the GHG emissions in these systems were produced in the final effluent storage ponds or in the intermediate manure storage before transportation in the case of collective treatment plants. In addition, methane leakages (estimated in 2% of the methane produced, according to the Swiss Quality Management Biogas Handbook) also had an important role in the GHG emissions in anaerobic systems. Collective treatment plants are sustainable, despite the higher GHG emissions due to transportation of substrates to the biogas plants. The GHG emissions' reduction in anaerobic treatment plants is due, to a large extent, to the

recovery of the biogas to produce heat and electrical power, avoiding the use of fossil fuels, and also to the very low methane production potential of the digested effluent. Differences in GHG emissions reported by García-González et al. (2016) for the different anaerobic digestion treatment plants were basically due to solid and nitrogen content in raw manure, as well as the differences in transportation distances from farms to the treatment plants. Another fact to consider is that as anaerobic digestion does not reduce N; the digestate will need further post-treatment or a land application planning. In this case, emissions derived from the fuel consumption during transport and land application of digestate will be the same as from raw manure. The digested product presents a lower dry matter content and thus a lower potential for CH_4 formation. However, when the digested manure had not been fully digested in the biogas plant, higher CH_4 emissions from digested than from untreated slurry during subsequent storage were observed (Clemens et al. 2006). Therefore, in anaerobic technologies, the hydraulic retention time must be long enough to exploit the potential for gas production without increasing GHG emissions during subsequent storage and field application (Clemens et al. 2006; Riaño and García-González 2015). A higher reduction percentage of GHG emissions (90%) has been calculated for manure composting systems (García-González et al. 2016).

In the case of dairy farms, several works have also evidenced the positive impact of the introduction of a biogas production plant. The storage of liquid manure for long periods of time without processing contributes to the most of GHG emissions during dairy manure management. The implementation of manure treatment technologies allows facilities to reduce emissions significantly, mostly through anaerobic digestion (Aguirre-Villegas and Larson 2017). Reduction of up to 50% of GHG emissions related to dairy manure management has been reported (Amon et al. 2006). In this case, the mitigation of GHG emissions is greatly due to the prevention of GHG emissions from undigested slurry storage (Battini et al. 2014). When producing electricity through anaerobic digestion, GHG emissions can be further reduced by replacing on-farm fossil fuel-based processed (Aguirre-Villegas et al. 2015). However, the mitigation of GHG is highly dependent on the fossil source to be replaced (Junior et al. 2015). In addition, it is important to point out that the poor management of on-farm digesters can compromise the environmental advantages of anaerobic digestion (Brunn et al. 2014). Thus, depending on the type of fossil fuel that is replaced by biogas, a poor management of biogas plants could led to the release to the atmosphere of between 3 and 51% of the biogas produced, which can have a great impact on global warming.

Despite the development of both aerobic and anaerobic manure treatment technologies, capital investment has been identified as the most important challenge facing implementation of cleaner treatment technologies, since these prove very costly compared to conventional manure practices (Vanotti et al. 2008). Fortunately, by adopting the Kyoto protocol, new programmes have been developed aimed at reducing anthropogenic emissions of GHG. Such programmes can help offset the higher installation costs of cleaner technologies and, therefore, stimulate their adoption by farmers (Vanotti et al. 2008).

10.4.2 Wastewater and Sewage Sludge

Municipal wastewater collection and treatment in wastewater treatment plants (WWTP) contribute to GHG emmisions due to biological degradation, being N_2O and CH_4 the main GHG contributors as it has been highlighted by the IPPC guidelines (IPCC 2014). According to Mannina et al. (2016), WWTP are one of the most important sources of anthropogenic CH_4 emissions, releasing close to 9% of total CH_4 emissions to the atmosphere. Regarding N_2O emissions, the US Environmental Protection Agency (USEPA 2006) estimated that WWTP are the sixth largest N_2O contributor; hence, these emissions must be controlled. Anaerobic digestion is one of the strategies to reduce these emissions. According to Wang et al. (2016), reductions of GHG emissions between 24 and 76% could be expected after utilization of biogas from anaerobic digestion of sludge in municipal WWTP.

Conventional processes for municipal wastewater treatment facilities, mainly based on nitrification and denitrification to remove nitrogen and organic matter simultaneously, are high energy and chemical intensive. Power is needed for running pumps and air blowers and for heating, and chemicals are mainly required for pH and alkalinity adjustment, phosphorous removal or other processes such as coagulation/flocculation. Electricity and chemicals have intrinsic carbon footprints, corresponding to the GHGs generated during their manufacturing and transport. In this way, the most significant contribution to GHG emissions in conventional processes for municipal wastewater treatment is these indirect emissions. In addition, the operation of WWTP results in direct emissions of GHG generated during the biological processes, such as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O).

Technology innovation has a great potential for reducing energy consumption and GHG emissions from WWTP. A simultaneous reduction of energy consumption and an increment in energy recovery from wastewater would be important elements for achieving carbon neutrality (Wang et al. 2016). Caker and Stenstrom (2005) compared GHG production by aerobic and anaerobic treatment systems, including anaerobic wastewater treatment by processes such as the upflow anaerobic sludge blanket reactor and anaerobic filters. This study concluded that for very low strength wastewaters (less than 300 mg biochemical oxygen demand (BOD)/L), aerobic processes will emit less greenhouse gas. At higher strengths, anaerobic wastewater treatment would be more favourable, and the crossover point depends on the relative efficiency of the aerobic system. A technology to economically recover dissolved CH_4 from process effluents could make anaerobic wastewater more suitable in reducing GHG at all influents. Additionally, the combination of autotrophic processes to remove nitrogen, such as the partial nitrification and Anammox processes or microalgal-based technology, with anaerobic digestion could be an alternative. Using these technologies, oxygen requirements of aerobic processes are minimized while methane production is maximized (Campos et al. 2016).

Industrial wastewaters contain higher BOD and suspended solid (SS) concentrations than municipal wastewaters, leading to higher GHG production per m^3 of wastewater treated (Shahabadi et al. 2009). Taking into account GHG emissions, Shahabadi et al. (2009) recommended the combination of an aerobic reactor with anaerobic solid digestion with the recovery and use of the produced biogas for industrial wastewaters and specifically for food processing wastewater.

To minimize the GHG emissions in the WWTP, two main strategies may be implemented: (1) to prevent GHG emissions through a modification of the operation scheme in order to minimize the existing emissions, but this strategy may incur in important costs. (2) To use the current operation scheme of the WWTP and modify the operational conditions to decrease emissions and/or implement carbon capture and treat the gaseous streams. This strategy has remarkably lower impact than the first one (Parravicini et al. 2016).

10.4.3 Municipal Wastes

Disposal and treatment of municipal wastes are significant contributor to GHG emissions. The most extended system of municipal solid waste disposal is landfilling and that it is expected to increase due to the replacement of open dumping by landfilling in developing countries (Baldasano and Soriano 2000; Lou and Nair 2009). GHG emission of conventional landfills is highly dependent on waste composition, among other variables (Lou and Nair 2009). Studies suggest a large variation of GHG emission factors, varying between 1.3 and 2.0 t CO_2 eq per tonne of waste (Baldasano and Soriano 2000; Lou and Nair 2009). Landfill gas capture for flaring or combustion to recover energy is the most common mitigation strategy, showing a great potential for GHG reduction compared with conventional landfilling. Gas capture would led to a global warming potential (GWP) reduction of up to 58% of the total landfill's global warming potential (Liamsanguan and Gheewala 2008). Composting is considered a simple and effective way of treating the organic fraction of municipal wastes, while reducing GHG emissions. In this case, aerobic bacteria transform the organic matter to mostly CO_2 instead of CH_4, reducing the GWP of the landfill. Comparing with conventional landfills, a reduction of CH_4 concentration could achieve 90% in some cases (Cossu 2003; Lou and Nair 2009). In spite of this advantage, energy consumption associated with aeration is likely to be considerably higher than the operational requirement of conventional landfill. However, when the global GHG emissions are considered (decomposition and operational emissions), landfills appear to have a heavier impact on GHG emissions than composting (Lou and Nair 2009).

Anaerobic digestion presents advantage over composting, incineration or combination of digestion and composting mainly because of its improved energy balance (Edelmann et al. 2000). For instance, according to Liu et al. (2012) work, GHG reduction reaches 114 and 523 kg CO_2 eq per ton of waste for anaerobic digestion with power generation and bio natural gas compared with landfill baseline, respectively.

Finally, some authors have highlighted the importance of the implementation of an integrated municipal management system combining different treatments, as opposite to the use of one single process, for the reduction of GHG emissions (Baldasano and Soriano 2000). Thus, the calculated emission factor for landfilling is 1.97 t CO_2 eq per ton of waste, whereas the combination of sorting, wet biogasification, incineration and landfilling allows a reduction of 40% of GHG emissions. A combination approach is the best way to extract the material (e.g. recovery of nutrients as fertilizers) and energetic recycling potential of the different fractions of municipal wastes, to get the most out of these wastes.

10.5 Strategies to Increase GHG Mitigation During AD

Anaerobic organic matter degradation results in GHG formation. Carbon dioxide (CO_2) and methane (CH_4) are the main components of biogas, usually in a proportion of 25–50 and 50–75%, respectively (Wellinger et al. 2013). Methane is a valuable resource that can be easily converted into renewable energy but it is the second most prevalent GHG after CO_2. AD plants can be divided based on the substrate type into four categories (Deremince and Königsberger 2017). These are agricultural (energy crops, agricultural residues and catch crops), sewage (sewage sludge), landfill (biogas collected from organic waste disposal areas) and others (biowaste and municipal waste, household waste and industrial waste). Besides the potential GHG emissions from the AD plant itself, a variety of potential GHG emissions related to AD facilities have been identified (Fig. 10.5) (Burg et al. 2018). These include (1) waste disposal, (2) transportation to AD plant, (3) waste storage before AD, (4) AD plant, (5) conversion of biogas to energy and (6) digestate management before and during land application. In order to identify strategies to mitigate all the potential GHG emissions related to AD, this section reviews the potential sources of GHG emissions in AD facilities.

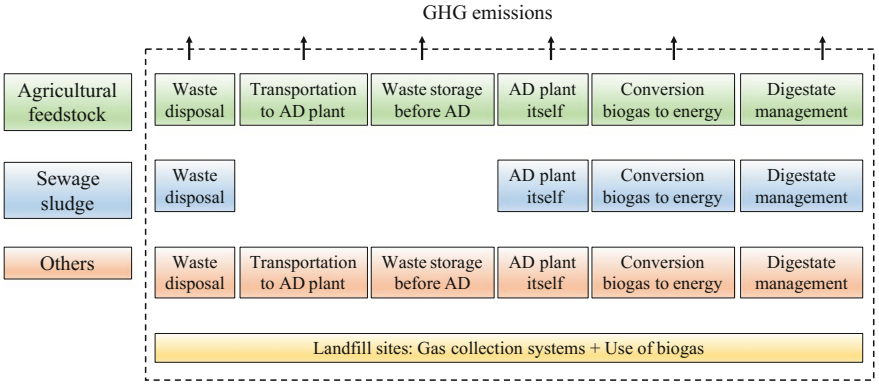

Fig. 10.5 Potential sources of GHG emissions in AD facilities. Adapted from Burg et al. (2018)

10.5.1 Agricultural AD Plants

GHG emissions in agricultural AD facilities can be divided into emissions from (1) waste disposal, (2) transportation of agricultural/livestock waste to AD plants, (3) agricultural/livestock waste storage before AD, (4) the AD plant itself, (5) conversion of biogas to energy and (6) digestate management. Manure is the main feedstock in agricultural AD plants, and most of the gas emissions in agricultural AD plants are related to manure disposal. These gases include CH_4, N_2O and CO_2. Methane production during manure management depends on the anaerobic conditions present in the farm. These emissions are higher if manure is treated as a liquid than if it is treated as a solid by-product. In the case of N_2O, it is released when the denitrification process is not completed. That is in the presence of anaerobic conditions, warm temperatures and carbon availability. The animal housing itself, more specifically littered systems, can be another source of N_2O. The Best Available Techniques (BAT) reference document for the intensive rearing of poultry or pigs compiles a wide variety of techniques that can be used to minimize GHG emissions during livestock waste management. These techniques include good housekeeping, nutritional management, efficient use of water and energy or on-farm manure processing, among others (Santonja et al. 2017). Carbon dioxide emissions related to transportation vary depending on the vehicle, but a standard emission factor of 0.43 kg CO_2/km can be applied to roughly calculate the emissions related to transportation (Burg et al. 2018). These emissions could be diminished by minimizing the distances from agricultural waste sources to AD facilities. The degradation of manure during storage prior to AD feeding could lead to both GHG emissions and potential energy losses. For instance, a study evaluating the CH_4 emissions pattern during pig slurry storage under Mediterranean conditions in summer recommended a maximum storage period of 30–35 days to prevent significant storage-related CH_4 emissions (Moset et al. 2012). Also the use of wooden lids placed on the slurry tank was found to reduce the net total GHG emissions in untreated cattle slurry and with anaerobically treated slurry (Clemens et al. 2006), and in general, some authors indicated that a solid cover or the presence of a surface crust on slurry stores reduced CH_4 emissions (Clemens et al. 2006; Husted 1994; Sommer et al. 2000).

The AD plant itself also accounts for the total GHG emissions. According to the Swiss Quality Management Biogas Handbook, a 2% of the annual amount of biogas produced can be assumed as emissions. In this vein, frequent controls should be done in order to identify biogas leakages (Liebetrau et al. 2017). A variety of available devices and methods for measuring emissions from AD plants as well as a review of different experiences in AD facilities is compiled in Liebetrau et al. (2017). Another potential source of emissions is the conversion of biogas to energy. Electrical energy and thermal energy are produced to ensure energy for the operation of the AD plant. The energy is produced by burning some of the produced CH_4, while CO_2 is released to the atmosphere. The use of this energy is not always optimized and up to 50% of the produced thermal energy sometimes goes to waste

(Szabó et al. 2014). Therefore, an effective utilization of the produced heat energy could be a strategy for GHG mitigation in this phase. Moreover, a control of the engine settings of the cogeneration unit should be regularly carried out to ensure complete combustion (Liebetrau et al. 2017). Finally, digestate management prior to field application is one of the main sources of GHG emissions. Two strategies are proposed to mitigate these emissions: (1) covering the digestate tank and (2) applying any aerobic post-treatment to avoid methanogenic activity (Liebetrau et al. 2017). Extended information about the role of digestate management on the reduction of GHG emissions can be found in Sect. 6.

10.5.2 Sewage Sludge AD Plants

Activated sludge treatment followed by AD of the sewage sludge in the same facility is among the most used technologies for sewage treatment nowadays. GHG emissions in sewage sludge AD facilities can be divided into emissions from (1) waste disposal, (2) the AD plant itself, (3) conversion of biogas to energy and (4) digestate management. Regarding waste disposal, GHG emissions are related to wastewater treatment and they include CH_4 and N_2O (Parravicini et al. 2016). N_2O is mainly produced in the activated sludge tank, as a product of the nitrification–denitrification processes. According to Parravicini et al. (2016), N_2O emissions from this tank account for a 26% of the estimated 36 kg CO_2 eq/year for a WWTP with AD. The optimization of the operational conditions of these processes is highlighted as the main strategy to reduce N_2O in WWTP-AD plants. Regarding CH_4, the sludge line in the WWTP is the main source of emissions before the sludge is fed to the AD plant. Reducing the time of sludge storage in tanks prior to AD would contribute to GHG mitigation. Regarding biogas conversion to energy, the efficiency of this process presents a high dependency on the country. For example, in Germany up to a quarter of the electricity and heat that is consumed in the WWPT is obtained from the produced biogas. On the contrary, in Brazil, most of the gas is nowadays burned and, therefore, not converted into bioenergy (dos Santos et al. 2016). In this country, the economic viability of electricity production from biogas depends on financial initiatives or consortia between neighbouring cities to build a centralized AD plant (dos Santos et al. 2016). AD is used as the last stabilization step for the primary and secondary streams obtained after activated sludge treatment of municipal wastewater. For this reason, the obtained product presents less risk of GHG emissions if compared to the digestate obtained in agricultural AD plants.

10.5.3 Landfills

GHG emissions from landfill sites include CH_4, CO_2 and small amounts of N_2O. A huge amount of biodegradable material ends up in landfill sites. This material is a potential source of CH_4 that is collected and mainly used for energy purposes. During the initial phase, landfill waste is not sealed and the biodegradable fraction experiences aerobic and anaerobic degradation. Consequently, CH_4 and CO_2 are emitted. Then, when the landfill is sealed, CH_4 production is increased and it is here where biogas can be collected, being this measure the main strategy to mitigate CH_4 emissions in landfill sites (EUROSTAT 2014). If the gas cannot be used to produce energy, it must be flared (Directive EC Waste Landfill 1999). The landfill directive includes some measures for landfill gas control and GHG emissions minimization, such as (1) lining of the landfill base and sides to create a low permeable barrier to subsurface gas flow or (2) surface sealing including impermeable mineral layers and gas drainage layers. Moreover, a variety of strategies has been proposed by the European Commission in order to maximize biogas recovery while minimizing CH_4 emissions. These strategies include (1) starting biogas collection as soon as possible, right after the deposit of the waste, (2) minimizing the area of waste not sealed, (3) installing gas collection systems as soon as possible, (4) sealing all landfill infrastructure such as leachate or gas wells to prevent gas leaks, (5) regular monitoring for all sealing systems to detect possible leaks and (6) regular maintenance and optimization of collecting systems. After biogas collection, this should be utilized and the maximum amount of energy should be obtained. Some techniques to optimize biogas utilization from landfill sites are (1) introduction of the treated CH_4 into the gas mains, (2) combined heat and power utilization, (3) direct use of the gas as a fuel or (4) electricity generation from biogas.

