Chapter 3
Enzyme-Mediated Enhanced Biogas Yield

Thamarys Scapini, Aline Frumi Camargo, Fábio Spitza Stefanski, Natalia Klanovicz, Rafaela Pollon, Jessica Zanivan, Gislaine Fongaro and Helen Treichel

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

T. Scapini (✉) · A. F. Camargo · F. S. Stefanski · N. Klanovicz
R. Pollon · J. Zanivan · G. Fongaro · H. Treichel
Laboratory of Microbiology and Bioprocess, Department of Environmental Science and Technology, Federal University of Fronteira Sul, Erechim, Brazil
e-mail: thami.scapini01@gmail.com

G. Fongaro
Department of Microbiology, Immunology and Parasitology (MIP), Laboratory of Applied Virology, Federal University of Santa Catarina, Florianópolis, Brazil

H. Treichel and G. Fongaro (eds.), *Improving Biogas Production*, Biofuel and Biorefinery Technologies 9,
https://doi.org/10.1007/978-3-030-10516-7_3

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

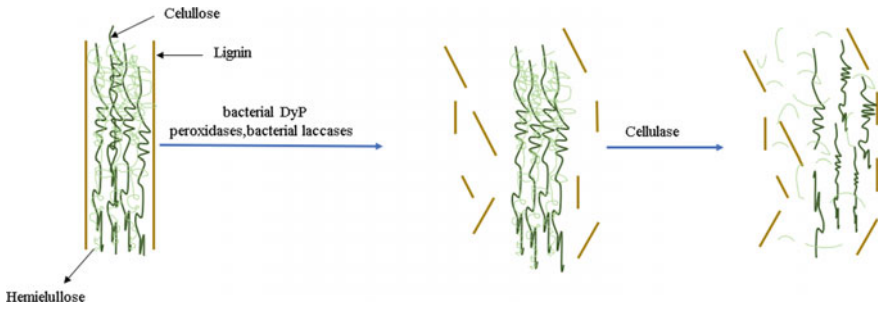