10.5.4 Other AD Plants

Other AD plants include biogas plants treating municipal organic waste, household waste and industrial organic waste. In many cases, organic household and industrial wastes are co-digested with manure in manure-based biogas plants. The potential GHG emissions sources are similar to those in agricultural AD plants: (1) waste disposal, (2) transportation of waste to AD plants, (3) waste storage before AD, (4) the AD plant itself, (5) conversion of biogas to energy and (6) digestate management. GHG emissions related to organic household disposal include the collection system, the frequency and the waste composition. An optimization of the separation and collection of organic materials in origin together with a selection of types of waste collected are proposed for reducing GHG emissions (Yoshida et al. 2012). In the case of organic industrial waste, the emissions due to the collection are reduced. Emissions related to transportation (CO_2) are mainly dependent on the vehicle, and they could be diminished by minimizing the distances from the waste

sources to AD facilities. Potential emissions related to waste storage before AD, the AD plant itself and conversion of biogas to energy, as well as the strategies to reduce these emissions, are similar to those in agricultural AD plants. In this case, the use of this digestate as fertilizer is subjected to its content of pollutants and its application is ruled by each country legislation (Al Seadi and Lukehurst 2012).

As a summary, the main potential sources of GHG emissions in AD facilities are identified. Organic waste storage (including manure, digestate, organic household waste) is the main source of GHG emissions in agricultural and industrial AD facilities. Covering the storage tanks could be a strategy to mitigate those emissions. In the case of WWTPs, the main source of GHG emissions lies on the activated sludge tank, being the optimization of the operational conditions the main strategy to counteract those emissions. In the case of landfill sites, the spontaneous anaerobic fermentation of the disposed organic matter results in CH_4 and CO_2 emissions. To reduce these emissions, the collection of this CH_4 is of major importance, as well as to produce energy or further upgrade it to be used in vehicles.

10.6 The Role of Digestate Management on the Reduction of GHG

Digestate contains a high amount of organic matter and nutrients. Its use as organic fertilizer is gaining great interest day by day due to its economic and environmental advantages. Among others, these benefits include the energy and GHG emission savings, if compared to the production of inorganic fertilizers. However, there are GHG emissions related to management of digestate that should be evaluated. These indirect emissions are mainly produced during the storage, transportation and land application and soil degradation of the digestate. In this sense, the quality of the digestate is of major importance for its application as a fertilizer; since, for example, the most mineralized the digestate the less N_2O emissions will generate, as less degradable volatile solids in the digestate will decrease O_2 demand in the soil and therefore less N_2O will be emitted. Other characteristics of digestate are also important as specific chemical composition (i.e. content of nutrients, moisture, pH); safety standards according to the current legislation for each country (including pathogens, heavy metals and organic pollutants). In this vein, and due to a high risk of chemical contamination, digested sewage sludge or digestate obtained from industrial feedstock is only allowed to be used as a fertilizer in some European countries (Al Seadi and Lukehurst 2012). The present section briefly describes potential GHG emissions in these different steps.

10.6.1 GHG Emissions During Digestate Storage at the AD Plant

The main use of digestate is agricultural application as fertilizer. Since digestate is produced during the whole year and fertilization must be done during the growing season, a storage tank in the AD facility is needed. Alternatively, a storage tank for digestate can be placed close to the fields where it will be applied. Generally, the digestate is stored in uncovered tanks for up to 180 days from which GHG, such as CO_2 and CH_4, are emitted to the atmosphere (Menardo et al. 2011). Moreover, it can be dewatered to separate solid and liquid fractions for its easy handling and transportation (Zeshan and Visvanathan 2014). N_2O emissions during digestate storage are not expected to be a significant source of the total N_2O emissions from biogas plants, as the anaerobic conditions in the tanks prevent its production (Holly et al. 2017). However, digestate storage is a great contributor to CH_4 emissions from anaerobic systems. CH_4 emissions from digestate are not well quantified, with only few studies providing data. For instance, Baldé et al. (2016) estimated that annual emissions from earthen digestate storage were about 12% of CH_4 produced within the digester, thus counteracting GHG emission reductions that are usually assigned to AD. Gioelli et al. (2011) reported that the digestate storage accounts for about 27% of total CO_2-eq emissions generated during anaerobic processes. In spite of these high emissions, CH_4 emissions from digestate storage are substantially lower compared to untreated manure storage. Specifically, a reduction of 85% was obtained by Maldaner et al. (2018), when comparing total annual CH_4 emissions from untreated manure with the digestate tank at the same farm (accounting for 6.6 and 1.0 kg m^{-3} y^{-1}, respectively). Amon et al. (2006) also found that anaerobic digestion reduced GHG emissions by 60% from the untreated slurry due to the reductions of CH_4 emissions. Zeshan and Visvanathan (2014) calculated a decrease of about 75% in the GHG emission potential of digestate compared with organic fraction of municipal solid waste.

The reduction in CH_4 emissions from the digestate tanks is not only related to the degradation of part of the organic matter, but also due to an increase in the less digestible form of organic matter in the digestate (Maldaner et al. 2018). Indeed, the digestate organic matter content and its quality greatly affect to CH_4 emissions during storage. Both the amount and the quality of the organic matter are influenced by the technical and operating parameters of the biogas plant. Thus, in biogas plants operating at high organic loading rates (OLR) and at short hydraulic retention times (HRT), the digestate still contains a considerable amount of undigested organic matter that it is gradually digested during storage. Under such conditions, and if the storage tank is uncovered, a considerable amount of CH_4 could be released to the atmosphere. In these cases, the collection of CH_4 during digestate storage could be economically viable operating at high OLR and at low HRT. Besides, covering storage tanks offers an opportunity to reduce GHG emissions to the atmosphere while capturing residual digestate methane (Kaparaju and Rintala 2006; Menardo et al. 2011). On the contrary, the operation at low OLR and at very long HRT results in negligible emissions from digestate (Menardo et al. 2011).

Other factors encourage enhanced CH_4 production after AD. Specifically, the sludge layer in the storage tank is a key contributor to CH_4 emissions, since it could contain as much volatile solids as the annual discharge from the anaerobic digester (Baldé et al. 2016). Another determining factor controlling CH_4 emissions during digestate storage is the environmental temperature and that of the digestate entering the storage tank (Clemens et al. 2006; Sommer et al. 2007; Maldaner et al. 2018; Menardo et al. 2011). For instance, CH_4 emissions during summer were approximately 50% higher than during winter for biodigesters fed with a mixture of manure and crops (Liebetrau et al. 2013). Likewise, Rodhe et al. (2015) found negligible emissions from digestate in the winter. In addition, it is noteworthy that due to heating during anaerobic digestion, the high digestate temperature can enhance CH_4 emissions.

Minimizing the retention time during storage will reduce GHG emissions, since CH_4 emissions will be avoided. In addition, during storage part of the ammonia is volatilized due to favourable conditions of pH. The losses of N would decrease the potential value of digestate as fertilizer hence reducing GHG savings from fertilizer substitution by digestate (Zeshan and Visvanathan 2014). Digestate solid–liquid separation also affects to GHG emissions. Particularly, the effect of solid–liquid separation would depend on the type of biogas feedstock. Thus, Holly et al. (2017) found that a solid–liquid separation following anaerobic digestion reduced 68% of CH_4 emissions in digestate storage, compared with raw dairy manure storage. Perazzolo et al. (2015) observed that mechanical separation of anaerobically digested cattle slurries reduced GHG emissions by 40%, while on digested pig slurries no significant effect was observed.

As an overall, the operation of anaerobic digestion plants as well as digestate management is of key importance for minimizing CH_4 during digestate storage in biogas plants. Some potential best practices can be adopted for reducing GHG emissions including regular storage emptying, digestate solid–liquid separation and storage tank covering (Baldé et al. 2016).

10.6.2 GHG Emissions from Digestate During Transportation and Land Application

Emissions during digestate transportation and land application are highly dependent on the distance to the fields from the AD plant and if the digestate is further treated to reduce water content (Møller et al. 2009). This is due to the low dry matter content of digestate (<10%), that often makes storage and transportation expensive. Møller et al. (2009) have proposed a global warming factor in the range of 0.9–1.9 kg CO_2-eq per tonne of wet waste and 1.5 kg CO_2-eq per tonne of wet waste for transportation and land application of the digestate.

After land application, the biodegradation of the digestate begins, resulting in CO_2 and N_2O emissions. Emission coefficients for CO_2–C and N_2O–N are in the

range 0.86–0.96 of the C and 0.013–0.017 of the N applied to the soil, respectively, depending on environmental conditions, soil characteristics and other parameters related to agriculture, such as the application technique (Møller et al. 2009). Comparative studies have evaluated GHG emissions from land application of digested and undigested manure; however, obtained data are very variable. Clemens et al. (2006) compared GHG emissions from untreated and anaerobically digested cattle slurry after land application, and they concluded that there were no significant differences between both types of slurry with annual emissions of 4.15–8.1 kg CO_2 eq. per m^3. Indeed, they found that GHG emissions from slurry storage are more important than emissions after field application. On the contrary, some authors report between 17 and 71% lower N_2O emissions from land application of digestate, compared with that from undigested manure, depending among others on the soil characteristics (Börjesson and Berglund 2007; Chantigny et al. 2007). This reduction has been attributed to the lower content in easily degradable C in digested feedstock, hence less energy source for denitrifier bacteria (Nkoa 2014; Vallejo et al. 2006; Rochette et al. 2000). Moreover, several works have concluded that digestate presents a higher risk of N_2O emissions than undigested manure. This higher risk could be due to the higher ammonium content of the digestate. Thus, Thomas and Hao (2017) indicated that N_2O emissions from soil receiving digestate were 4.3 and 3.6 times higher than the emissions of the separated solids and cattle manure, respectively. In addition, other studies have found higher N_2O emissions from soil amended with digestate than those from a soil amended with inorganic fertilizers. For example, Pampillón-González et al. (2017) evaluated GHG emissions during the growth of wheat cultivated in soil amended with digestate and concluded that although emissions of CO_2 and CH_4 were not significantly affected by fertilization, cumulative N_2O emissions increased by five times compared to urea-amended soil. The variability in results found in the literature highlights the importance of conducting additional research that explores the GHG emissions after digestate land application.

Several management practices can be adopted in order to minimize GHG emissions from land application of digestate. Spring application would mitigate N_2O emissions via the reduction of the amount of substrates necessary for the accomplishment of N_2O-related freeze–thaw processes (Nkoa 2014). Agricultural practices that enhance soil aeration and a good drainage would also mitigate N_2O emissions after the application of anaerobic digestates (Nkoa 2014).

Land application of digestate also would replace the use of inorganic fertilizers and, consequently, the GHG emissions from fertilizer manufacturing would be avoided (Pampillón-González et al. 2017). The average emission values for the production of nitrogen, phosphorous and potassium fertilizer calculated from Boldrin et al. (2009) are 8.9 kg CO_2-eq/kg N, 1.8 kg CO_2-eq/kg P and 0.96 kg CO_2-eq/kg K. The substitution of inorganic fertilizer will depend on the concentration and availability of nutrients in the digestate; therefore, it is important to characterize the composition of digestate before its use. Some other advantages that can lead to GHG reductions are the increment of water retention in the soil (thus reducing irrigation), the reduction of the requirement of herbicides or

biocides, the improvement of soil structure or the reduction of the erosion (Møller et al. 2009). These savings are not yet well quantified; however, it is worth noticing that these induced effects on soil would address to important benefits for global warming. In any case, the use of digestate as a fertilizer for land application implies a high reduction of GHG emissions when compared with a scenario in which digestate is disposed in a dumpsite (Zeshan and Visvanathan 2014).

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Chapter 11
Digester Slurry Management: The "One Health" Perspective

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Abstract The increasing demand for food, energy and natural resources has stimulated the use of anaerobic biodigestion, aiming at the treatment of biomass derived from anthropic activities with potential for biogas production. Digestate is rich in nutrients for soil fertilization purposes, with a potential direct impact on the safety of human, animal and environmental health, within the "One Health" scope. "One Health" deals with the set of strategies applied to human and animal medicine, combined with the conservation of the environment. This chapter will address the management and recycling of digestate in agriculture, considering chemical and microbiological contaminants (pathogens) from an One Health approach.

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11.1 Digestate Use and Management

Anaerobic digestion produces, together with biogas, a residual material called digestate. The digestate presents a high amount of nutrients such as nitrogen (N), phosphorus (P) and potassium (K), as well as organic matter, which could be beneficial for agricultural purposes as biofertilizer (Barbanera et al. 2018). However, the digestate also presents a high moisture content and is not fully stabilized when leaving the digester, as well when applied without proper treatment into the ground, which can generate phytotoxic and odor concerns (Alburquerque et al. 2012; Arab and Mccartney 2017). For this reason, the digestate needs to be managed properly and receives specific treatment before its implementation on the ground, in order to avoid environmental problems and threats to public health (Alburquerque et al. 2012), due to the potential for emissions of ammonia and nitrate, leaching of heavy metals and the presence of pathogens (Barbanera et al. 2018).

Sanitary safety is a relevant factor that impacts on environmental, animal and human health, the three pillar of the concept "One Health"; the set of studies related to the area of human and animal medicine with the conservation and development of the environment. In this context, the concept of "Unique Health", with an innovative character, is defined as an addition of values and knowledge of human and animal health, to economize and improve environmental services, being possible through the joining of areas, professionals and institutions, according to the (WHO), Food and Agriculture Organization (FAO) and World Organization for Animal Health (OIE) (Nguyen-Viet et al. 2015).

Recycling has been the most widely used technique in the management of anaerobic digestion and its derivatives while adding value to the product and closing the cycle of matter. In Brazil, recycling is a technique which is the main priority of the National Politic of Solid Wastes (PNRS) to ensure the management of municipal solid residues (Brazil 2010). However, certain quality characteristics, stability, and hygiene must be met for the sustainable recycling of digestate in the environment (Alburquerque et al. 2012).

An option to improve the quality and stability of the solid fraction of digestate is through composting (Arab and Mccartney 2017). The composting process can be improved by direct microbial inoculation; the digestate can be applied as inoculant instead of acquiring or preparing commercial microbial cultures, being, therefore, more advantageous economically (Arab et al. 2017). The addition of digestate in windrow composting of organic municipal waste fresh and/or partially stabilized may increase the rate of reaction of the composting and decreases the time for the compound to achieve stability in 30–36% with addition of 20–40% of digestate (% ww) (Arab and Mccartney 2017). Both the composting and anaerobic digestion

processes are mediated by a range of different microorganisms. Bacteria play an important role in the thermophilic and post-aeration phases and fungi are essential in the maturation phase. For this reason, the digestate should be added in the process of composting in adequate quantity, in order to ensure uniformity of microbial species. The use of 40% (wet weight basis) of digestate in the composting of municipal organic waste revealed that mixing between the two substrates (organic waste and digestate) led to a favorable condition for microbial species present (Arab et al. 2017).

Bustamante et al. (2012) studied composting by digestate (obtained from anaerobic digestion of cattle slurry and silage) and residues of grapevine pruning, as bulking agent. The results showed that the organic matter of digestate is mineralized, increasing electrical conductivity, as well as the humification index of germination during the composting, allowing the humification of organic matter in the absence of phytotoxins. The compounds reached appropriate degrees of stability and maturity, physical properties suitable for use as fertilizer for crops, and also the suppression of the phytopathogen *Fusarium oxysporum* f. sp. *melonis*. However, the salinity and the concentrations of Cu and Zn present in the composted material from digestate and various bulking agents (wheat straw, grapes, etc.), limited its application in agriculture (Bustamante et al. 2013). Similarly, the composting of solid digestate leads to the accumulation of nutrients (P, K, Mg and Ca) and heavy metals (Cd and Cr) due to the organic matter degradation during composting (Knoop et al. 2018). The digestate can replace the mineral fertilizer on the production of sida (*Sida hermaphrodita*—Malvaceae), maize (*Zea mays* L.—Poaceae) and alfalfa (*Medicago sativa* L.—Fabaceae), showing a positive effect of digestate in biomass production of plants (Barbosa et al. 2014), and the quantities of macro-element present on digestate are comparable to mineral fertilizer (Koszel and Lorencowicz 2015) and, therefore, it can be used as fertilizer for crops and food products. In addition, the use of digestate as biofertilizer in agriculture has been also evaluated by ecotoxicological tests, including direct (using plants and earthworms) and indirect tests (based on aquatic organisms and luminescent bacteria). Experiments with earthworms showed no serious negative effects for mixtures containing up to 15% (w/w referring to the dry matter) of digestate. Tests with plants did not show negative effect when lower concentrations than 20% (w/w) of digestate were applied. The indirect tests showed a LC_{50} value of 13.61% (v/v) for *Daphnia magna* and no toxicity to *Artemia* sp. and *Vibrio fischeri*. These results encourage the use of the digested as fertilizer in agriculture (Pivato et al. 2016).