Fig. 3.1 Degradation of lignocellulosic material by enzymes

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

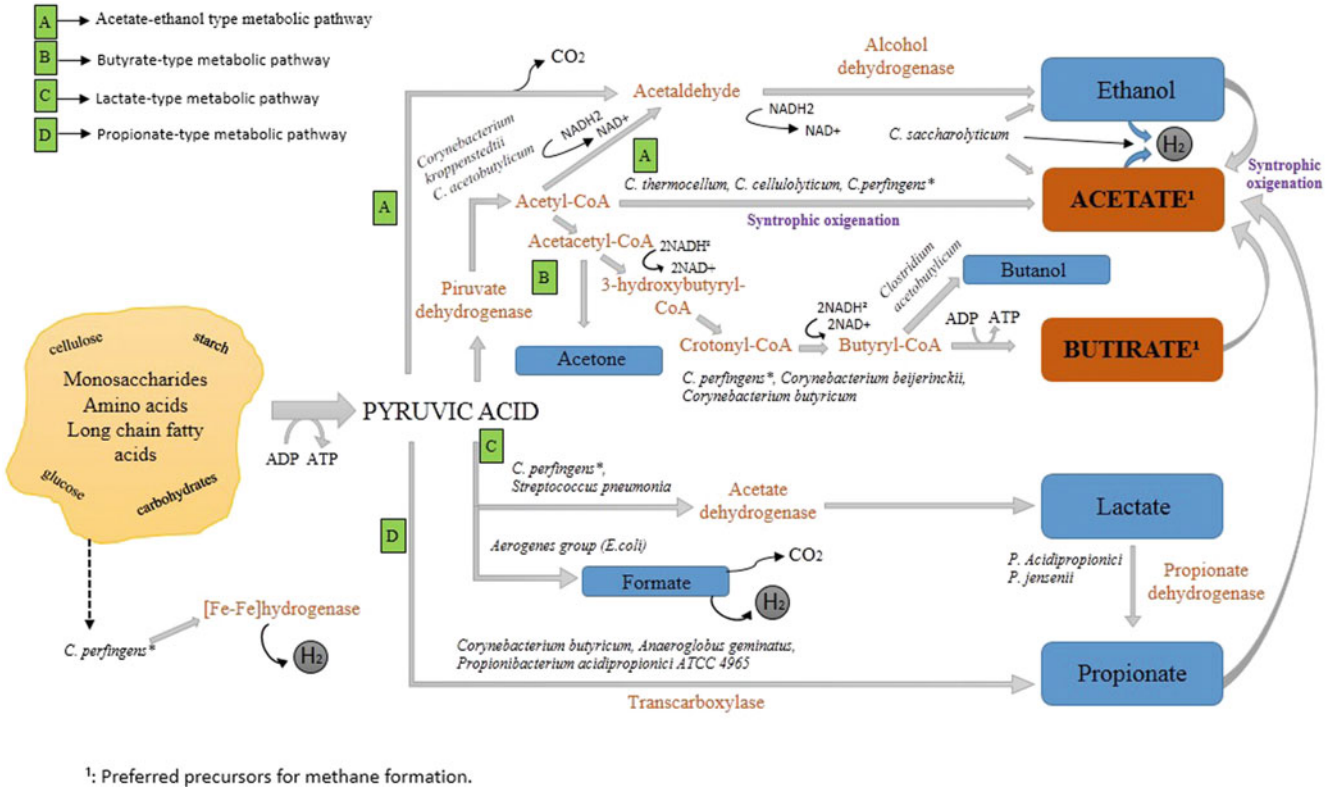

Fig. 3.2 Acidogenic metabolic pathways and some of the principal microorganisms involved in the production of the compounds. *Database* Murray et al. (1982), Svensson et al. (1992), Kaji et al. (1999), Hoskins et al. (2001), Hwang et al. (2001), Carlier et al. (2002), Guedon et al. (2002), Chen et al. (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

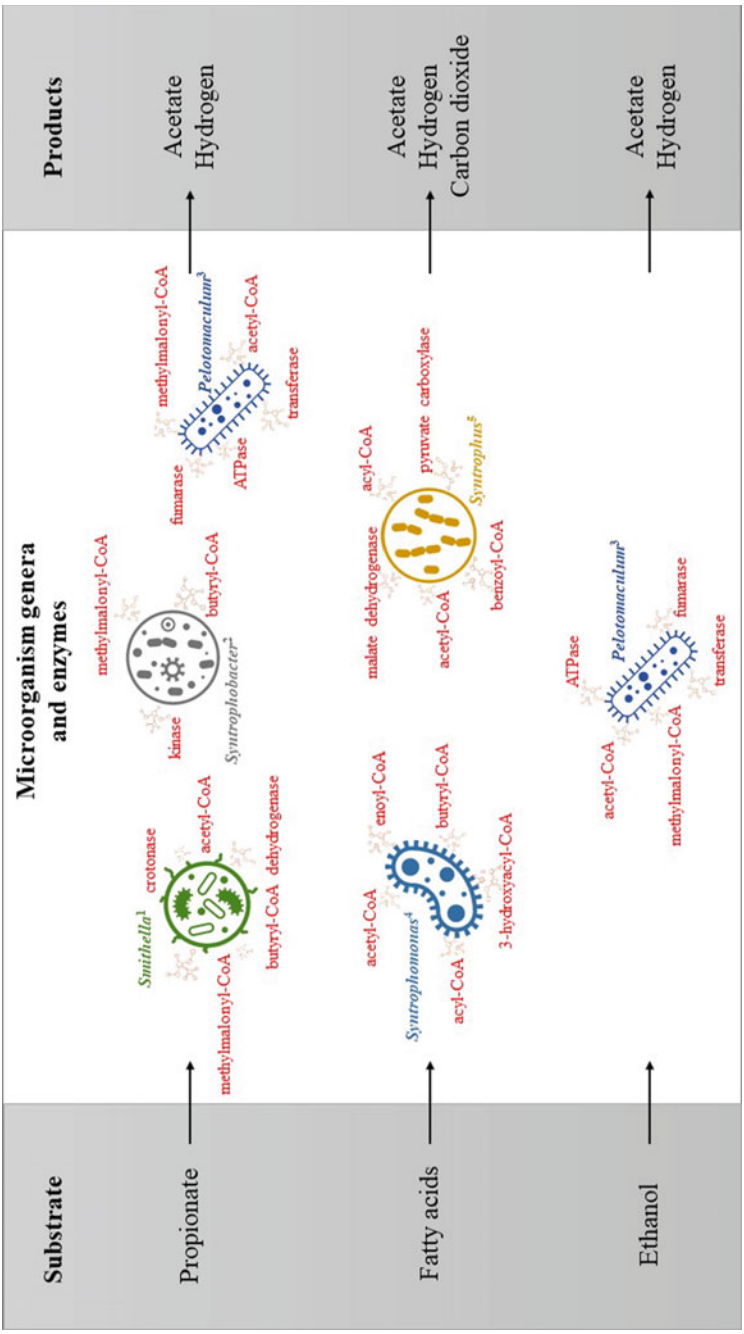

Fig. 3.3 Overview of the principal interactions in acetogenesis between substrates, microorganisms and enzymes (shown in red) and the principal products obtained. *Database of interactions* Imachi et al. (2002, 2007), Gerardi (2003), Kosaka et al. (2006), Jha et al. (2011), Christy et al. (2014), Venkiteshwaran 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).