However, the production of biofertilizers from digestate is hampered by legislative issues. In spite of derivatives of digestate present similar characteristics to the mineral fertilizers, the legislative framework has not encouraged the marketing of fertilizers of biological origin (Bolzonella et al. 2018). Therefore, few studies have evaluated other applications of digestate (Table 11.1).

From a bioengineering point of view, the algae and cyanobacteria could be integrated into a sewage treatment effluent, to treat both the effluent as digestate (solid and liquid), while producing products of industrial interest (Arias et al. 2017). Arias et al. (2017), for example, evaluated the use of a blend of urban and digestate

Table 11.1 Different applications of digestate presented in current literature

Origin of digestate	Fraction of the digestate utilized	Application	References
Mixture of cow manure (27% VS), cheese whey (15% VS), poultry manure (23% VS), olive pomace (2% VS) and corn silage (33% VS)	Solid	Production of an enzyme (exo- and endo-glucanase, xylanase, β-glycosidase, and laccase)	Musatti et al. (2017)
Food waste	Liquid	Production of biochar by pyrolysis	Opatokun et al. (2017)
Sewage sludge and source-segregated biodegradable waste	Liquid	Nitrogen removal of old landfill leachate	Peng et al. (2018)
Cattle slurry mixed with energy crops (maize silage and triticale silage)	Liquid	Biofertilizer	Riva et al. (2016)
Sargassum horueri	Solid	Phenol production	Wei et al. (2018)

secondary effluent as a source of nutrients to grow and select cyanobacteria from a joint consortium of microalgae (green algae of the genus *Chlorella* and *Stigeoclonium*) and cyanobacteria (cf. *Oscillatoria* sp., cf. *Aphanocapsa* sp. and *Chroococcus* sp.) on a photobioreactor. The authors reported removing an average of 96% of total ammoniacal nitrogen (TAN), 95% of dissolved reactive phosphorus ($P - PO_4^{3-}$) and 91% of nitrate ($N - NO_3^-$). In a similar study, *Chlorella vulgaris* was grown in liquid digestate diluted from anaerobic digestion of swine manure and maize, to reduce concentrations of nutrients and their toxicity. The results showed that a significant reduction of the toxicity (82, 88 and 100%) for the organisms tested (*R. subcapitata*, *L sativum* and *D. magna*, respectively), with a high removal efficiency (>90%) of ammonia, total nitrogen and phosphate (Franchino et al. 2016).

Barbanera et al. (2018) studied the production of bio-oil from digestate by microwave-assisted liquefaction held in polyethylene glycol (PEG) and glycerol, using sulfuric acid as a catalyst. Bio-oil yield of 59.38%, with a heating value of 28.48 MJ/kg, was obtained in optimum conditions. This result indicates the possibility of the use of digestate for production of biofuels through a process that is economically viable, whose operational time is reduced due to heating by microwave. The production of pellets and briquettes from digested pulp solid fraction (DSF) is also possible and economically feasible. The costs of production of briquettes and pellets with DSF are approximately four times smaller than the production on sawdust and the calorific power is similar (8.3–16.7 MJ/kg, depending on the moisture content) (Czekala et al. 2018). In addition, the pelleting is an

Table 11.2 Effect of stripping, drying, membranes, and vacuum evaporation in the treatment of digestate

Treatment	Effect	References
Vacuum evaporation	Condensed water can be applied as dilution water for digestion, for irrigation of crops or cleaning of floors. It can also be released into surface waters, after the adequacy to the regulations of patterns of release	Chiumenti et al. (2013)
Drying	Removes the water from the digestate through the grille. It is necessary amount of energy to total removal of water from the digestate, which corresponds to 90% of the earth. Ammonia nitrogen can be removed with steam bath or kept in the digestate if it is acidified through the addition of mineral acids	Bolzonella et al. (2018)
Stripping	Ammonia (NH_3) is stripped and physically transferred from the aqueous to the gas phase	Limoli et al. (2016)
Membranes	Liquid phase of digestate is treated in ultrafiltration (UF) and reverse osmosis (RO) systems. The concentrate is rich in both macro and micronutrients. There is reduction of initial digestate volume	Bolzonella et al. (2018) Ledda et al. (2013)

effective method to eliminate the presence of *Clostridium* spp. of digestate of milk production (Pulvirenti et al. 2015). The heat treatment can also eliminate the *Escherichia coli* present in the digestate (Solé-bundó et al. 2017).

Other studies have focused their attention for nutrient recovery of digestate through treatment technologies as the stripping, drying, membranes (Bolzonella et al. 2018) and vacuum evaporation (Chiumenti et al. 2013). The characteristics of these techniques are summarized in Table 11.2.

11.2 Unwanted Impurities and Pathogens in Digestate

The use of digestate as fertilizer is an efficient way to recycle materials and reduce the use of mineral fertilizers (Yang et al. 2017). Several raw materials are used for anaerobic digestion resulting in digestate such as animal waste, lignocellulosic waste, human waste and food waste (Al Seadi et al. 2013). The limitations of the use of the digestate are dependent on the origin and the way in which the raw material is collected, making it fundamental so that no harmful effects to the environment arise due to the quality of the material, such as pH, high organic matter content and non-material biodegradable substances such as heavy metals and antibiotics (Al Seadi et al. 2013; Yang et al. 2017). In addition, the digestate must have high quality for application as fertilizer, and therefore, the pathogens, chemical and physical impurities and pollutants must be controlled (Al Seadi et al. 2013).

11.2.1 Impurities

The addition of trace nutrients, such as iron, copper, zinc, and nickel, in anaerobic digesters is essential for the synthesis of essential coenzymes in methanogenic pathways to increase the efficiency of the anaerobic digestion of food residues. They are also added in low concentrations in the animal rations in order to increase the productivity, being frequently found in the manure (Zhang et al. 2015; Yan et al. 2018). However, when the concentrations of these compounds exceed, inducing overdoses in the digesters, can cause toxic effects on the microorganisms of the digestion process, resulting in loss of microbial resources, impairing the quality of the final digestate, increasing the difficulty of the process, and increasing the concentration of these metals in the digestate that impair its use as biofertilizer when disposed in environment (Ortner et al. 2014; Zhang et al. 2015). Bioaccumulate potential in the digestate is related to non-biodegradability of the metals, and can be found in the solid and liquid fractions, in reducible and oxidizable forms (Yan et al. 2018).

The supplementation of anaerobic digesters with small doses of heavy metals to increase the biogas production and quality is still a major challenge, facing contradictions between the increase of the economic yield and the great risk of environmental impacts due to the high load of these compounds that have carcinogenic characteristics and even in low concentrations can cause serious damage to animal health and environment (Zhang et al. 2015). Excessive levels of heavy metals (Cu, Zn, Mn, As, Cd and Pb) in the digestate have been reported in the solid and liquid fractions of a digestate that had the substrate of anaerobic digestion of pig manure (Li et al. 2018). It should be noted that the analyzes of the study in question were carried out during the stabilization period of the digestate, and the Cu, Zn, As and Pb concentrations showed a significant increase in concentrations during the period, which may have occurred due to the reduction of the volume of the digestate due to the loss of water by evaporation during storage, which caused the highest concentration of the metals in the volume of digestate. This fact is of extreme importance for the analysis of the digestate as biofertilizer, since the reduction of the amount of water in the medium concentrates the nutrients and impurities, bringing greater risks if disposed of in the environment.

The presence of antimicrobials and hormones in the digestate is linked to the therapeutic use in livestock (Bloem et al. 2017; Kemper 2008). Antibiotics act selectively against microorganisms, and when these compounds are found in the environment, the environmental microbiota can be affected, losing their activity due to low or no resistance to this type of substances (Bloem et al. 2017; Insam et al. 2015). Approximately 200,000 tons of antibiotics are used globally, only in the livestock sector, number that tends to increase (Bloem et al. 2017; Hirsch et al. 1999; Kummerer 2009). The inappropriate and excessive use of antimicrobials can cause to remain in the digestate even after the digestion process, contributing to the appearance of antimicrobial resistant bacteria (ARB). In addition, using the digestate as a fertilizer, another serious environmental problem can happen (Bloem et al. 2017;

Kemper 2008; You and Silbergeld 2014); the negative effect on the soil functions and organisms (Jechalke et al. 2014), and since the plants haven capacity to absorb these compounds (Bloem et al. 2017; Chowdhury et al. 2016; Wang et al. 2016), these compounds can be detected in the food chain (Bloem et al. 2017). Only a few studies have analyzed the elimination of antimicrobial compounds during the digestion process (Arikan et al. 2006; Cheng et al. 2018b; Ratsak et al. 2013; Spielmeyer et al. 2014), and even low concentrations are transported to the environment, which causes concern, since these antibiotics are not diluted and have low leaching capacity (Bloem et al. 2017; Cheng et al. 2018a, b).

The residual hormones in the digestate act very similar to antimicrobials and represent a significant source of pollution (Cheng et al. 2018b; Ebele et al. 2017; Speltini et al. 2011). Toxicological analysis on materials containing residues of these substances have demonstrated a risk to human health and the environment, due to a number of factors, including: endocrine disruption in the environment microbiota (Adeel et al. 2017; Ronquillo and Hernandez 2017), effects on the growth, reproduction and behavior of several species, such as fish, plants and bacteria, even in low concentrations (De Cazes et al. 2014) and even has been associated to breast and prostate cancer (Adeel et al. 2017).

The levels of ammonia present in the digestate are also essential for the possibility of subsequent application. When used as fertilizer, the greater nutrient availability is a key factor in improving soil quality (Nkoa 2013). However, when the digestate, also contains impurities, such as heavy metals, and particularly antimicrobials and hormones, the use of ammonia no longer exerts its nutritional function properly, since the synthesis of ammonia in the soil is carried out by a specific group of microorganisms sensitive to the antimicrobials and hormones (Odlare and Pell 2009; Pell et al. 1998; Risberg et al. 2017), resulting in losses of nitrogen through the volatilization of ammonia and nitrate leaching (Al Seadi et al. 2013).

In this scenario, the incorrect management of the digestate can cause serious environmental and human health problems, particularly when it also included impurities such as heavy metals, antimicrobials, hormones, among others. Researchers are looking for alternatives to remove these compounds from the final effluent of digestion process, and some current technologies including advanced oxidation, ultraviolet light and ozone, demonstrated effectiveness for the removal of antibiotics present in the digestate from swine manure (Ben et al. 2009, 2011; Qiang et al. 2006). Despite the relevance of these studies, the removal techniques are of high energy cost, besides generating secondary byproducts with polluting potential (Cheng et al. 2018b; Liu et al. 2009).

11.2.2 Pathogens

Among the potential pathogens present in digesters, enteric pathogens are the most abundant. Bacteria, as *Salmonella* and diarrheagenic types of *Escherichia coli*,

Vibrio and *Campylobacter* are studied due to infectious potential by contaminated water and food. *E. coli*, a biomarker model of global fecal contamination, includes commensal and interactive types to the intestinal microbiota of man and animals; however, some varieties can also contain virulence determinants. Those include diarrheagenic *E. coli* such as Enteropathogenic (EPEC), Enterotoxigenic (ETEC) Enteroinvasive (EIEC), Enterohemorrhagic (EHEC) and Enteroaggregative *E. coli* (EAEC) (Al-Badaii and Shuhaimi-Othman 2015). Similarly, some protozoa can be associated, particularly those that they are waterborne pathogens, such as *Cryptosporidium* spp., *Giardia* spp. and *Ascaris* spp. They are the most resistant in the environment, against the processes of treatment and disinfection of matrices, like water, sewage, and effluent. (Centers for Disease Control and Prevention 2013; Leal et al. 2013).

Enteric viruses can be found in high concentrations in digestate from anaerobic treatment. These viruses are resistant to extreme pH, high temperatures, salinity, and natural ultraviolet (UV) radiation. They also have a rapid adsorption capacity on the solid particles dispersed in the environment, favoring their stability. Among other viral pathogens that could be present in slurry, hepatitis E virus (HEV) and rotaviruses (RVs) are remarkable due to their zoonotic potential (Delahoy et al. 2018). Hepatitis E is an acute and self-limiting viral disease with a mortality rate of less than 1%. However, in pregnant women and immunocompromised individuals, this disease may become chronic and may progress to cirrhosis of the liver, with mortality rates reported up to 25% (Meng 2010). The etiological agent belongs to the family *Hepeviridae*, genus *Orthohepevirus* and is responsible for causing outbreaks mainly in emerging countries due to poor sanitary conditions. It has recently been discovered that some genotypes of the virus are zoonotic (Park et al. 2016). Studies in industrialized countries showed a high prevalence of seropositive individuals, and sporadic cases of hepatitis E in these places were related to the consumption of game meat and mainly to pork products (viscera—mainly liver, other derivatives). The contact of humans with pigs carrying the virus is also related to a higher seroprevalence, having an impact on public health, since the pigs act as asymptomatic reservoirs. HEV Genotype 3 is often reported as a cause of hepatic illness in humans in Americas and is ubiquitous in swine populations and was reported both in swine slurry and pork byproducts (Heldt et al. 2016). RVs are members of the *Reoviridae* family (Suzuki and Hasebe 2017). Although there are vaccines to prevent the infection in humans, RV is still among the most important etiological causes of diarrhea worldwide, and the infection by new zoonotic types may not be avoided by the current immunogens (Cuffie et al. 2016). The generation of new RV types is common due to the possibility of mutation and reassortment of the 11 segments of double-stranded RNA, which makes these viruses highly variable. Animal RVs are a public health concern due to their potential for genetic exchange with human RVs and the consequent generation of viruses with enhanced zoonotic potential. Since co-infections by different animal and human RV types are a prerequisite for reassortment events, the proper management of slurry to avoid new human infections is mandatory (Delahoy et al. 2018).

It is also noteworthy in the "One Health" context that the evolution of zoonoses is highly due to antimicrobial resistance, becoming a global problem. Antimicrobials are widely used in animal farms to prevent infections and also as animal growth promoters (FAO 2015; CDC 2013). Resistance to antimicrobial drugs is characterized by the ability of microorganisms to resist the effects of a chemotherapeutic agent which it is normally susceptible to. The transmission of the antimicrobial resistance can be increased by the selective pressure due to the presence of antimicrobials in the environment, which enhances the magnitude and spread of the resistance (Haese and Silva 2004). Both antimicrobials and enteric pathogens are present in the animal and human digestates and can disseminate resistant microorganisms, as well as select antimicrobial resistance genes.

11.2.2.1 Control of Zoonotic and Resistant Pathogens

The incorrect management of animal waste can be a serious issue on human health by facilitation of the transmission of zoonotic diseases, with serious economic (losses in animal production) and environmental impacts (contamination of facilities and final products). Other environmental side effects are related to the infiltration and contamination of water and groundwater, the unpleasant odor, the potential damage to the autochthonous fauna and flora (Manyi-Loh et al. 2016). Proper management of livestock and derived slurry, the supply of adequate access to clean water and feed consumption, as well as the temperature and ventilation control systems are necessary parts of an integrated control plan to avoid the spread of zoonotic pathogens in farms (Hodgson et al. 2016). Farm sanitation and strict biosecurity measures are also needed to reduce the spreads of pathogens in animals' excreta (Staggemeier et al. 2015). Other measures like avoidance of runoff from animal housing and storage facilities are also relevant part of the process (Manyi-Loh et al. 2016).

Human and animal pathogens are usually inactivated over time due to a combination of factors such as pH, temperature, humidity, carbon content, nutrient availability, microbial antagonistic behavior, among others (Semenov et al. 2007). The natural inactivation rate is usually slow and unreliable, since the different factors inherent to environmental changes, such as seasonal ones, are not controlled. For these reasons, the storage and the treatment of human and animal excreta must be effectively carried out, since it is possible to quantify the inactivation factors as well as to control these factors (Sidhu et al. 2001). Among the classically recognized factors with potential for inactivation of enteric pathogens such as temperature, solar radiation (UV), pH variation, turbidity, organic composition of the matrix, presence of predatory microorganisms, aggregation between the microorganisms themselves or with particles, the temperature is considered the most important factor (Bertrand et al. 2012).

Functional procedures for the removal of antibiotics from digestate have been studied. Among the physical and chemical methodologies used for this purpose are chemical oxidation and biodegradation (destructive methods), adsorption and

membrane techniques (nondestructive processes). The adsorption of the adsorbent on the surface of the solid (adsorbent) (Sawyer et al. 2002) is considered a potential method in the removal of different classes of antibiotics. For this purpose, aluminum oxide can be used to adsorb amoxicillin (Putra et al. 2009) or tetracycline (Chen and Huang 2010).

11.3 Final Considerations

The global demand for food as well as soil infertility and water contamination have stimulated studies aimed at the reuse of effluents, as digestate, for biofertilization purposes. However, many challenges are encountered in the safe management of this digestate, being the sanitary and agronomic aspects very relevant. It is necessary to develop strategies applied to the actual productive conditions, aiming at obtaining valued and sanitary products safe from a "One Health" perspective. To establish a global safety standard on "One Health" context, studies involving chemical and microbiological risk analysis are required, considering different exposure situations and implications for human and animal health. From the determination of contamination limits, effective and economically feasible strategies for inactivation of infectious agents that can trigger disease should be established.

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Chapter 12
Closing the Loop on Biogas Plants: Recycling Digestate and Sludge on Agriculture and Microbial Risk Assessment

Maria Elisa Magri, Priscila Carlon, Luiza Jofily Miranda Cruz and Leonardo Dalri-Cecato

Abstract Management of human and animal wastes is among the major constraints towards the sustainable development of human settlements, where we demand increasing amounts of clean water, food, and energy. The aim of most sanitation solutions is to keep waste away from the generation site, such as households or animal stalls. The misconception that wastes have no useful purpose has resulted in unsustainable systems. However, the recovery of energy and agricultural use of the organics and nutrients contained in excreta and solid waste can improve soil structure and fertility, increasing productivity, reducing the dependency of resource-demanding chemical fertilizers, and thus contributing to food security. Treatment plants for waste anaerobic biodigestion can be applied in that context, moving from "treatment" plants to become "resource recovery" plants. The recovery of biogas in those plants for energy production is highly valuable, and added value can be obtained by the recycling of the biodegradation products—accumulated sludge and digestate. Those fractions should be treated sufficiently to inactivate pathogens to a certain extent. The quantitative microbial risk assessment is an effective approach to estimate risks, which can be applied to any scenarios of recycling liquid fractions from biogas reactors in agriculture.

Keywords Waste · Risk assessment · Nutrient recycling · Pathogens · Biogas · Sludge · Digestate · QMRA

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12.1 Introduction—Risks and the Resource Recovery Concept

Management of human and animal wastes is nowadays among the major constraints towards the sustainable development of human settlements, where we demand increasing amounts of clean water, food, and energy. Water pollution is caused largely by inappropriate discharge of human and animal wastes into water bodies. It is well known that faecal sludge, either from human or animal systems, has considerably high pollution potential, and its mismanagement is a common reality in many regions, especially in low- and mid-income countries.

The aim of most sanitation solutions is to keep waste away from the generation site, such as households or animals stalls. The misconception that wastes have no useful purpose has resulted in unsustainable systems. However, the recovery of energy and agricultural use of the organics and nutrients contained in excreta can improve soil structure and fertility, increasing productivity, reducing the dependency of resource-demanding chemical fertilizers, and thus contributing to food security. These benefits can be obtained since excreta is treated sufficiently to inactivate pathogens and make it safe.

Innovations on resource recovery are urging, with special focus on integrated waste management, responding to the need of development of sustainable resilient energy and sanitation systems for areas where poor infrastructure, water scarcity, and limited energy supply restrain the capacity for economic growth. These initiatives would contribute to long-term sustainability of cities in both its urban and rural areas and climate-compatible activities for the development.

Treatment plants for waste anaerobic biodigestion can be applied in that context, moving from "treatment" plants to become "resource recovery" plants. The recovery of biogas in those plants for energy production is highly valuable, and even added value can be obtained by the organics and nutrient recycling of other degradation products of the process that consist of the accumulated sludge and the digestate outcomes from the bioreactors.

But what is the risk associated? That is always a raised question when talking about recycling of human- and animal-derived wastes, since they have a high concentration and a very diverse pathogen content of importance for public health.

Microbial risk associated with the biogas fraction is very low when compared with the liquid fractions of sludge and digestate since pathogens stay in those fractions. The objective of this chapter is to present the quantitative microbial risk assessment as an effective approach to estimate risks, which can be applied to any scenarios of recycling liquid fractions from biogas reactors, operating with human and/or animal manure in agriculture.

12.2 Risk Analysis General Concepts

Risk analysis is an effective tool used by most diverse fields including economy, business, engineering, environmental, and human health. This tool works as a systematic and preventive approach through which is possible to minimize, control, and avoid risks, as well as to aid decision-making (Haas et al. 2014).

The process of risk analysis includes risk assessment, risk management, and risk communication. Risk assessment can be qualitative or quantitative and is intended to characterize and estimate all the potential risks involved during a process. Quantitative risk assessment associates numeric values to the risk and through probabilistic calculus provides an overview of the risks (WHO 2016).

12.2.1 Quantitative Microbial Risk Assessment (QMRA)

Quantitative microbial risk assessment consists of the application of risk assessment principles with emphasis on microbial risk and the aim to estimate health effects associated with exposure of an individual to a pathogenic microorganism in different scenarios (Haas et al. 2014). Through the use of systematic information applied to a mathematical model, QMRA enables a preventive management of microbiological risk contamination.

QMRA methodology is supported by the World Health Organization (WHO), which describes the process and steps in "Quantitative Microbial Risk Assessment: Application for Water Safety Management", published in 2016. The methodology is divided into four steps—hazard identification, dose–response model, exposure assessment, and risk characterization—which will be presented below.

(1) Hazard identification

Hazard identification is the first step of QMRA and has the main purpose to identify the microbial agent, all diseases associated with this pathogen, and the spectrum of human illnesses.

For this purpose, epidemiological and microbiological studies need to be accessed in order to obtain all necessary information about the microbial agent—pathogenicity, virulence, and infectivity—and about the human response to the microorganism (Haas et al. 2014).

(2) Dose–response model

According to Weir et al. (2017), the choice of an adequate dose–response model is essential for a successful analysis. The dose–response model describes the relationship between the level of microbial exposure and the probability of this exposure to affect human health.

The dose–response models are expressed by mathematical functions and are based on experimental data. Until now, some dose–response models were

developed and each of them has a parameter which best describes the microbial agent concerned (Haas et al. 2014).

The following describes the two most useful models: the exponential dose–response model and the beta-Poisson dose–response model.

Exponential dose–response model

The exponential dose–response model is characterized by the assumption that each microorganism has an independent and constant probability to survive (*r)* and consequently to cause an infection in a host. In other words, this model does not take into consideration some variabilities that could interfere in pathogen behaviour. Equation (12.1) describes this model.

$$\mathrm{Pd} = 1 - \exp -r \times d \tag{12.1}$$

P(d) risk of infection;
d dose ingested by the individual;
r specific parameter of each microorganism, which represent the probability to survive and infect the host.

Beta-Poisson dose–response model

The beta-Poisson model differs from the exponential model as it assumes that some variations in pathogen–host probability to survive may occur. According to Haas et al. (2014), this variation may appear due to host characteristics, such as gender, age, or immunity or due to the diversity of pathogen ability.

In mathematical terms, the parameter "*r*" of Eq. (12.1) is no longer constant and varies according to a probability distribution represented by the parameters α *e* β. Given this, the risk of infection is calculated using Eq. (12.2).

$$\mathrm{Pd} = 1 - 1 + d - \alpha \tag{12.2}$$

P(d) risk of infection;
d dose ingested by an individual;
α *e* β parameters that express the survival probability distribution of each microorganism.

(3) Exposure assessment

Exposure assessment is intended to determine the population exposed to the risk (adults, children or immunocompromised people), the routes of transmission (air, soil, water, ingestion, inhalation, contact), the exposure scenario, and the distribution of the microorganism concentration (WHO 2016; Haas et al. 2014).

In order to estimate the dose ingested by an individual, the distribution of the microorganism concentration must be known. Ideally, these values should be

determined through laboratory analysis for each case; however, the process of quantification for most pathogens can be very challenging and expensive. Therefore, in these cases the use of a comprehensive database with the most similarity as possible is recommended (Haas et al. 2014). The dose ingested by an individual is given by Eq. (12.3).

$$\text{Dose} = v \times c \tag{12.3}$$

d dose ingested by an individual;
v volume ingested per exposure;
c microorganism concentration.

The determination of exposure scenario enables the identification of the contact level between the individual and the microbial agent, as well as the exposure frequency. These parameters have a high influence on the risk of infection. Moreover, once established the exposure scenario it is possible to know the average ingested volume in that case. The values of volume ingested can be found in the literature, which is normally based on experimental volunteer studies (WHO 2016).

(4) Risk characterization

The risk characterization is the last step of the QMRA and consists in the integration of all data obtained on the previous steps, with the intent to estimate the probability of the risk to occur as well as its magnitude. According to Haas et al. (2014), during this characterization all the variabilities and uncertainties are taking into consideration. The uncertainties can be related to the dose–response model chosen, model parameters, ingested volumes, and microorganisms concentrations.

Usually, to conduct the characterization the Monte Carlo simulation is used—a mathematical tool, which simulates various scenarios using probability distribution and as a result shows all possible scenarios and the probability of people to get infected when exposured to pathogens present in the scenarios. The results are expressed through probability of infection, disease or death, or through disability-adjusted life years (DALY), a measure that expresses all wasted years as a consequence of health problems due to microbiological contamination (Haas et al. 2014).

12.3 Nutrients in Human and Animal Manure and Microbial Pathogen Content for Risk Assessment

In general, human and animal excreta present a large variety of primary and secondary macronutrients, which would characterize them as compound biofertilizers. Table 12.1 presents a complete characterization of excreta regarding macro- and micronutrients content.

The biofertilizers that could be produced as digestate and sludge in biogas plants, considering the percentage of macro- and micronutrients in human and animal

Table 12.1 Characterization of human and animal excreta regarding macro- and micronutrients, presented as fresh and dried human faeces, stored urine, faecal sludge from wastewater treatment plant, and swine manure digestate and sludge from anaerobic biodigesters for biogas production

Characteristics	Human faeces[1] mg kg^{-1} (% db)[a]		Human urine[2] mg L^{-1} (% db)[a]		Faecal sludge[3] (% db)[a]	Swine manure digestate[4] (% db)[a]	Swine manure sludge[4] (% db)[a]
Organic carbon	46,620	(45)[b]	2448	(2.5)	16.7	0.14	4.32
Sulphur	2.9	0.46	510	0.5	0.23	0.02	0.6
Phosphorus (P_2O_5)	14,490	2.3	530	0.5	1.25	0.012	0.06
Total nitrogen	5040	5[b]	6834	6.8	1.83	0.24	0.28
Potassium (K_2O)	10,206	1.6	1824	1.8	0.02	0.05	0.09
Boron	<63	<0.01	Nd	Nd	0.01	<0.0001	<0.004
Cobalt	4.4	0.0007	<0.1	<0.0001	0.0007	–	–
Cooper	38	0.006	0.03	0.00003	0.02	<0.0001	–
Iron	1455	0.23	0.09	0.0001	1.43	0.01	0.06
Magnesium	10,275	1.6	52	0.052	0.15	0.05	1
Manganese	3213	0.51	<0.01	<0.00001	0.01	0.005	0.005
Molybdenum	<6.3	<0.001	<0.1	<0.0001	<0.0001	<0.0001	<0.0001
Nickel	2.5	0.0004	<0.1	<0.0001	21.6	<0.0001	–
Zinc	214	0.034	0.34	0.00034	0.07	0.007	0.007

[a]Calculated on a dry basis (db)
[b]Organic carbon and total nitrogen measured from fresh human faeces (Magri 2013)
[1]Human faeces collected from urine-diverting dry toilet (Magri 2013)
[2]Human urine collected from urine-diverting dry toilet (Magri 2013)
[3]Dry faecal sludge from anaerobic wastewater treatment plant (Kafer 2015)
[4]Digestate and sludge collected from anaerobic digester treating swine manure (Fongaro 2016)

excreta, can be compared to organic fertilizers, but are less effective in terms of concentrations when compared to chemical fertilizers.

At the same time that human and animal manure have high concentrations of carbon and nutrients, the concentrations of metals are small, which is an advantage for the reuse of excreta (Vinnerås 2002; Albihn and Vinnerås 2007).

While manure application in agriculture has its benefits, its management, especially its storage, represents a main limiting factor, given that long storage periods can lead to nutrients and carbon losses. Castellanos-Navarrete et al. (2015) conducted a study in western Kenya regarding manure utilization in farms and indicated that poor manure management led to low nutrient cycle efficiencies, indicating that long periods of storage contribute to nitrogen and phosphorus losses by volatilization and leaching. The authors also noticed carbon losses up to 51% in

the farms monitored, which agrees with previous researches that indicate that poor storage conditions can lead to losses between 30 and 55% of carbon. However, half of the farms counteracted the carbon losses through biomass additions.

Nutrient losses by leaching can lead to a series of environmental impacts, especially on water bodies. One way of reducing this kind of impact is to apply the Ecological Recycling Agriculture (ERA), which is an organic agriculture that aims at closed nutrients cycles (Granstedt et al. 2008), and has potential to decrease nitrogen surplus in agricultural soils and its leaching due to increased nutrient efficiency (Granlund et al. 2015). Granlund et al. (2015) carried a study regarding Finnish agriculture catchments and simulated a theoretical crop rotation developed to represent ERA cultivation, with considered N fixation, mineralization, and manure as nitrogen sources. The authors observed reductions up to 33% in nitrogen losses on fields working in ERA when compared to those from conventional agriculture, which is mostly based on chemical fertilizers. Manure utilization in agriculture can also increase soil organic carbon, improve soil physical and biological properties, and lead to reduced carbon losses, especially when farmyard manure is applied (Baldivieso-Freitas et al. 2018).

The study carried by Castellanos-Navarrete et al. (2015) shows the importance of manure application in agriculture, once 43% of the total nitrogen inputs into the maize fields studied came from storage manure. Yet these amounts were insufficient to prevent major nutrient depletion in most farms analysed, it was still a considerable contribution to soil quality, especially in situations similar to those observed in the study, where at many times farmers lack resources to work and struggle to overcome difficulties. Thereby, they observed that composted manure provided the largest N inputs to the soil and similar amounts of carbon to those coming from crop residues left in the fields.

Although manure application has its advantages, Baldivieso-Freitas et al. (2018) indicate that research should consider studies about the effects of applying more stabilized organic matter, as it may be better to enhance soil quality and increase the organic matter contents in the soil. Given that, anaerobic digestion is a good way of treating animal manures in order to produce biogas, once they provide the adequate organic substrate. In addition, it is possible to mix horticultural fruit wastes with the manure, once they cannot be processed alone. Iocoli et al. (2019) carried a study in which different animal manures and onion waste were treated by anaerobic digestion, and each manure and its products of digestion and co-digestion had their fertilizing properties evaluated. The authors could notice large differences in the composition between the unprocessed wastes while the digestates had similar characteristics, which complies with the capacity of anaerobic digestion to generate more uniform products.

In this sense, it is possible to infer that the digestate is a more uniform fertilizer than the unprocessed manures. However, a high hydraulic retention time must be applied in the biodigester, in order to promote full degradation of the organic matter, as lower HRT may lead to the production of an unstable digestate, which can cause unpleasant smells, storage problems, and negative impacts on crops (Iocoli et al. 2019).

In addition to the above characteristics, excreta may contain pathogenic microorganisms and micropollutants (i.e. drugs residues and hormones). The highest concentration of pathogens is found in the faeces fractions, while the highest concentration of micropollutants in the urine fraction of excreta.

Inactivation of pathogens is recognized as a limiting factor for the reuse at any scale. Similarly, the risk posed by micropollutants in the environment, especially in the aquatic environment, is recognized as a serious environmental problem, although its consequences are still poorly understood (Carrington et al. 1991; Winker 2010; Fatta-Kassinos et al. 2011). However, from another perspective the presence of micropollutants in low concentrations is not considered a limiting factor for recycling, since it is believed that the excreta application to the soil ends up promoting an additional barrier against its direct release in the water bodies.

Enteric pathogens are excreted mainly on faeces of infected organisms, which can be human and/or animal. Some pathogens of relevance to public health are presented in Table 12.2, as well as its reservoir (e.g. human or animal faeces), the diseases each pathogen develops on human, the concentrations that are excreted per gram of faeces, the duration of the shedding of the pathogens on faeces, and the infectious dose for the disease to develop in another person. All of the presented pathogens are zoonotic, i.e. pathogens that are able to infect both animals and humans. The knowledge and collection of data on pathogen content in the biofertilizers produced from excreta are an essential step for conducting the risk analysis.

Disease propagation depends on factors such as microorganisms' survival in the environment and the required infectious dose to infect a susceptible individual. Pathogens' survival in the environment varies according to each group and species: virus, for example, are not able to multiply outside a host; however, they are able to maintain at a stable concentration or decrease over time. Protozoa are also unable to multiply in the environment, but they are highly resistant, even to most disinfectants. As for bacteria, some groups are able to multiply in the environment, while others are able to persist or decrease, depending on factors like nutrient availability and temperature (Leclerc et al. 2008). Pathogens' infectious dose represents the amount of organisms necessary to cause an infection; i.e. the lower the infectious dose, less microorganisms are necessary to cause an infection (Griffin and Tauxe 1991).

The amount of microorganisms excreted from an infected organism, as well as the duration of shedding, varies depending on the pathogen and the host. However, for most pathogens presented in Table 12.2, the amount excreted in faeces is considerably high, what changes for each pathogen is the duration of shedding, which varies from days to weeks. Furthermore, the infectious dose is lower for some pathogens, such as *Escherichia coli*, *Shigella spp.*, Rotavirus, and *Giardia intestinalis*, while higher for others. This means lower doses of these microorganisms are needed to develop an infection. These pathogens should get extra attention when conducting QMRA studies on reuse of human and animal excreta for agriculture. It is also important to evaluate zoonotic pathogens, as they are of greater importance to public and animal health.