References

Abdelsalam E, Samer M, Attia YA, Abdel-Hadi MA, Hassan HE, Badr Y (2017) Effects of Co and Ni nanoparticles on biogas and methane production from anaerobic digestion of slurry. Energy Convers Manag 141:108–119

Abdel-Shafy HI, Mansour MSM (2014) Biogas production as affected by heavy metals in the anaerobic digestion of sludge. Egypt J Petrol 23:409–417

Abedi D, Zhang L, Pyne M, Perry Chou C (2011) Enzyme biocatalysis, chap 1. In: Comprehensive biotechnology, pp 15–24

Achinas S, Achinas V, Jan G, Euverinka W (2017) A technological overview of biogas production from biowaste. Engineering 3:299–307

Ács N, Bagi Z, Rákhely G, Minárovics J, Nagy K, Kovács KL (2015) Bioaugmentation of biogas production by a hydrogen-producing bacterium. Biores Technol 186:286–293

Ahmadi N, Darani KK, Mortazavian AM (2017) An overview of biotechnological production of propionic acid: from upstream to downstream processes. Eletron J Biotechnol 28:67–75
Al Seadi T, Rutz D, Prassl H, Köttner M, Finsterwalder T, Volk S, Janssen R (2008) Biogas handbook. University of Southern Denmark Esbjerg, Niels Bohrs
Amha YM, Anwar MZ, Brower A, Jacobsen CS, Stadler LB, Webster TM, Smith AL (2018) Inhibition of anaerobic digestion processes: application of molecular tools. Biores Technol 247:999–1014
Aronson EL, Allison SD, Helliker BR (2013) Environmental impacts on the diversity of methane-cycling microbes and their resultant function. Front Microbiol 4
Azman S, Khadem AF, Van Lier JB, Zeeman G, Plugge CM (2015) Presence and role of anaerobic hydrolytic microbes in conversion of lignocellulosic biomass for biogas production. Crit Rev Environ Sci Technol 45:2523–2564
Azman S, Khadem AF, Plugge CM, Stams AJ, Bec S, Zeeman G (2017) Effect of humic acid on anaerobic digestion of cellulose and xylan in completely stirred tank reactors: inhibitory effect, mitigation of the inhibition and the dynamics of the microbial communities. Appl Microbiol Biotechnol 101:889–901
Baserba MG, Angelidaki I, Karakashev D (2012) Effect of continuous oleate addition on microbial communities involved in anaerobic digestion process. Biores Technol 106:74–81
Batstone DJ, Keller J, Angelidaki I, Kalyuzhnyi SV, Pavlostathis SG, Rozzi A, Sanders WTM, Siegrist H, Vavilin VA (2002) The IWA anaerobic digestion model no. 1 (ADM1). Water Sci Technol 45(10):65–73
Bensaid S, Ruggeri B, Saracco G (2015) Development of a photosynthetic microbial electrochemical cell (PMEC) reactor coupled with dark fermentation of organic wastes: medium term perspectives. Energies 8:399–429
Bharathiraja B, Sudhargarsana T, Jayamuthunagai J, Praveenkumar R, Chozhavendhan S, Iyyappab J (2018) Biogas production—a review on composition, fuel properties, feed stock and principles of anaerobic digestion. Renew Sustain Energy Rev 90:570–582
Brémond U, Buyer R, Steyer J, Bernet N, Carrere H (2018) Biological pretreatments of biomass for improving biogas production: an overview from lab scale to full-scale. Renew Sustain Energy Rev 90:583–604
Buyukkamaci N, Filibeli A (2004) Volatile fatty acid formation in an anaerobic hybrid reactor. Process Biochem 39:1491–1494
Carlier JP, Marchandin H, Jumas-Bilak E, Lorin V, Henry C, Carrièrre C, Jean-Pierre H (2002) Anaeroglobus *geminatus* gen. nov., sp. nov., a novel member of the Family Veillonellaceae. Int J Syst Evol Microbiol 52:983–986
Cazier EA, Trably E, Steyer JP, Escudié R (2015) Biomass hydrolysis inhibition at high hydrogen partial pressure in solid-state anaerobic digestion. Biores Technol 190:106–113
Chaganti SR, Kim D-H, Lalman JA (2011) Flux balance analysis of mixed anaerobic microbial communities: effects of linoleic acid (LA) and pH on biohydrogen production. Int J Hydrogen Energy 36:14141–14152
Chen X, Sun Y, Xiu Z, Li X, Zhang D (2006) Stoichiometric analysis of biological hydrogen production by fermentative bacteria. Int J Hydrogen Energy 31:539–549
Chen Y, Cheng JJ, Creamer KS (2008) Inhibition of anaerobic digestion process: a review. Biores Technol 99:4044–4064
Chen Y, Luo J, Yan Y, Feng L (2013) Enhanced production of short-chain fatty acid by co-fermentation of waste activated sludge and kitchen waste under alkaline conditions and its application to microbial fuel cells. Appl Energy 102:1197–1204
Chen JL, Ortiz R, Steele TWJ, Stuckey DC (2014) Toxicants inhibiting anaerobic digestion: a review. Biotechnol Adv 32:1523–1534
Chen C, Guo W, Ngo HH, Lee D, Tung K, Jin P, Wang J, Wu Y (2016) Challenges in biogas production from anaerobic membrane bioreactors. Renewable Energy 98:120–134
Choi J, Han S, Kim H (2015) Industrial applications of enzyme biocatalysis: current status and future aspects. Biotechnol Adv 33(7):1443–1454