Table 12.2 Pathogenic microorganisms present on faeces and its characteristics, sources, diseases, and infectious dose

Microorganism	Reservoir and source	Disease	Zoonotic disease	Concentration excreted or detected	Duration of shedding	Infectious dose	Reference
Escherichia coli O157	Human and animal faeces	Enteritis	Yes	Human: 10^8 bacteria/g faeces Calves: 10–10^7 c.f.u./g faeces	7–13 days	<700	Ashbolt (2004), Ottosson (2003), de Roda Husman and Schets (2010), Coia (1998), Besser et al. (2001), Tuttle et al. (1999)
Salmonella spp.	Human and animal faeces	Salmonellosis	Yes	Human: 10^4–10^{10} bacteria/g faeces	2–7 days	10^3	Ashbolt (2004), Ottosson (2003), Blaser and Newman (1982), Haas et al. (2014)
Shigella spp.	Human and animal faeces	Shigellosis	Yes	Human: 10^6 bacteria/g faeces	30 days	10–100	Ashbolt (2004), Ottosson (2003), de Roda Husman and Schets (2010), Levine et al. (1973)
Vibrio cholerae	Human faeces and freshwater zooplankton	Cholera	Yes	Human: 10^6–10^9 bacteria/mL of rice-water stool; 10^2–10^5 bacteria/g faeces	3–5 days	Diarrhoea: 10^8–10^9, Cholera diarrhoea: 10^{11}	Ashbolt (2004), Ottosson (2003), de Roda Husman and Schets (2010), Smith et al. (1961), Hornick et al. (1971), Dizon et al. (1967)
Hepatitis E virus	Human and animal faeces	Infectious hepatitis; miscarriage, and death	Yes	Pigs: 2.7–6.2 × 10^6 genomic copies/g faeces	30 days	$10^{4.5}$	Ashbolt (2004), Ottosson (2003), Tsarev et al. (1992), Kasorndorkbua et al. (2004), Meng et al. (1998)
Rotavirus	Human and animal faeces	Enteritis	Yes	Human: 10^{10} infectious particles/g faeces Swine digestate: 7–10 GC/L	4–6 days	10–100	Ashbolt (2004), Ottosson (2003), de Roda Husman and Schets (2010), Fongaro et al. (2014)

(continued)

Table 12.2 (continued)

Microorganism	Reservoir and source	Disease	Zoonotic disease	Concentration excreted or detected	Duration of shedding	Infectious dose	Reference
Cryptosporidium parvum	Water, human, and other mammal faeces	Cryptosporidiosis	Yes	Human: 16^6–10^7 oocysts/g faeces	1–2 weeks	8–1042	Ashbolt (2004), Ottosson (2003), de Roda Husman and Schets (2010)
Giardia intestinalis	Water and animal faeces	Giardiasis	Yes	Cattle: 3.10^3 cysts/g faeces	Highest intensities 2–6 weeks (age of lambs)	10–100	Ashbolt (2004), Ottosson (2003), de Roda Husman and Schets (2010)
Ascaris lumbricoides	Human and pig faeces	Ascaridiasis	Yes	Human: 10^4–10^5/g faeces	–	–	Ashbolt (2004), Ottosson (2003), Haas et al. (2014)

12.4 Inactivation of Pathogens During the Biogas Production—Anaerobic Bioreactors

The natural inactivation of pathogens is usually a slow process, and because of that, it is necessary to apply treatments that seek the disinfection in effluents and other wastes, such as human faeces, animal manures, and digestate from anaerobic biodigesters (Semenov et al. 2007; Sidhu 2001).

During the process of anaerobic biodigestion, 90% of the enteric bacteria and viruses can be inactivated (Fongaro et al. 2014). However, it is important to emphasize the importance of applying high-efficiency treatments, in order to minimize 99.9% of most pathogens, depending on the initial pathogenic load and the purpose of the recycling, keeping in mind the safety when recycling the digestate.

Methods based on alkalinity are among the main treatments used to reduce pathogens in environmental matrices, which consist of adding alkaline compounds such as ash and lime in the waste. The efficiency of this process is mainly due to the elevation of the pH that alkalinizes the cellular cytosol, as well as interferes with protein activity, inactivating microorganisms (Magri et al. 2013; Chandran et al. 2009).

As an effect of alkali treatment, non-ionized ammonia—NH_3—is generated, which is an important biocidal agent. Therefore, wastes with high ammonia concentrations, as sludges and digestates from human and animal manure biodegradation, have good potential for ammonia sanitization. It is observed that this treatment needs to be performed outside the biogas reactor. The mechanism of pathogen inactivation mediated by NH_3 is due to the solubility of ammonium in the lipids, which facilitates its entry and diffusion in the cells, being able to act in cell destabilization, membrane destruction, and protein denaturation (Emmoth et al. 2011; Bujozek 2001). There is little research about the virucidal mechanism of NH_3. However, it is reported that it leads to a cleavage in viral genetic material and small viral structural changes. Thus, viruses can be prevented from entering the host cell as well as from replicating (Decrey et al. 2015). In addition to NH_3, the carbonates (CO_3^{2-}) formed as a consequence of pH and chemical equilibrium, probably due to organic matter decomposition, are also reported by their biocidal action (Magri 2013; Chandran et al. 2009).

As an example, it is possible to mention Fongaro et al. (2014) that studied pathogen inactivation based on free ammonia in swine digestate in Brazil, reaching inactivation in the order of 7 log 10 and 4 log 10 in 23 days of treatment for inactivation of enterobacteria and enteric viruses, respectively.

The use of heat in hygienic or disinfection processes is widely used, once it leads to structural protein denaturation, enzymatic inactivation, and nucleic acids denaturation, thus irreversibly preventing pathogens' replication in excreta and digestates, being widely used (Fong and Lipp 2005). Maheshwari et al. (2004) applied temperatures of 50 °C in order to inactivate adenovirus in substrata used for biodigestion and reported a significant inactivation of 4.0 log 10 decay of HAdV 5 in 10 min of exposure.

Some of the most important parameters to consider during the thermal inactivation process are the decimal reduction time (DT), which expresses the time required to reduce a logarithmic unit at the concentration of viable cells at a given temperature (*T*), and the thermal coefficient (*z*), which represents the temperature difference required for the reduction of a logarithmic unit in the DT value. They are both related to the degree of heat resistance of a microorganism in a given matrix (Wigginton et al. 2012; Pecson et al. 2007). However, it should be noted that the use of heat requires energy expenditure that could be used for other purposes.

In this context, the application of economically feasible techniques to reduce pathogens in environmental matrices, such as those generated from the biodigestion process, is extremely important for the reduction of microbiological risks in the nutrients recycling in this process.

12.5 Scenarios and Data of Risk Exposure on the Biogas Production Chain and Risk Management

In the biogas treatment plants, as well as in the sites where the recycling of excess sludge and digestate is done, there are several possible routes where microbial risk exists in different extents. Here, we focus on the risk posed by sludge and digestate during its application on land for agricultural purposes since that is considered the most sustainable use in the context of the circular economy.

The production of digestate and sludge and the possible general routes of risk exposure to human and animals are represented in the series of Figs. 12.1, 12.2, 12.3, 12.4, 12.5, 12.6.

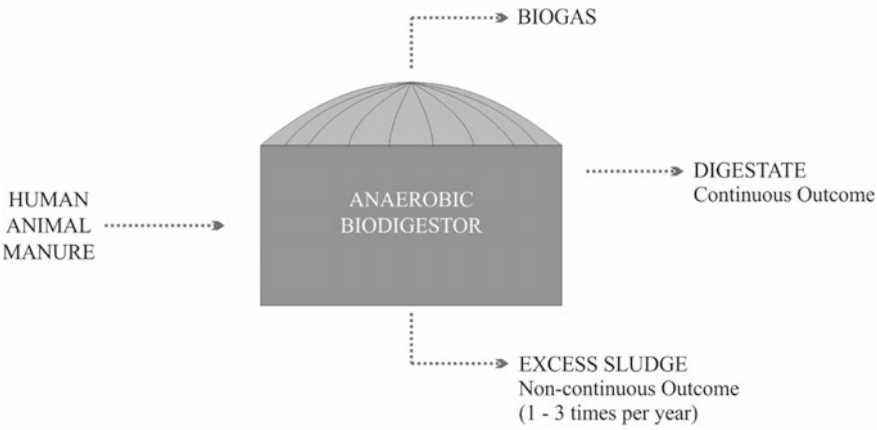

Fig. 12.1 Digestate and excess sludge formed during the anaerobic digestion and biogas generation process

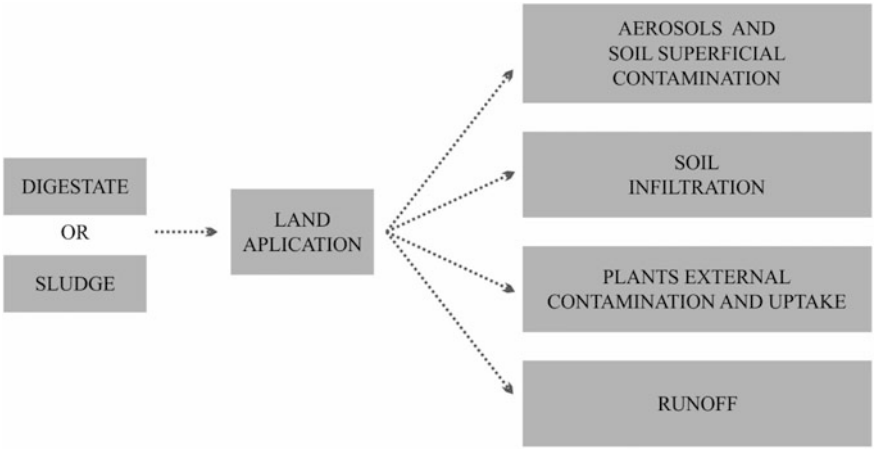

Fig. 12.2 General view of main exposure paths and sources of microbial contamination after land application of digestate or sludge

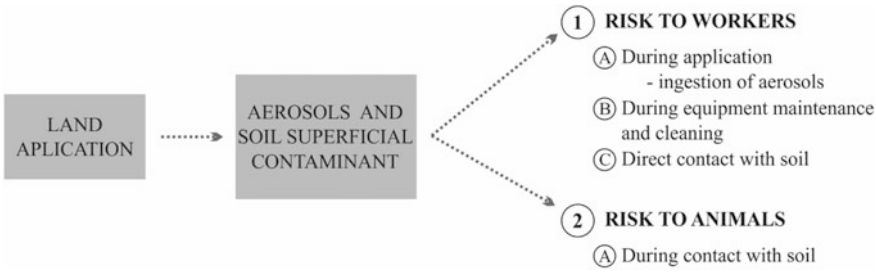

Fig. 12.3 Exposure routes related to the generation of aerosols and superficial contamination of soil during land application of digestate and/or sludge

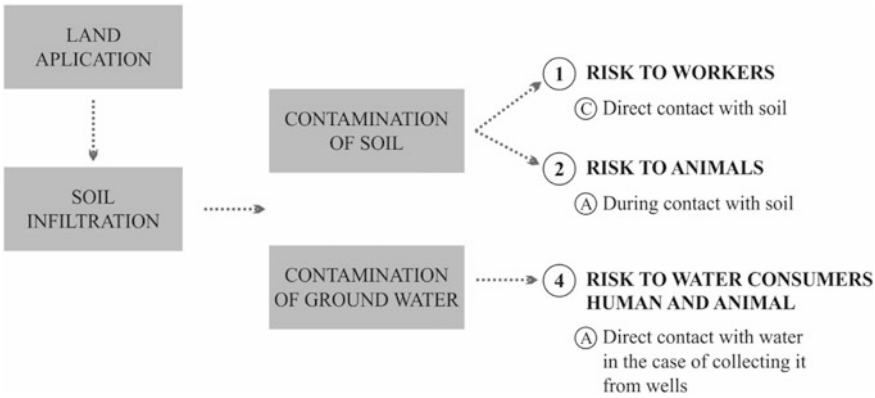

Fig. 12.4 Exposure routes related to soil infiltration of digestate and/or sludge with consequent soil and groundwater contamination

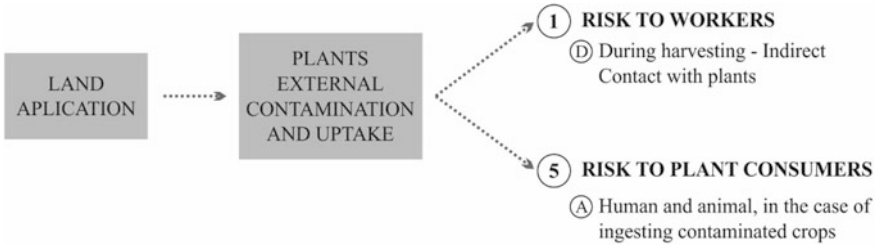

Fig. 12.5 Exposure routes related to the contamination of the external and internal parts of plants after application of digestate and/or sludge

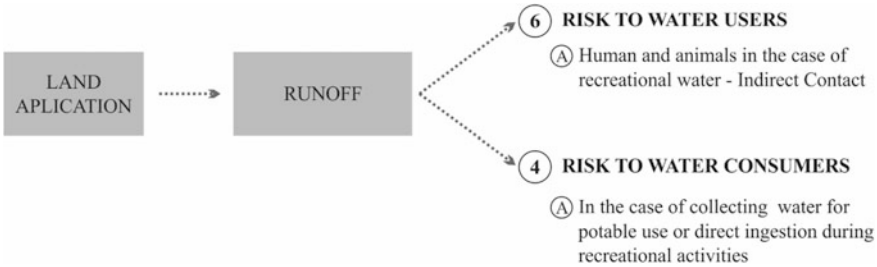

Fig. 12.6 Exposure routes related to the contamination of water bodies as a consequence of rain events promoting the runoff in the fertilized area with digestate and/or sludge

During manure application on soil, some main routes of exposure of humans and animals can be highlighted: exposure to aerosols and soil contaminated superficially (Fig. 12.3); exposure to contaminated groundwater by soil infiltration of excreta (Fig. 12.4); exposure to contaminated plants (crops) (Fig. 12.5); and exposure to superficial water contaminated by agricultural runoff (Fig. 12.6).

The ingestion of contaminated soil and aerosols is normally referred to as indirect contact and accidental ingestion. Workers (farmers in that case) accidentally ingest soil as a consequence of hands to mouth gesture (faecal–oral route), and hands can get contaminated during soil labour, harvesting crops, or still during maintenance of equipment and tools used. Julian et al. (2018) simulated (100 simulations per farmer) and predicted final *Escherichia coli* concentrations across farmers for both hands of 1.4 ± 1.3 log 10 CFU/cm^2. In this case, *E. coli* indicates the potential for a physical adhesion of enteric pathogens in hands. Comparing the concentration found for Julian et al. (2018) with the concentration of *E. coli* in human faeces of 10^8 log 10 (Table 12.2), each cm^2 was contaminated with approximately 0.0001% of *E. coli*, which might be sufficient for illness, in the case of pathogenic *E. coli*.

Moazeni et al. (2017) estimated the infection risk for farmers in contact with contaminated soil irrigated with an effluent containing 12–16 pfu/mL of

enterovirus. The probability of infection was 8.8×10^{1} per person per year for farmers, which is about 2 log higher than the tolerable infection risk of 2×10^{3} pppy targeted in the study.

The modelling for assessing risk can be conducted also from the opposite perspective. For instance, Kobayashi et al. (2017) targeted the tolerable burden of disease of 10^{-4} and 10^{-6} for disability-adjusted life year loss per person per year (DALYS) and calculated a threshold reduction level for norovirus gastrointestinal illness (GII) that would be required for the use of a sewage effluent for agricultural irrigation. That approach is widely used on decision-making and management processes, as well, for legislation.

Understanding the behaviour of enteric bacteria following application to soils is an important element in predicting exposure to adjacent water, and the development of regulatory guidelines to manage the risk of faecal contamination of water from soils that have received manures (Haas et al. 1999).

The chemical composition of livestock wastes and the treatment applied after the biogas reactor are some of the elements that will influence the dynamics of surviving enteric bacteria following application to soils (Topp et al. 2009).

Topp et al. (2009) compared risk for application of fresh untreated manure and treated manure for which a 3-log reduction was achieved prior to land application. In the absence of treatment, the risk of infection (expressed as a probability of risk of infection per event, as a point estimate) from Cryptosporidium was 1.75×10^{4} and from Campylobacter 1.27×10^{2}. In contrast when considering treated livestock waste that had a 1000-fold reduction in pathogen content, the risk from Cryptosporidium was 1.75×10^{7} and that from Campylobacter was 1.27×10^{5}.

Herein, we can state as an important evaluation, the multi-barrier approach for managing risk from shed microorganism. An effective multi-barrier strategy has three major components, according to Topp et al. (2009): (1) managing herd health to minimize the acquisition, potentiation, and release of zoonotic pathogens into the manure; (2) management of the manure during storage to effect a reduction in pathogen content prior to release into the broader environment; and (3) application of the material to land at a judicious rate, and under suitable land, climate, and crop conditions to minimize the off-site movement of contaminants into adjacent surface or groundwater.

The multi-barrier approach was at first considered to water supply systems, aiming to assure safe drinking water, and since then, people are beginning to shift their focus from compliance monitoring to the more holistic approach.

During the application of biofertilizer on soil, there is also a risk related to aerosols, as cited above. According to Courault et al. (2017), the risk decreases with increasing distance from the emission source and that wind speed has a great impact on atmospheric dispersion.