Chojnacka A, Szczęsny P, Błaszczyk MK, Zielenkiewicz U, Detman A, Salamon A, Sikora A (2015) Noteworthy facts about a methane-producing microbial community processing acidic effluent from sugar beet molasses fermentation. PLoS ONE 10

Chotwattanasak J, Puetpaiboon U (2011) Full scale anaerobic digester for treating palm oil mill wastewater. J Sustain Energy Environ 2:133–136

Christy PM, Gopinath LR, Divya D (2014) A review on anaerobic decomposition and enhancement of biogas production through enzymes and microorganisms. Renew Sustain Energy Rev 34:167–173

Collins MD, Falsen E, Akervall E, Sooden B, Alvarez A (1998) *Corynebacterium kroppenstedtii* sp. nov., a novel Corynebacterium that does not contain mycolic acids. Int J Syst Bacteriol 48:1449–1454

Cooney M, Maynard N, Cannizzaro C, Benemann J (2007) Two-phase anaerobic digestion for production of hydrogen-methane mixtures. Biores Technol 98:2641–2651

Coral J, Karp SG, Vandenberghe LPS, Parada JL, Pandey A, Soccol CR (2008) Batch fermentation model of propionic acid production by propionibacterium acidipropionici in different carbon sources. Appl Biochem Biotechnol 151:333–341

De Bok FAM, Stams AJM, Dijkema C, Boone DR (2001) Pathway of propionate oxidation by a syntrophic culture of *Smithella propionica* and *Methanospirillum hungatei*. Appl Environ Microbiol 67(4):1800–1804

Dereli RK, Ersahin ME, Ozgun H, Ozturk I, Jeison D, Van der Zee FP, Van Lier JB (2012) Potentials of anaerobic membrane bioreactors to overcome treatment limitations induced by industrial wastewaters. Biores Technol 122:160–170

Desbois AP, Smith VJ (2010) Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. Appl Microbiol Biotechnol 85:1629–1642

Dong X, Plugge CM, Stams AJM (1994) Anaerobic degradation of propionate by a mesophilic acetogenic bacterium in coculture and triculture with different methanogens. Appl Environ Microbiol 60:2834–2838

FAO Agricultural Services Bulletin—128 (1997) Methane production. In: Renewable biological systems for alternative sustainable energy production. ISBN 92-5-104059-1

Ferry JG (2011) Acetate kinase and phosphotransacetylase. In: Methods in enzymology, vol 494

Fotidis IA, Karakashev D, Angelidaki I (2014) The dominant acetate degradation pathway/methanogenic composition in full-scale anaerobic digesters operating under different ammonia levels. Int J Environ Sci Technol 11:2087–2094

Fournier GP, Gogarten JP (2007) Evolution of acetoclastic methanogenesis in *Methanosarcina* via horizontal gene transfer from cellulolytic *Clostridia*. J Bacteriol 190(3):1124–1127

Garvie EI (1980) Bacterial lactate dehydrogenases. Microbiol Rev 44:106–139

Gerardi MH (2003) The microbiology of anaerobic digesters. In: Wastewater microbiology series. Wiley, 177 p

Girbal L, Soucaille P (1994) Regulation of *Clostridium acetobutylicum* metabolism as revealed by mixed-substrate steady-state continuous cultures: role of NADH/NAD ratio and ATP pool. J Bacteriol 176:6433–6438

Girbal L, Vasconcelos I, Saint-Amans S, Soucaille P (1995) How neutral red modified carbon and electron flow in *Clostridium acetobutylicum* grown in chemostat culture at neutral pH. FEMS Microbiol Rev 16:151–162

Gonzalo G, Colpa DI, Habib DI, Fraaije MW (2016) Bacterial enzymes involved in lignin degradation. J Biotechnol 236:110–119

Graham DE, White RH (2001) Elucidation of methanogenic coenzyme biosyntheses: from spectroscopy to genomics. Nat Prod Rep 19(2):133–147

Grochowski LL, White RH (2010) Biosynthesis of the methanogenic coenzymes. In: Comprehensive natural products II, pp 711–748

Guedon E, Desvaux M, Petitdemange H (2002) Improvement of cellulolytic properties of *Clostridium cellulolyticum* by metabolic engineering. Appl Environ Microbiol 68:53–58

Gupta KK, Aneja KR, Rana D (2016) Current status of cow dung as a bioresource for sustainable development. Biores Bioprocess 28:1–11

Halpern J (1985) Mechanisms of coenzyme B12-dependent rearrangements. Science 227:869–875
Hoskins J, Alborn WE Jr, Arnold J, Blaszczak LC, Burgett S, DeHoff SB, Strem ST, Fritz L, Fu DJ, Fuller W, Geringer C, Gilmour R, Glass JS, Khoja H, Kraft AR, Lagace RE, LeBlanc DJ, Lee LN, Lefkowitz EJ, Lu J, Matshushima P, McAhren SM, McHenney M, McLeaster K, Mundy CW, Nicas TI, Norris FH, O'Gara M, Peery RB, Robertson GT, Rockey P, Sun PM, Winkler ME, Yang Y, Young-Bellido M, Zhao G, Zook CA, Baltz RH, Jaskunas SR, Rosteck PR Jr, Skatrud PL, Glass JI (2001) Genome of the bacterium *Streptococcus pneumoniae* strain R6. J Bacteriol 183:5709–5717
Houwen FP, Plokker J, Stams AJM, Zehder AJB (1990) Enzymatic evidence for involvement of the methyl-malonyl-CoA pathway in propionate oxidation by *Syntrophobacter wolinii*. Arch Microbiol 155:52–55
Huang KX, Huang S, Rudolph FB, Bennett GN (2000) Identification and characterization of a second butyrate kinase from *Clostridium acetobutylicum* ATCC 824. J Mol Microbiol Biotechnol 2:33–38
Hussain AA, Abdel-Salam MS, Abo-Ghalia HH, Hegazy WK, Hafez SS (2017) Optimization and molecular identification of novel cellulose degrading bacteria isolated from Egyptian environment. J Genet Eng Biotechnol 15:77–85
Hwang S, Lee Y, Yang K (2001) Maximization of acetic acid production in partial acidogenesis of swine wastewater. Biotechnol Bioeng 75:521–529
Iannotti EL, Fischer JR, Sievers DM (1982) Characterization of bacteria from a swine manure digester. Appl Environ Microbiol 43:136–143
Imachi H, Sakai S, Ohashi A, Harada H, Hanada S, Kamagata Y, Sekiguchi Y (2000) Cultivation and in situ detection of a thermophilic bacterium capable of oxidizing propionate in syntrophic association with hydrogenotrophic methanogens in a thermophilic methanogenic granular sludge. Appl Environ Microbiol 66:3608–3615
Imachi H, Sakai S, Ohashi A, Harada H, Hanada S, Kamagata Y, Sekiguchi Y (2002) *Pelotomaculum thermopropionicum* gen. nov., sp. nov., an anaerobic, thermophilic, syntrophic propionate-oxidizing bacterium. Int J Syst Evol Microbiol 52:1729–1735
Imachi H, Sakai S, Ohashi A, Harada H, Hanada S, Kamagata Y, Sekiguchi Y (2008) *Pelotomaculum propionicicum* sp. nov., an anaerobic, mesophilic, obligately syntrophic, propionate-oxidizing bacterium. Int J Syst Evol Microbiol 57:1487–1492
Jackson BE, Bhupathiraju VK, Tanner RS, Woese CR, McInerney MJ (1999) *Syntrophus aciditrophicus* sp. nov., a new anaerobic bacterium that degrades fatty acids and benzoate in syntrophic association with hydrogen-using microorganisms. Arch Microbiol 171:107–114
Jha AK, Li J, Zhang L (2011) Research advances in dry anaerobic digestion process of solid organic wastes. Afr J Biotech 10(65):14242–14253
Kaji M, Taniguchi Y, Matsushita O, Katayama S, Miyata S, Morita S, Okabe A (1999) The hydA gene encoding the H_2 evolving hydrogenase of *Clostridium perfringens*: molecular characterization and expression of the gene. FEMS Microbiol Lett 181:329–336
Kanai M, Ferre V, Wakahara S, Yamamoto T, Moro M (2010) A novel combination of methane fermentation and MBR—Kubota submerged anaerobic membrane bioreactor process. Desalination 250:964–967
Kandylis P, Bekatorou A, Pissaridi K, Lappa K, Dima A, Kanellaki M, Koutinas AA (2016) Acidogenesis of cellulosic hydrolysates for new generation biofuels. Biomass Bioenerg 91:210–216
Kolbl S, Forte-Tavčer P, Stres B (2017) Potential for valorization of dehydrated paper pulp sludge for biogas production: addition of selected hydrolytic enzymes in semi-continuous anaerobic digestion assays. Energy 126:326–334
Kosaka T, Uchiyama T, Ishii S, Enoki M, Imachi H, Kamagata Y, Ohashi A, Harada H, Ikenaga H, Watanabe K (2006) Reconstruction and regulation of the central catabolic pathway in the thermophilic propionate-oxidizing syntroph *Pelotomaculum thermopropionicum*. J Bacteriol 188(1):202–210
Lee HS, Salerno MB, Rittmann BE (2008) Thermodynamic evaluation on H_2 production in glucose fermentation. Environ Sci Technol 42:2401–2407