For the exposure route presented in Fig. 12.5, the consumption of contaminated water or plants can be understood as direct contact. The barriers applied for reducing the risk in this situation vary from cleaning vegetables before consumption to the application of water treatment processes.

Moazeni et al. (2017) estimated the risk for lettuce consumers and as result a lower level of infection and disease burden (about 10^{-3}) but higher than the guideline threshold of 10^{-4} DALY pppy (WHO).

Mok et al. (2014) evaluated the risks for both situations, eating food washed and non-washed. The median probability of infection for washers ranged from 2.94×10^{-8} to 1.51×10^{-3}, and the median probability of illness per dose ranged from 1.35×10^{-16} to 3.52×10^{-7}. For non-washers, the probabilities were one order of magnitude higher. Other important factors that can influence risk in the cited situations are: type of vegetable, type of irrigation system, temperature and type of soil, time for consumption after harvesting, and "resting" period after manure application (Mok and Hamilton 2014; Amoah et al. 2018). For instance, consumption of lettuce grown on sludge-amended soil will result in probable infections but harvest after 30 days between sludge application and harvest in the study presented by Amoah et al. (2018) gave median probability infection risks with a risk level similar to the WHO tolerable risk value (10^{-4}).

For the scenario of agricultural runoff and consequent superficial water contamination, several parameters can interfere with the risk extent. Sensitivity analysis is usually performed to investigate how variability of the outputs can be apportioned quantitatively to different sources of variability in the inputs. Clarke et al. (2017) show that the parameter of importance that affected the variance in model predictions for ingestion of contaminated river water with *E. coli* was time in the stream, which highlights the importance of residence time of bacteria in that environment. The time that bacteria stay in the stream allows their contact with environmental inactivation factors such as temperature, pH, and photolysis, which may in turn influence the growth or die-off rates. The other parameters of importance were the water intake and initial counts in surface runoff.

12.6 Final Considerations

The recovery of biogas for energy production in treatment plants for waste anaerobic biodigestion is already a reality in many countries. Conventional plants are moving from "treatment" plants to become "resource recovery" plants, and even added value can be obtained by the organics and nutrient recycling of the accumulated sludge and digestate outcomes from the bioreactors. However, for conducting a safe nutrient recycling in agriculture, the pathogen content is a limiting factor. In that context, we presented the quantitative microbial risk assessment as an effective approach to estimate risks, which can be applied to any scenarios of recycling liquid fractions from biogas reactors in agriculture.

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Chapter 13
Current Efforts for the Production and Use of Biogas Around the World

Aline Viancelli, William Michelon and ElMahdy Mohamed ElMahdy

Abstract Biogas is a renewable energy source that can be generated from the digestion of a variety of organic materials and waste. Organic wastes used for biogas include animal manure, human excreta and other agricultural wastes, slaughterhouses and food industries residues or even urban solid waste. However, in some developed countries it has been used corn, barley, sunflower and sorghum as other energy sources. Biogas systems differ strongly between locations, form, cost structure and usage patterns. This difference is mainly related to the development condition of the country. When implemented properly, biogas systems can serve multiple purposes. Digesters are considered a clean and alternative technology that can help distant communities with their energy necessities by improving living conditions or even economical source. Considering this, the present chapter will be addressed: (i) Biogas production around the world; (ii) Feeding material used in different continents to generate biogas; (iii) usage of biogas produced.

Keywords Biogas substrates · Bioenergy · Anaerobic digestion
Renewable energy

13.1 Biogas Production Around the World

Biogas is the product of a biologically mediated process resulting from anaerobic digestion. Biogas consists mainly of methane (CH_4) around 50–70% and carbon dioxide (CO_2) in a concentration of 30–50%, where the concentration of CH_4 and CO_2 in biogas depends mainly on the substrate type (Angelidaki et al. 2018).

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In addition to CH_4 and CO_2, biogas also contains slight amounts of N_2 (0–3%), H_2O (5–10%), O_2 (0–1%), H_2S (0–10,000 ppmv) and NH_3 (Angelidaki et al. 2018; Muñoz et al. 2015).

Biogas systems differ strongly between locations, form, cost structure and usage patterns. This difference is mainly related to the development condition of the country. An increasing number of biogas plants in operation have emerged worldwide, in recent years. Biogas production is concentrated in Europe and in the United States (REN21 2018) however, the majority of anaerobic digesters are implemented in Asia, where the most of them have been used in rural communities for cooking and lighting (KC et al. 2014; Vasco-Correa et al. 2018). In Europe the number of biogas plants has increased (Fig. 13.1), with more than 14,500 biogas systems implemented, and the number is increasing (European Biogas Association 2014; Grando et al. 2017).

The clear leaders are Germany and Sweden, with digestors on the agricultural scale, with around 9000 of them, and its goal is to have about 10,000–12,000 digester by 2020 (Wilkinson 2011). However, other countries such as United Kingdom, Switzerland, United States, South Africa and Brazil also have built biogas plants (Fig. 13.2) (Angelidaki et al. 2018). In 2013, the number of biogas plants in Hungary, Czech Republic, Slovakia and Poland increased by 18% (Grando et al. 2017). Otherwise, in Latin America, it has increased the number of domestic biogas systems, especially in rural areas (Garfi et al. 2016; Grando et al. 2017).

In Africa the installation of biogas digesters were done in many countries such as Botswana, Cote d'Ivoire, Burkina Faso Burundi, Ethiopia, Rwanda, Senegal, Ghana, Guinea, Lesotho, Kenya, Namibia, South Africa, Nigeria, Zimbabwe and Uganda. The Africa Biogas Partnership Programme (ABPP) was created aiming to provide access to energy services in some African countries as Tanzania, Ethiopia, Uganda Kenya and Burkina Faso (SNV 2010).

In the United States there is a panorama for the anaerobic digestion industry increase with the potential to generate energy to 1.1 million houses using manure from 8000 dairy and hog farms (Vasco-Correa et al. 2018), nowadays there

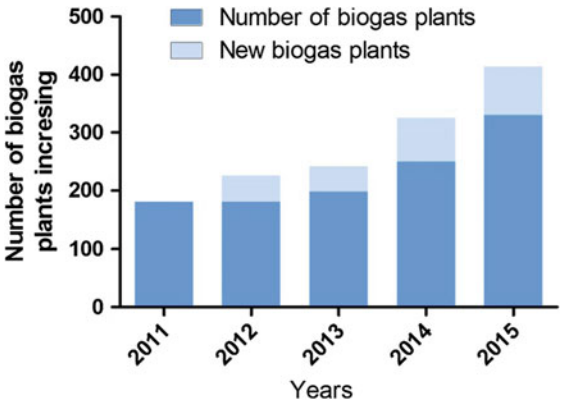

Fig. 13.1 Increasing biogas plants over years, all around the world (Angelidaki et al. 2018)

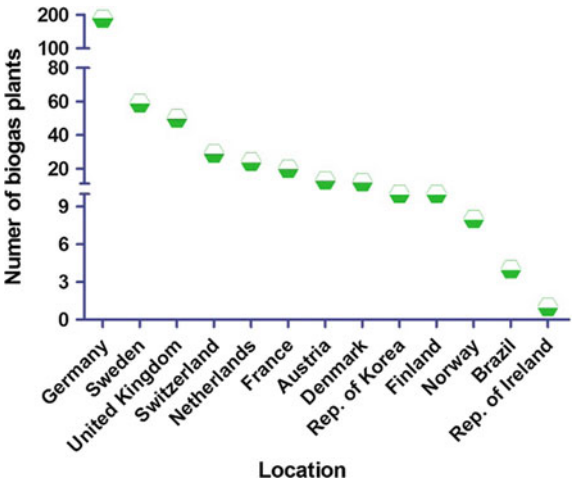

Fig. 13.2 Location and number of existing biogas plants (Angelidaki et al. 2018)

are approximately 250 real scale anaerobic digesters, around 1250 wastewater treatment plants and 38 industrial plants (Vasco-Correa et al. 2018). In addition, there are nearly 2500 wastewater treatment plants with capacity to produce biogas, however, a huge number are not using the biogas produced (American Biogas Council 2017; Vasco-Correa et al. 2018).

North America has increased the interest in implementation and use of anaerobic digestion. Canada has more than 100 biogas systems. In addition, Mexico also increased the interest in biogas plants implementation and in the use of existing biogas for energy generation rather than burning, is increasing (Alemán-Nava et al. 2015; CBA 2017).

In the Latin America despite the significant potential of anaerobic digestion, its operation has been slow. To fill this gap, was created in 2009 the Network for Biodigesters in Latin America and the Caribbean (RedBioLAC), aiming to improve information of this tools in the country (Garfí et al. 2016). RedBioLAC has helped in the installation of several agricultural and household biogas power plant in Ecuador, Costa Rica, Nicaragua, Mexico and Peru; besides, in Bolivia more than 1000 household biogas plants were implemented (Kapoor and Vijay 2013; Grando et al. 2017).

13.2 Feeding Material Used in Different Continents to Generate Biogas

Anaerobic digestion could be used for treatment of liquid and solid waste, and all around the world, many different substrates have been used in anaerobic digestion for biogas production (Fig. 13.3): agricultural waste, agricultural crops (barley, sunflower and sorghum), manure, human excreta, municipal solid waste, food

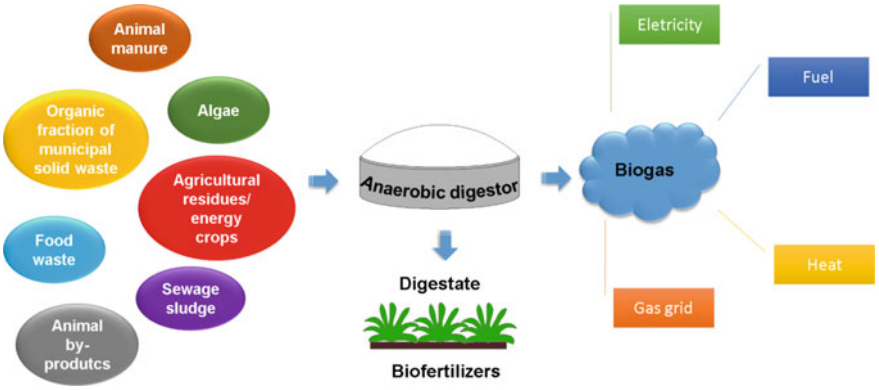

Fig. 13.3 Schematic representation of substrates used for biogas production and its application

residues, effluent and industrial effluents (from the most different origins, as food industry, wine sector, cassava), almost all materials with high organic load could be used (Krzywika and Szwaja 2017; Guerini Filho et al. 2018).

According to the Global Intelligence Alliance (2010), the potential materials for biogas production worldwide are: 75% in crops, manure and by-products; 17% in municipal wastewater and industrial effluent; and 8% in manure treatment plants. However, according to Global Methane Initiative (2018), the actual distribution of biogas plants, are majority represented by systems fed with municipal solid waste, followed by agriculture manure and wastewater reuse (Fig. 13.4).

Sometimes the substrates need to pass by a pre-treatment step, for example microalgae (Passos et al. 2014), poultry litter (Costa et al. 2012), corn stalks (Venturini et al. 2018) and corn stover (Bondesson et al. 2013); or be co-digested, when materials are put together aiming to improve the plant efficiency and synergistic effects (Luostarinen et al. 2011), for example swine carcass/swine manure (Tápparo et al. 2018), manure/lignocellulosic materials (Tsapekos et al. 2017), food residues and straw (Yong et al. 2015).

Europe has mainly two operation systems for digesters: "centralized" systems and "agricultural scale" digesters. Centralized system or set codifies the livestock manure of various farms with other organic materials, such as food, domestic and agricultural waste. In this model, a part of the digested is returned to farms where can be utilized as fertilizer, or sold to other producers (Holm-Nielsen et al. 2009; Wilkinson 2011; Vasco-Correa et al. 2018). Denmark is a innovator in the development of "centralized" or biogas systems, with about 150 biogas plants and 20 centralized systems, and they plan to upsurge their capability by 50% by 2020 (Holm-Nielsen et al. 2009; European Biogas Association 2015). These centralized plants have large capacity digesters up to 8000 m^3 (Nielsen and Angelidaki 2008). In Denmark the main substrate is manure with other co-substrates added to increase yield (Skovsgaard and Jacobsen 2017).

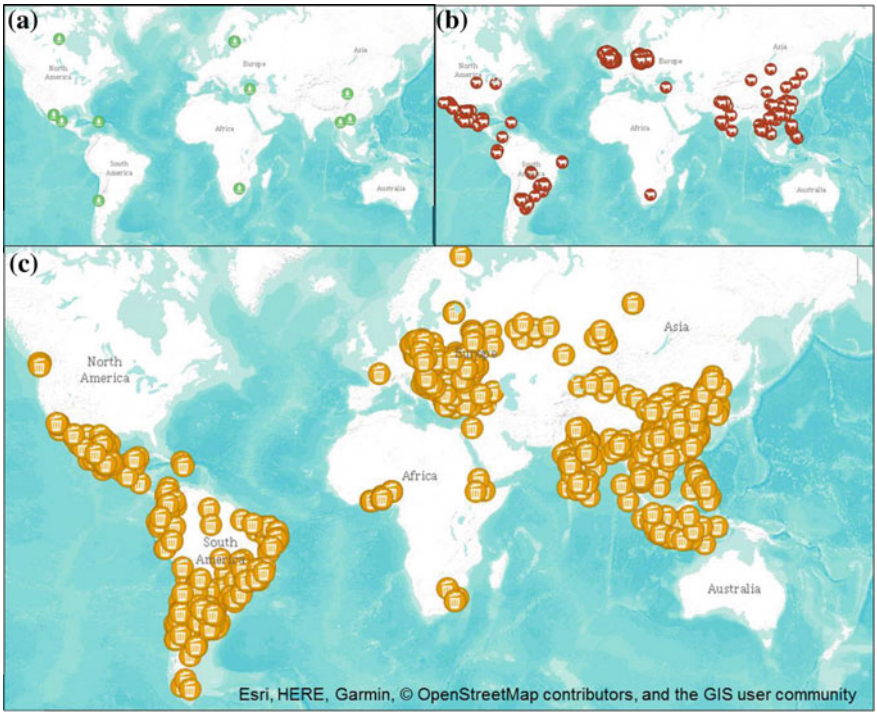

Fig. 13.4 Global distribution of materials used as biogas substrates: **a** wastewater reuse; **b** agriculture manure; **c** municipal solid waste (Global Methane Initiative 2018)

Biogas digesters have utilized a huge diversity of substrates along of African countries from north to south such as industrial effluent such as sugar cane bagasse (lignocellulosic waste from sugar mills and agricultural processing), animal dung and human excreta, chicken and dairy farms, public latrine, industrial wastewater and municipal solid wastes in north of Africa, as in Egypt (Tuesorn et al. 2013). Human and animal excreta are considered the most common feedstock material for anaerobic digestion and biogas production especially in central and south of Africa using the Chinese and Indian digester model (Omer and Fadalla 2013).

Anaerobic digestion use large quantities from energy crops such as corn, barley, sunflower and sorghum in sub-Saharan Africa, and have been used for warmth and energy production and the effluent can be used after recycling as a minor eco-friendly biofertilizers for agriculture purposes (Al Seadi et al. 2008). Jatropha (*Jatropha curcas* L.), is considered a promising crop for Africa's Biofuel Production in almost nine African countries, as Ghana, Madagascar, Burkina Faso, Lesotho, Malawi, Namibia, South Africa, Swaziland and Zambia, and was successfully used to produce biogas (Hendroko et al. 2015; Jabłoński et al. 2017). In Egypt, there is a huge amount of biomass with potential for using in biogas production (up to 40 million ton) however about 52% of this biomass has been direct

burned. The prevalent form of biomass use in Egypt is biogas in rural areas, where substrates used are rice straw and maize (Bakker et al. 2013; Cooper and Laing 2017). Nevertheless, the amount of biogas produced depends on many issues as the feedstock material amount, the substrate, the time and temperature of digester (Amigun et al. 2012).

In the United States, anaerobic digestion is well established in terms of using manure sludge as a substrate, with about 90% of anaerobic digestion plants implemented in the last years, where most of them (86%) use dairy manure as the main raw material (Edwards et al. 2015; Vasco-Correa et al. 2018). However, some researchers have highlighted the potential of using municipal solid waste, yard waste, paper and paper board (Linville et al. 2015).

In Latin America, it has been used food waste, in small-scale systems. However, there is an enormous potential for using agriculture residue, manure (swine and cattle) vinasse and cassava (REN21 2018).

13.3 Use of Biogas Produced

The biogas sector differs considerably in different parts of the world. Plant size ranges from small-scale households to large plants. Biogas is used in other ways in large parts of the world (IGU 2015). When implemented properly, biogas systems can serve multiple purposes, once that it could be used directly or in diverse ways; biogas could be used for heat, however the most frequent usage is for electricity generation (Vasco-Correa et al. 2018, 2018). Besides, in the context of circular economy, the digestate could be reused as biofertilizer to cultivate crops that could be used on biogas generation.

In 2000, the global energy generation from biogas was about 280,000 TJ and reached almost 1.3 million TJ by 2014, with an typical annual increase of 13.2% in of biogas production (International Energy Agency 2016; Vasco-Correa et al. 2018). In 2013, biogas generation was projected at about 59 billion m^3, with almost half of what has being produced in the European Union (World Bioenergy Association 2017; Vasco-Correa et al. 2018). The global installed capacity for energy production (Fig. 13.5) shows that the capacity increased all around the world in the last 10 years, with Europe been the leader, followed by North America, Asia, South America, Africa and Central America and Caribbean (IRENA 2018).