Leigh JA (2011) Growth of methanogens under defined hydrogen conditions. In: Methods in enzymology, vol 494
Li Y (2013) An integrated study of microbial community in anaerobic digestion systems. The Ohio State University, Graduate Program in Environmental Science, 208 p
Li J, Sun K, He J, Chen Q (2011) Using an amylase pretreatment of pig manure to enhance biogas production. National Engineering Center of Solid Waste Resources Recovery in Kunming University of Science and Technology. https://doi.org/10.1109/appeec.2011.5748924
Li L, He Q, Ma Y, Wang X, Peng X (2015a) Dynamics of microbial community in a mesophilic anaerobic digester treating food waste: relationship between community structure and process stability. Biores Technol 189:113–120
Li X, Chen Y, Zhao S, Chen H, Zheng X, Luo JY, Liu Y (2015b) Efficient production of optically pure L-lactic acid from food waste at ambient temperature by regulating key enzyme activity. Water Res 70:148–157
Lim JT, Ge T, Tong YW (2018) Monitoring of microbial communities in anaerobic digestion sludge for biogas optimization. Waste Manag 71:334–341
Liu Y, Whitman WB (2008) Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Ann N Y Acad Sci 1125:171–189
Liu Y, Balkwill DL, Aldrich HC, Drake GR, Boone DR (1999) Characterization of the anaerobic propionate-degrading syntrophs *Smithella propionica* gen. nov., sp. nov. and *Syntrophobacter wolinii*. Int J Syst Bacteriol 49:545–556
Liu D, Liu D, Zeng RJ, Angelidaki I (2006) Hydrogen and methane production from household solid waste in the two-stage fermentation process. Water Res 40:2230–2236
Liu C, Yang J, Zhang S, Guo J, Li Z (2008) Bacterial diversity comparison of anaerobic sludge from full-scale wastewater treatment bioreactors. J Biotechnol 136:S610–S630
Ma J, Zhao QB, Laurens LLM, Jarvis EE, Nagle NJ, Chen S, Frear CS (2015) Mechanism, kinetics and microbiology of inhibition caused by long-chain fatty acids in anaerobic digestion of algal biomass. Biotechnol Biofuels 8:141
Mathai PP, Zitomer DH, Maki JS (2015) Quantitative detection of syntrophic fatty acid-degrading bacterial communities in methanogenic environments. Microbiology 161(6):1189–1197
McInerney MJ, Wofford NQ (1992) Enzymes involved in crotonate metabolism in *Syntrophomonas wolfei*. Arch Microbiol 158:344–349
McInerney MJ, Rohlin L, Mouttaki H, Kim U, Krupp RS, Rios-Hernandez L, Sieber JR, Struchtemeyer CG, Bhattacharyya A, Campbell JW, Gunsalus RP (2007) The genome of *Syntrophus aciditrophicus*: life at the thermodynamic limit of microbial growth. PNAS Microbiol 104(18):7600–7605
Müller N, Worm P, Schink B, Stams AJ, Plugge CM (2010) Syntrophic butyrate and propionate oxidation processes: from genomes to reaction mechanisms. Environ Microbiol Rep 2:489–499
Murray DW, Khan WA, van den Berg L (1982) *Clostridium saccharolyticurn* sp. nov., a saccharolytic species from sewage sludge. Int J Syst Bacteriol 32:132–135
Nodar R, Acea MJ, Carballas T (1992) Poultry slurry microbial population: composition and evolution during storage. Biores Technol 40:29–34
Otín CL, Bond JS (2008) Proteases: multifunctional enzymes in life and disease. J Biol Chem 283:30433–30437
Pereira MA, Pires OC, Mota M, Alves MM (2005) Anaerobic biodegradation of oleic and palmitic acids: evidence of mass transfer limitations caused by long chain fatty acid accumulation onto the anaerobic sludge. Biotechnol Bioeng 92:15–23
Plugge CM, Dijkema C, Stams AJM (1993) Acetyl-CoA cleavage pathway in a syntrophic propionate oxidizing bacterium growing on fumarate in the absence of methanogens. FEMS Microbiol Lett 110:71–76
Prasertsan P, Khangkhachit W, Duangsuwan W, Mamimin C, O-Thong S (2017) Direct hydrolysis of palm oil mill effluent by xylanase enzyme to enhance biogas production using two-steps thermophilic fermentation under non-sterile condition. Int J Hydrogen Energy 42:27759–27766

Ren N, Wang B, Huang JC (1997) Ethanol-type fermentation from carbohydrate in high rate acidogenic reactor. Biotechnol Bioeng 54:428–433

Rincón B, Portillo MC, González JM, Borja R (2013) Microbial community dynamics in the two-stage anaerobic digestion process of two-phase olive mill residue. Int J Environ Sci Technol 10:635–644

Rivera-Salvador V, López-Cruz IL, Espinosa-Solares T, Aranda-Barradas JS, Huber DH, Sharma D, Toledo JU (2014) Application of anaerobic digestion model no. 1 to describe the syntrophic acetate oxidation of poultry litter in thermophilic anaerobic digestion. Biores Technol 167:495–502

Ruiz-Sánchez J, Campanaro S, Guivernau M, Fernández B, Prenafeta-Boldú FX (2018) Effect of ammonia on the active microbiome and metagenome from stable full-scale digesters. Biores Technol 250:513–522

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor

Sanchez S, Demain AL (2017) Useful microbial enzymes—an introduction, chap 1. In: Biotechnology of microbial enzymes, pp 1–11

Sarmiento FB, Leigh JA, Whitman WB (2011) Genetic systems for hydrogenotrophic methanogens. In: Methods in enzymology, vol 494

Sauer U, Eikmanns BJ (2005) The PEP-pyruvate-oxaloacetate node as the switch point for carbon flux distribution in bacteria. FEMS Microbiol Rev 29(4):765–794

Seedorf H, Fricke WF, Veith B, Brüggemann H, Liesegang H, Strittmatter A, Miethke M, Buckel W, Hinderberger J, Li F, Hagemeier C, Thauer RK, Gottschalk G (2008) The genome of *Clostridium kluyveri*, a strict anaerobe with unique metabolic features. Proc Nat Acad Sci USA 105:2128–2133

Selling R, Hakansson T, Bjornsson L (2008) Two-stage anaerobic digestion enables heavy metal removal. Water Sci Technol 57:553–558

Seon JY, Lee T, Lee SC, Pham HD, Woo HC, Song M (2014) Bacterial community structure in maximum volatile fatty acids production from alginate in acidogenesis. Biores Technol 157:22–27

Shah FA, Mahmood Q, Maroof Shah M, Pervez A, Ahmad Asad S (2014) Microbial ecology of anaerobic digesters: the key players of anaerobiosis. Sci World J 2014:1–21

Sieber JR, Sims DR, Han C, Kim E, Lykidis A, Lapidus AL, McDonnald E, Rohlin L, Culley DE, Gunsalus R, McInerney MJ (2010) The genome of *Syntrophomonas wolfei*: new insights into syntrophic metabolism and biohydrogen production. Environ Microbiol 12(8):2289–2301

Sillers R, Chow A, Tracy B, Papoutsakis ET (2008) Metabolic engineering of the non-sporulating, non-solventogenic *Clostridium acetobutylicum* strain M5 to produce butanol without acetone demonstrate the robustness of the acid-formation pathways and the importance of the electron balance. Metab Eng 10:321–332

Silva SA, Cavaleiro AJ, Pereira MA, Stams AJM, Alves MM, Sousa DZ (2014) Long-term acclimation of anaerobic sludges for high-rate methanogenesis from LCFA. Biomass Bioenergy 67:297–303

Silva FMS, Mahler CF, Oliveira LB, Bassin JP (2018) Hydrogen and methane production in a two-stage anaerobic digestion system by co-digestion of food waste, sewage sludge and glycerol. Waste Manag 76:339–349

Silvestre G, Rodríguez-Abalde A, Fernández B, Flotats X, Bonmatí A (2011) Biomass adaptation over anaerobic co-digestion of sewage sludge and trapped grease waste. Biores Technol 102:6830–6836

Speece RE, Boonyakitsombut S, Kim M, Azbar N, Ursillo P (2006) Overview of anaero-bic treatment: thermophilic and propionate implications. Water Environ Res 78(5):460–473