In Denmark, Finland, Iceland, Norway and Sweden biogas usage is still partial, with Sweden and Denmark taking the lead in their production. While these countries have similar characteristics, the biogas usage is diverse: in Denmark, biogas has been used in coproduction plants, whereas in Sweden it is generally converted to motor fuel (Nordic Energy Research 2010). Anaerobic digestion full-scale farm plants generally present a digester capacity of 200–1200 m^3, and are frequently built on dairy or swine farms (Weiland 2003), where animal manure from different farms are co-digested with agricultural wastes and crops (Wilkinson 2011; Vasco-Correa et al. 2018).

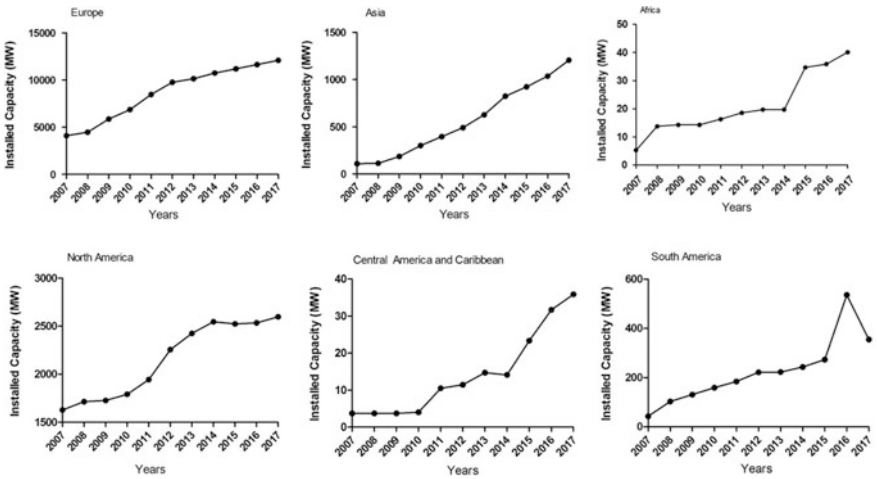

Fig. 13.5 Installed capacity (MW) from all biogas plants installed around the world

In Poland, the potential energy production (39.44 PJ) from biogas could be able to cover 7.5% of the country's energy requirement (Igliński et al. 2012; Grando et al. 2017). Besides the huge amount of challenges on power generation, biogas industry increased in recent years, because Poland could cover about 47% of domestic request for natural gas (Bielski and Marks-Bielska 2015; Grando et al. 2017). Sweden stands out as the country that mainly updates biogas for use as a vehicular fuel (International Gas Union 2015).

Bio-power production (electricity) reached 33 MW in Africa while 10.4 GW in Europe. Africa has large volumes of wastes but biogas production is still less developed than in other regions all over the world. Most of the biogas plants are being used for both cooking and lighting along African countries.

Asia has the highest number of biogas systems implemented, majority of them as domestic digesters that have been used in rural area for cooking and lightning (KC et al. 2014; Vasco-Correa et al. 2018). Latin America rural areas could also use household digesters, but this technology has only been successfully installed in latest years (REN21 2018) with increasing numbers of household biogas plants mainly for cooking and heating (Garfi et al. 2016).

The United States showed shy implementation of anaerobic digestion, with about 2100 current operating plants (American Biogas Council 2017), however, the operating plants use biogas generated for electricity generation and fuel boilers (Edwards et al. 2015).

Full-scale biogas systems have been constructed to use palm oil mills wastes generated in farms in Argentina, Colombia and Honduras (Kapoor and Vijay 2013). Brazil had 127 biogas plants using agroindustry residues, biosolids, sewage, and landfill gas, producing about 584 billion m^3 biogas/year, representing 3835 GWh

Table 13.1 Biogas production profile around the world

Countries/ regions	Feeding material	Biogas generated or potential (m^3/year)	Destination of biogas	References
European Union	Sewage sludge, landfill, manure, Agriculture crops and waste	18,207 mil	Electricity, heat	European statistics (2017)
China	Wastewater, food waste	15 billion	Electricity, cooking, lighting	IRENA (2018)
India	Lignocelulotic, agriculture crops, food waste, wastewater	10 billion	Cooking	Ministry of New and Renewable Energy (2014)
Africa	crops, wastewater	12.8 billion	Organic solid wastes	Rupf et al. (2015)
United States	Manure, landfill, wastewater	18.5 billion	Electricity	US EPA (2017)
Brazil	manure, industrial residues, biowaste, sewage sludge	584 billion	Electricity	REN21 (2018)
Uruguay		54–84 million	Electricity	López (2016)
Colombia		6000 million		López and Borzaccooni (2017)

of energy in 2015 (International Energy Agency 2016; REN21 2018; Scarlat et al. 2018) In the last years, the biogas electricity production capacity increased from 196 MW in 2015 to 450 MW in 2016 (IRENA 2018) (Table 13.1).

13.4 Final Remarks

Biogas has a huge potential as an alternative energy source, and also could help with many different waste/residues destination. However, the improvement of this technology will depend on the interactions between diverse areas as agriculture, rural development, energy production and policies. Besides, it is of special concern the technology transference know-how and adaptation to the local conditions, once that the well working state of anaerobic digestors is directly influenced by environmental parameters as temperature.

Otherwise, biogas production is a ecofriendly technology, that has the advantage of generate a digestate rich in nutrients, that make its and excellent biofertilizer or soil conditioner.

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Chapter 14
An Overview About of Limitations and Avenues to Improve Biogas Production

Helen Treichel, Sergio Luiz Alves Junior, Caroline Müller and Gislaine Fongaro

Abstract Worldwide, biogas production has been successfully happening in rural and urban areas, catering to livestock and industry. However, there are great obstacles to be overcome and public policies to be developed aiming at the materialization of biogas plants for green energy purposes and recycling of nutrients. In this context, this chapter will discuss the main challenges encountered worldwide in the biogas chain, highlighting the scenario and innovations on biogas chain and the legal and administrative framework/incentives for biogas production and uses.

Keywords Innovations · Bioenergy · Biotechnology · Green energy Administrative framework

14.1 Scenario and Innovations on Biogas Chain

The need to mitigate greenhouse gas (GHS) emissions to climate change control and global energy demands has boosted and stimulated production and biogas. The Paris Agreement, signed at the 21st Conference of the Parties (COP-21) in December 2015, continued the global actions to mitigate GHS emissions, where

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countries signatories of the agreement have entered into national commitments called the National Contribution, to reduce its GHG emissions and prevent of climate change (UNFCCC 2017).

In this context, the biogas is a clean and green source of energy that can contribute to the reduction of carbon footprints. However, small part of the biogas production potential is used in the world, and the usual energy from biogas will depend of composition and properties of biogas, according to feedstock types and their pretreatment and digestion systems model, considering temperature, pH and retention time as main components. Policies for regulation and encouragement of biogas production and use are essential to foster this green energy chain.

This chapter discusses trends and challenges in the chain of biogas, with a view of perspectives (pretreatment, new systems and methods) on the production and yield of biogas, as shown in Fig. 14.1, which outlines key points in this chain.

Biogas production can be carried out from a wide diversity of raw materials, combined or not. The choice for feedstock or substrate should take into account regional availability and potential. This leads to several forms of process optimization for the various substrates (Sun et al. 2015). In addition, the conduction forms are also variable, including different metabolic profiles from the employed microorganisms. Such differences range from the way of obtaining energy and carbon (since the production of methane can occur by secondary fermentation of acetate in chemoorganotrophs or by consumption of H_2 and CO_2 in chemolitotrophs) to the optimum temperature for microbial metabolism.

Regarding the efficiency and yield gain in the biogas production, the concentration of methane has a prominent place, given the extreme importance of CH_4 for the potential of biogas application and valorization. In addition, some impurities can have significant negative impacts on the utilization system, such as corrosion,

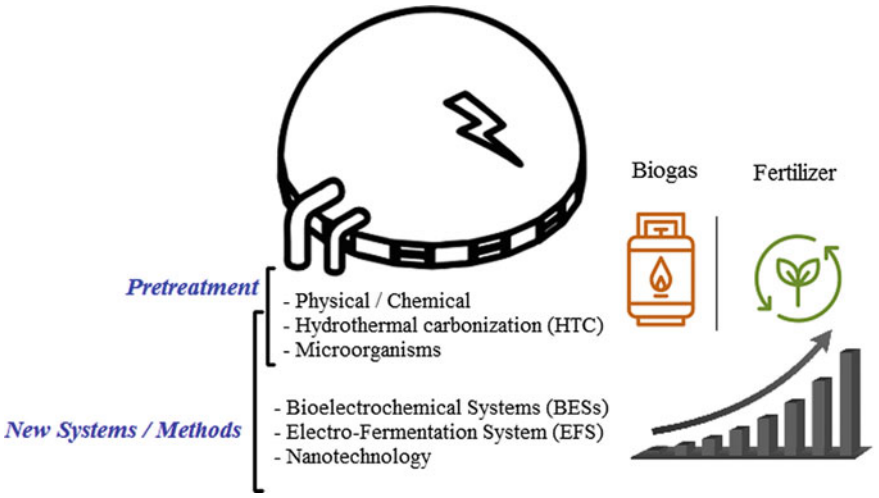

Fig. 14.1 Key points in the biogas chain described in this chapter

uncontrolled emissions and increased risk to human health. In this sense, different biogas cleaning and upgrading technologies have shown to be very promising and have attracted great interest from the bioenergy industry (Sun et al. 2015). Using thermophilic upflow reactors, Bassani et al. (2017) obtained an upgrade from 23 to 96% in the methane content, with the totality of H_2 and CO_2 externally provided being converted to CH_4. With two upflow reactors in series or with bubble column reactors, and with recirculation of the gas produced (for reuse of H_2 and CO_2 by hydrogenotrophic methanogenesis metabolism), Kougias et al. (2017) upgraded the methane content to 98%. More recently, Bu et al. (2018), using a biogas bio-upgrading technique with coke oven gas injection under thermophilic and extreme-thermophilic conditions, verified not only gain in methane yield, but also reduction of lag phase by one-fifty. However, there must be considered the financial, energy and environmental costs for biogas upgrading, in order to verify the feasibility of each strategy employed. More than simply choosing the cheapest technology, it is necessary to select the most appropriate for each circumstance, since the greatest advances are obtained when the technology employed is site-specific and case-sensitive (Sun et al. 2015).

14.1.1 Co-digestion and Pretreatment

Recent studies describe several kinds of co-digestion as alternative technologies for increasing biogas yield in biodigesters (Adelard et al. 2015). In addition to the use of municipal waste, food and animal waste, co-digestion can be performed using crops and animal manure, which is one of the major stakes in increasing biogas production (Wangliang et al. 2016). On the other hand, this strategy is not just a simple mixture; it is necessary to measure the capacity of biogas production and it is imperative to analyze the proportions of each substrate added in order to ensure the highest biomethane potential possible. Valenti et al. (2018), for example, tested six different feedstock mixtures and, when they evaluated their biomethane potential, technical feasibility and economic feasibility, authors verified a difference of up to 100% between the potential of the different tests performed.

Taking into account the co-digestion with lignocellulosic biomass, the pretreatment of the feedstock can contribute greatly with the optimization of the process, in such a way that different research groups have looked for economically viable alternatives to reach this purpose (Paudel et al. 2017). Recently, Thomas et al. (2018) observed an increase of up to 37% on the biochemical methane potential (BMP) using lime (CaO) as a pretreatment of *Miscanthus* biomass. On the same track, Venturin et al. (2018), using swine manure and corn stalk as substrate, detected a 22% increase in the final volume of biogas and a reduction of more than 60% in the time required for digestion, when the lignocellulosic biomass was pretreated with hydrogen peroxide.

The hydrothermal carbonization (HTC) is also an important low-cost alternative for pretreatment of raw materials. It has recently been found that the addition of

hydrochar on food waste was able to promote a 2.5-fold increase in methane specific yield (Zhao et al. 2018). In the same line, Gómez et al. (2018), using swine manure as feedstock, obtained a 39% increase in methane production when digestions were supplemented with biochar. It is worth noting that this same benefit was observed, by the same authors, when the raw material employed was pretreated with microwaves. The latter strategy, however, may instill higher production costs.

Still keeping the pretreatment of raw materials as a central point, there are studies focused on the availability of carbohydrates (for conversion to biogas) through swelling agents that facilitate the digestion process of polymers such as cellulose (Hewetson et al. 2016; Shiga et al. 2017). The crystallinity of this polysaccharide is one of the main limiting factors for an efficient hydrolysis of the lignocellulosic biomass and its consequent conversion to biogas. Thus, crystal disruption and the breakdown of hydrogen bonds are necessary to allow the access of enzymes or other catalysts to the cellulose structure, facilitating the hydrolysis of the glycosidic bonds between the glucose monomers. Zhang et al. (2018) have shown that the use of moderate acids such as phosphoric acid and trifluoroacetic acid can promote a more than twofold increase in glucose yield during cellulose hydrolysis with a commercial enzyme.

There are also examples of pretreatments employed in processes that use only one substrate. Lu et al. (2018) employed EDTA to remove organic-bonding metals from sewage sludge and, through this pretreatment, obtained an expressive decrease of these metals in the substrate (from 5.1 to 1.4%). Besides, this assured a 48% increase in methane generation.

The feedstocks for biogas production are so variable that even wool and feather can be used. And even in this context, despite the few studies on the literature, there are already strategies to increase the methane yield through alkaline, thermal, enzymatic and biological pretreatments of these raw materials, which, if combined, can increase up to 20 times the yield of CH_4 (Forgács et al. 2013; Kabir et al. 2013; Patinvoh et al. 2016). In addition to these methods, Kuzmanova et al. (2018) have shown that, by reducing the size of the particles and consequently increasing their solubility and bioavailability, the use of liquid nitrogen (LN_2) can increase the methane yield from wool by more than 80%.

Pretreatments, however, can be economically, energetically and environmentally onerous. Although they may be used to increase biogas yield, the use of some pretreatment technologies can increase energy consumption (with uninteresting unbalance), cost (with the use of hydrolytic enzymes, for example) and even the carbon footprint in the process (Fan et al. 2018). In this sense, researchers have shown that the use of inoculums containing a consortium of interdependent microorganisms previously adapted and selected (as it is carried out in several other bioprocesses that rely on microorganisms) can be beneficial to the production of biogas and may be less expensive and more friendly environment.

14.1.2 Use of Inoculums Consortium—Microorganisms

The prevalence of some anaerobic microorganisms can affect hydrolytic and methanogenic activities, which provides different yield degrees to the process. It has already been verified that the inoculum performed with a selection of high hydrolysis efficiency bacteria and with methanogenic archaea increases the methane yield in biodigesters using seaweed biomass as substrate (Sutherland and Varela 2014), and that the inoculation reduced by half the lag phase of processes carried out with swine wastewater (Córdoba et al. 2016). Indeed, the selection and adaptation of microorganisms at the same time decreases diversity and increases specificity, which directly affects yield (De Francisci et al. 2015). Given the low cost of selection and maintenance of microorganisms and the non-energetic and environmental burden, this can be an interesting technology. In this sense, Gonzalez-Fernandez et al. (2018) have shown that in microalgae biomass, the use of previously adapted anaerobic microorganisms may prevent the application of pretreatments which, although may slightly increase the yield, can be costly.

Moreover, in the field of microorganism selection, the so-called bioaugmentation appears to be promising among low-cost technologies to increase the yield of biogas, opening up even the possibility of using genetically modified organisms (Nzila 2017). The literature presents several very recent works, with different microorganisms used, both fungi and bacteria, under different conditions of temperature and substrates. In biodigesters with cow manure, the bioaugmentation with an enriched cow rumen culture promoted a nearly sixfold increase in methane production (Ozbayram et al. 2018). In another work, the combination of pretreatment by steam explosion with the bioaugmentation by a cellulolytic bacterium (*Caldicellulosiruptor bescii*) increased in 140% the methane yield compared to the untreated birch in processes with lignocellulosic biomass (Mulat et al. 2018). Ferraro et al. (2018) found that a combination of anaerobic ruminal fungi and a pool of hydrogen-producing fermentation bacteria allowed an increase of up to 330% in wheat straw and mushroom spent straw when compared to the unaugmented condition.

14.1.3 Innovative Systems

Another innovative and very promising technology for the production of biogas with high yield is the Bioelectrochemical Systems (BESs). These low-cost systems are based on biological and electrochemical processes, which can be used to exploit waste to increase the generation of different products of interest, including biogas (Sasaki et al. 2010, 2018a; Schievano et al. 2016). In the last ten years, different BESs have been developed and, more recently, it has been verified that the presence of an anode and a cathode can control microbial fermentations by overcoming the thermodynamic limits of some metabolic pathways. In BESs, called

Electro-Fermentation System (EFS), the electrodes function as a supplementary electron source or sink, affecting both extracellular and intracellular oxidation-reduction potential. Thus, in addition to exerting significant effects on microbial metabolism and cellular regulation, an EFS also influences the interspecific interactions and the selection of bacteria in the processes (Moscoviz et al. 2016). Recently, Sasaki et al. (2018b) have demonstrated an increase in the proportion of methane in the biogas produced even in two-stage processes from these systems.