Stams AJM, Plugge CM (2009) Electron transfer in syntrophic communities of anaerobic bacteria and archaea. Nat Rev Microbiol 7(8):568–577

Svensson BH, Dubourguier HC, Prensier G, Zehnder AJB (1992) *Clostridium quinii* sp. nov., a new saccharolytic anaerobic bacterium isolated from granular sludge. Arch Microbiol 157:97–103

Thauer RK (1998) Biochemistry of methanogenesis: a tribute to Marjory Stephenson. Microbiology 144:2377–2406
Vanwonterghem I, Evans PN, Parks DH, Jensen PD, Woodcroft BJ, Hugenholtz P, Tyson GW (2016) Methylotrophic methanogenesis discovered in the archaeal phylum Verstraetearchaeota. Nat Microbiol 1
Vavilin VA, Rytov SV, Lokshina LY (1996) A description of hydrolysis kinetics in anaerobic degradation of particulate organic matter. Biores Technol 56:229–237
Venkiteshwaran K, Bocher B, Maki J, Zitomer D (2015) Relating anaerobic digestion microbial community and process function. Int J Microbiol Insights 8:37–44
Vital M, Howe AC, Tiedje JM (2014) Revealing the bacterial butyrate synthesis pathways by analyzing (meta)genomic data. MBio 5
Vrieze J, Hennebel T, Verstraete W (2012) Methanosarcina: the rediscovered methanogen for heavy duty biomethanation. Biores Technol 112:1–9
Wang J, Wan W (2009) Factors influencing fermentative hydrogen production: a review. Int J Hydrogen Energy 34:799–811
Wang K, Yin J, Shen D, Li N (2014) Anaerobic digestion of food waste for volatile fatty acids (VFAs) production with different types of inoculum: effect of pH. Biores Technol 161:395–401
Wang P, Wang H, Qiu Y, Ren L, Jiang B (2018) Microbial characteristics in anaerobic digestion process of food waste for methane production—a review. Biores Technol 248:29–36
Weiland P (2010) Biogas production: current state and perspectives. Appl Microbiol Biotechnol 85:849–860
Wofford NQ, Beaty PS, McInerney MJ (1986) Preparation of cell-free extracts and the enzymes involved in fatty acid metabolism in *Syntrophomonas wolfei*. J Bacteriol 167:179–185
Wood HG, Ljungdahl L (1991) Autotrophic character of acetogenic bacteria. In: Variations in autotrophic life. Academic Press Books, pp. 201–250
Xing J, Criddle C, Hickey R (1997) Effects of a long-term periodic substrate perturbation on an anaerobic community. Water Res J 31:2195–2204
Yatawara MDMDWMMK (2015) Generation of biogas from degradable organic wastes, lesson 32. In: Practical manual for GCE A/L biosystem technology teachers, pp. 227–236
Yen HW, Li RJ, Ma TW (2011) The development process for a continuous acetone–butanol–ethanol (ABE) fermentation by immobilized *Clostridium acetobutylicum*. J Taiwan Inst Chem Eng 42:902–907
Yi J, Dong B, Xue Y, Li N, Gao P, Zhao Y, Dai L, Dai X (2014) Microbial community dynamics in batch high-solid anaerobic digestion of food waste under mesophilic conditions. J Microbiol Biotechnol 24:270–279
Yoo M, Croux C, Meynial-Salles I, Soucaille P (2017) Metabolic flexibility of a butyrate pathway mutant of *Clostridium acetobutylicum*. Metab Eng 40:138–147
Yue Z, Yu H, Wang Z (2007) Anaerobic digestion of cattail with rumen culture in the presence of heavy metals. Biores Technol 98:781–786
Zhang C, Su H, Baeyens J, Tan T (2014) Reviewing the anaerobic digestion of food waste for biogas production. Renew Sustain Energy Rev 38:383–392
Zhao H, Li J, Liu J, Lü Y, Wang X, Cui Z (2013) Microbial community dynamics during biogas slurry and cow manure compost. J Integr Agric 12:1087–1097
Zhou M, Yan B, Wong JWC, Zhang Y (2017) Enhanced volatile fatty acids production from anaerobic fermentation of food waste: a mini-review focusing on acidogenic metabolic pathways. Biores Technol 248:68–78
Zhu J (2000) A review of microbiology in swine manure odor control. Agr Ecosyst Environ 78:93–106
Zhu H, Parker W, Basnar R, Proracki A, Falletta P, Beland M, Seto P (2009) Buffer requirements for enhanced hydrogen production in acidogenic digestion of food wastes. Biores Technol 100:97–102
Zinder SH (1993) Physiological ecology of methanogens. In: Methanogenesis, pp. 128–206