Nanotechnology also deserves notoriety among the strategies that promote high productivity and yield increase in biogas production. Quan et al. (2017) reported a molecular basket sorbent, based on tertiary amine supported over mesoporous silica, with high selectivity for removal of H_2S from the biogas, which ends up increasing the desired proportion of CH_4. In another recent study, Anjum et al. (2018) have synthesized nanotubes composed of carbon nitride and titania (C_3N_4/TiO_2) aiming to improve the increase visible light-mediated photocatalytic degradation of wastewater sludge. In this approach, they verified an increase of up to 60% in methane generation. From brewery wastewater, Carpenter et al. (2015) demonstrated that the addition of 0.25% nanoscale zerovalent iron (NZVI) to the bioreactors promoted a 28% increase in methane production and a 58% decrease in CO_2 release. It has also been verified that the addition of nanoparticles of trace metals to livestock manures biodigesters can increase the yield of biogas by 80% and by more than 100% the methane yield in this biogas (Abdelsalam et al. 2016).

14.1.4 Post-digestion

Finally, in addition to the concern with the yield gain in biogas production, there is also concern about the use and stability of the digestates. Although they are often used in agriculture (Tambone et al. 2010), unstable digestate may still have potential for extra biogas production, and thus, post-digestion may contribute not only to increased biogas production, but also with the reduction of environmental and health impacts, since these digestates may even promote higher proliferation of pathogens (Abdullahi et al. 2008).

Wojnowska-Baryła et al. (2018) point out the possibility of using the digestate for a psychrophilic post-digestion, which allows, in addition to an increase in methane production, a reduction of uncontrolled emission of this gas into the atmosphere—which would occur if unstable digestate were employed in agriculture without a post-digestion (thereby increasing the release of greenhouse gas into the atmosphere). In this work, the authors demonstrated the possibility of an additional of up to 27% in biogas productivity, using the same raw materials through post-digestion. Thus, post-digestions can generate not only a production gain, but also provide mitigation of process impacts.

14.2 Legal and Administrative Framework/Incentives for Biogas Production and Use

The main discussions on renewable energy production took place in the 1970s due to concerns about high GHG emissions, discussed at the Stockholm Conference in 1972, and the intense oscillation in the fossil fuels price during the oil crises of 1973 and 1979. Since then, discussions have been held with the aim of establishing government policies to stimulate the production and use of renewable energy throughout the world.

In the last four decades, Europe has emerged in production accounting for 72.3% of the total biogas produced in the world in 2016. Among the main biogas producers are Germany as the world leader (33,803 GWh), followed by the USA (13,466 GWh), Italy (8259 GWh), UK (7706 GWh) and Czech Republic (2590 GWh) (IRENA 2018b). High production in the Europe countries is due to biogas being considered one of the key technologies both to reach RED (Renewable Energy Directive) targets for renewable energies in 2020—renewable energy as 20% share of total energy consumption (Directive 2009/28/CE; EU 2009)—and to meet their requirements within the European organic waste management directive as energy source (Directive 2006/12/CE; EU 2006). In 2014, European Parliament also establishes regulations for the implementation of an infrastructure for alternative fuels (Directive 2014/94/UE; EU 2014). However, the incentives have been different for each country, since the final product should consider local needs and feedstock materials (Pfau et al. 2017), as will be summarized below.

Germany stands out not only because of the greater biofuel volume, but also because it started production more than four decades ago. A great example of biogas incentive policies has been observed in this country, where the first projects were operated in the 1970s by farmers mainly to use liquid and solid manure and feed leftovers in a useful way, to protecting the climate and avoid GHG emissions and to generate electricity and heat for its own operation ($\sim$70 kW; Markard et al. 2016). In 1991, the country adopted the feed-in tariff system (StrEG), which guaranteed the incentive of 6.5 eurocents/kWh for electricity generated (<500 kW) from landfill gas and sewage gas and 7.1 eurocents/kWh for biomass-based energy (<150 kW) (Wütenhagen and Bilharz 2006). In 2000, the StrEG system was updated within the Energy Renewable Sources Act (Erneuerbare-Energien-Gesetz—EEG), revised in 2004, which was mainly based on: (i) the right of grid connection for renewable energy facilities, (ii) the obligation for grid operators to preferentially purchase electricity based on renewables, and (iii) a minimum feed-in tariff to be paid for the generated electricity (Daniel-Gromke et al. 2018). This update marked a strong development of biogas through different rules for each renewable energy technology, as well as stimulating the use of energy crops, an important fact when the country was experiencing a reduction in agricultural production, closure of farms and availability of agricultural areas (Markard et al. 2016). Thus, Germany increased from approximately 100 to 4000 biogas plants between

1990 and 2008 (Koçar and Civaş 2013), and a new generation of larger mills (~300 kW) was introduced by the implementation of the feed-in tariff guarantee for a period of 20 years (Markard et al. 2016).

From 2009, the EU Biomass Action Plan was presented to intensify the energetic use of biomass, adopting new specific incentives like manure bonus (using animal manure), landscape bonus (garden and plant biomass) and biomass bonus (rejected crops or crop residue) to avoid further pressure on food prices (Britz and Delzeit 2013; Edwards et al. 2015). In the same year, EEG was revised adding a special bonus for substrates composed of at least 30% of animal waste and minimum use of heat in cogeneration (Markard et al. 2016). In the biomass-based electricity generation in Germany 2017, of a total of 51.4 billion kWh, 63.2% resulted from biogas production (AGGE-Stat 2018). Thus, after the consolidation of large plants, EEG 2017 changed the funding for renewable energy sources from a fixed tariff to a tender system (Daniel-Gromke et al. 2018). It has also been imposed the condition need-based and flexible electricity generation and limited use of grain and maize until 50% by weight, for food security (FNR 2017), which led Scandinavia, for example, to ban the use of energy crops for biogas production (EurObserv'ER 2017). Currently, German has more than 10,000 plants in operation, where biogas production occurs mainly from agricultural substrates (87.8%) to generate electricity (58.1%), heat (32.9%), flaring (8.0%) and vehicle fuel (IEA 2017a).

The USA, the world's second largest producer of biogas, has the anaerobic digestion industry well established in terms of utilizing sewage sludge as a substrate, most of which supply combined heat and power (CHP) units (Edwards et al. 2015). The energy security is considered a key driver fostering renewable energy and anaerobic digestion in the country. Initially, the country relies on two pieces of legislation: The renewable portfolio standard (RPS), which administers the selling of renewable energy credits, as feed-in tariffs and setting of renewable energy quotas, is paramount in providing financial incentives for anaerobic digestion. In 2014, the White House released its strategy to reduce methane emissions under the Climate Action Plan—Strategy to Reduce Methane Emissions to accelerate the adoption of biogas systems, with the goal of reducing GHG emissions across the sector's value chain by 25% by 2020 (USDA 2014). Thus, there is a growing trend to upgrade the gas to biomethane for use in transport, where it qualifies as an advanced biofuel. California ranks first among USA states for methane production potential from biogas sources (ABC 2015). This sector grew some 15% in 2017 (REN21 2018). Actually, the USA has in operation over 2200 sites producing biogas, of which 1269 water resource recovery facilities using an anaerobic digester (~860 currently use the biogas they produce), 652 landfill gas projects, 250 anaerobic digesters on farms, and 66 stand-alone systems that digest food waste (ABC 2018). Almost half of the biogas is used for electricity and half for heat production (IRENA 2017b).

Italy and UK stand out as the second and third largest European biogas producers, respectively, but with a production around 76.4% less than Germany. The biogas production in Italy is mainly used as electricity (78%) by commerce (55%) and industry (45%) (IRENA 2018b). In the country, support schemes for renewable

energy sources (RES) are managed by Gestore dei Servizi Energetici (GSE—Manager of Electricity Services), using green certificates system. The Ministerial Decree from IT (2008) and the decrees that preceded it have provides that the qualification of the powered by renewable sources (IAFR qualification) was necessary prerequisite for obtaining green certificate or for access to the all-inclusive tariff based on the net electricity produced and fed into the grid. The higher incentives were for biogases obtained from agriculture, animal husbandry and forestry (Law no. 99/2009). However, the main incentive to electricity generation by biogas production occurred in 2012 with the DM 6/7/2012 (IT 2012), which included a different feed-in tariff (*tariffa onnicomprensiva*, TO, in italian) and premium tariff, where plants with a capacity up to 100 kW and between 1 kW to 5 MW can access incentives directly, respectively; and tenders eligible for capacities above 5 MW (Jimeno 2015). The number of plants under the TO regime increased from 33 (21 MW) in 2008 to 1082 (803 MW) in 2015 (GSE 2015) and still in 2015 there were a total of 414 biogas plants that requested government incentives and produced a total power of 159 MW (Carlini et al. 2017). From 2016, the green certificate was extinguished and two types of incentives were offered: (i) an all-inclusive tariff (TO); and (ii) an incentive (I), calculated as the difference between a fixed value and the zonal energy hour price (GSE 2017). For systems with power up to 500 kW, it is possible to choose both modes alternatively, but systems with a capacity of more than 500 kW can instead access only the incentive. Following the Germany example, Italy directed higher incentives to small-medium size biogas plants (IEA 2016). In the last six years, the country has invested more than 4 billion Euros in more than 1700 biogas plants already built, including agricultural, sewage, waste and industrial subproducts (Maggioni 2017), of which about 65% an electric performance below 500 kW (GSE 2015). Currently, the National Energy Strategy has designed strong incentives for the production of biomethane in the country, which uses about 3 billion N/m^3 biomethane equivalent per year (Maggioni 2017). According to CIB (Consorzio Italiano Biogas), the country is able to generate a potential of 10 billion m^3 by 2030, of which at least eight from agricultural feedstock.

In the UK, the renewable support scheme is based on the Energy Act 2008 (Hermann and Hermann 2018) and managed by the Office of Gas and Electricity Markets (OFGEM). The regulations and financial incentives apply to biogas production include the Renewables Obligation (RO) and the feed-in tariff (FIT) for electricity, and the Renewable Heat Incentive (RHI) for heat production from biogas combustion and biomethane injection to the grid. The scheme FIT scheme came into effect in 2010 and aimed to support small-scale renewable energy sources plants (<5 MW) and the RO system (revised by the FTO 2012; UK 2012) was to support mainly plants above 5 MM, besides tax regulation mechanism (Maroulis 2015; Hermann and Hermann 2018). While Italy is an example of selective collection, composting of food and garden waste was incentive later for AD industries in UK (Jain et al. 2018), and mainly driven by its conversion into electricity until 2016. By 2015, British per capita biogas production was 404 kWh compared to 284 kWh in 2005, a 42% increase (Deremince and Königsberger 2017). Between

2015 and 2016, the Department for Business, Energy and Industrial Strategy reports that biogas electricity production from anaerobic digestion increased to 2.1 TWh or by 40% (EurObserv'ER 2017). In 2017, UK had 557 operational plants and capacity of 730 MWe, with 83.6% related only to electricity or to CHP plants. Electricity generated by small biogas plants (<500 kWe) and mostly biomethane production uses agricultural feedstocks. Medium- and large-scale biogas plants (>500 kWe) preferably use sewage, followed by agricultural and municipal/commercial waste as feedstocks (IEA 2017c). After a period of degressions in the FIT scheme (Maroulis 2015), new incentives are being directed toward upgrading biogas plants to biomethane, which is feasible in the UK because there is already an extensive gas distribution network. In October 2017, the UK adopted Clean Growth Strategy that targets government fundings of £2.5 billion mainly to accelerate the shift to low carbon transport (33%) and deliver clean, smart and flexible power (25%) (Damave 2018).

There have been an increasing number of countries, states or provinces adhering to the RED rules. Targets and policies for renewable energy had been established actually in more than 100 countries, a significant increase from 47 countries in 2007 (Song et al. 2014; REN21 2018). Germany, Austria, Denmark and Switzerland use more than 50% of the biogas produced in electricity. On the other hand, Finland and the Netherlands use most of it for heat generation and Sweden and Norway for biomethane (IEA 2016). Sweden leads Europe in the use of biogas fuel for vehicles (EurObserv'ER 2017). According to Energigas Sverige, 64% of total biogas output in 2016 (put at 2 TWh) was converted into biomethane, which was used almost exclusively for vehicle fuel. The country has 63 biogas enrichment plants that produced 1234 GWh of biomethane in 2016, and 13 plants that injected it directly into the country's two natural gas grids (EurObserv'ER 2017). The incentive to produce biomethane has been due to the need to reduce or even ban dependence on fossil fuels. In 2017, five countries announced their intention to ban sales of new diesel and petrol cars by 2030: India (Vidhi and Shrivastava 2018), the Netherlands and Slovenia (REN21 2018); and by 2040: France and the UK (EPRI 2017; IEEJ 2017). Since biomethane has a similar quality to natural gas, it is in fact a potentially substitute for fossil natural gas. Iran, China and Pakistan are the countries with greater number of natural gas vehicles (IRENA 2017b). Another incentive in the production and the consumption of biogas-derived electricity is the use and expansion of electric vehicles (Podkaminer et al. 2017).

In Asia, China has been producing biogas in a small scale (at household level), promoted by the government, since 1920. Currently, biogas production in rural areas of China comes from two primary sources: household biogas digester, and medium- and large-scale biogas plants. The Chinese government issued the renewable energy law and renewable energy prices and cost-sharing management trial procedures in 2005 to encourage various domestic enterprises to become involved in renewable energy development (revised by Song et al. 2014). In ten years, the offered financial incentive increased from 47 million dollars (in 2002) to 760 million dollars (in 2011) (Feng et al. 2012), which allowed the construction of 42 million small (8–12 m^3) household biogas digesters and 27 thousand medium-

and large-scale biogas plants in China between 2003 and 2013 (IGU 2015) and 850 for large livestock and poultry farms between 2001 and 2005. After 2009, China has enhanced its support for biogas engineering projects by offering subsidies from 25 to 45% of the whole cost of projects, setting up policies similar to feed-in tariffs to promote power generation through biogas plants, for improving the efficiency of biogas production and utilization (Gu et al. 2016). Large-scale biogas projects focused mainly on agricultural and industrial (including municipal) wastes. In 2016, China produced 1863 GWh of biogas, Asia's second largest producer, behind only Thailand (IRENA 2018b).

Significant growth is also estimated for the South-Central and South-Eastern Asian countries such as Bangladesh, Cambodia, Vietnam, Indonesia and Nepal (REN21 2018), where the biogas will continue to be limited to meeting the primary energy needs (light and cooking), mainly in rural areas. In Bangladesh, for example, the National Domestic Biogas and Manure Programme has been supporting the expansion of biogas technology in rural areas, and an estimated 80,000 small-scale systems that use animal waste are in operation (IEA 2017b). In Vietnam, the Biogas Programme for the Animal Husbandry Sector was launched in 2003 and facilitated the construction of nearly 250,000 small biogas digesters (IRENA 2018a).

As in Asia, most heat demand in Africa is for cooking, with the majority supplied from traditional biomass, which can have serious impacts on health and generally is not sustainably produced (IEA 2014). More than 58,000 biogas cookstoves were installed by the end of 2016, from Africa Biogas Partnership Programme "Progress tracker" Burkina Faso, Ethiopia, Kenya, Tanzania and Uganda since 2009 (REN21 2018). Globally, a cumulative total of more than 50 million biogas cookstoves had been installed as of year-end 2016, with about 126 million people using biogas for cooking (IRENA 2017a). However, investment in access to clean cooking in developing countries reaches a cumulative $20 billion over the period to 2030, providing cleaner cooking access for almost 900 million more people (IEA 2017b).

In Latin America, Brazil stands out as the largest producer of biogas (873 GWh, in 2015), followed by Argentina (120 GWh) and Peru (50 GWh) (IRENA 2018b). Incentives in Brazil started effectively in 2009 with the institution of National Policies for Climate Change (Federal Law nº 12.187/2009; Brasil 2009) and Solid Waste (Federal Law nº 12.305/2010; Brasil 2010), which included the low-carbon program and the incentive program for alternative sources of energy. In 2012, the Rio de Janeiro state (State Law nº 6361/12; RJ 2012) made mandatory the injection of 10% of the biogas from municipal solid waste into the piped gas local distribution network. In the following year, the state of São Paulo reduced the tax on the internal exits of biogas and biomethane, as an incentive in its production (Decreto nº 60.001/13; SP 2013). In 2015, along with other 194 countries, Brazil adheres to the Paris Agreement and commits to meet targets for reducing GHG emissions by 37% by 2025, compared to 2005. Brazil currently has (2017) 127 biogas plants in operation (Itaipu 2017), and 22 registered units (CIBiogás 2017), where most of

them (47%) utilized agricultural substrates and 34% used industry substrates, mainly for heat and electric power. However, in relation to the amount of biogas produced for energy purposes, 43% of it originates from sanitary landfills, 29% from agriculture substrates and 22% from industry (CIBiogás 2017).

14.3 Final Considerations

The current biogas scenario corroborates with decision-making and investment initiatives in world, aimed at reducing fossil-based emissions and increasing renewable energy. Even though the production and use of biogas have been considered feasible from the "sustainable economy" point of view, as discussed in this book, it is necessary news perspectives on cost reduction of deployment and operation of biogas units and political support to biogas production and use in the whole world.

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