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The significance of microbial community functions and symbiosis in enhancing methane production during anaerobic digestion: a review

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Abstract
The anaerobic digestion process used for methane production has been studied for decades but most studies focused on the optimisation of physico-chemical operating parameters. A holistic understanding of the role played by different microbial communities and their symbiotic associations in facilitating the breakdown of the organic substrates to form methane gas is very key and yet it has only received little attention. This review discusses the AD process and various traditional approaches that have been used to improve its efficiency. The major limitation of these approaches in failing to elucidate the actual roles played by the myriads of microorganisms within their communities and symbiotic associations, as a fundamental starting point for AD process control and optimisation was highlighted. A review of the AD microbial pathways so far known was done, followed by an introduction of the metagenomics coupled with metabolomics approach for a more intricate understanding of the biological processes that happen in AD systems. Progress in the application of this approach during the digestion of various organic substrates including animal manures was also reviewed and finally, prospects for the future use of multi-omics (metagenomics, transcriptomics and metabolomics) approach, were highlighted.

Keywords Anaerobic digestion · Methane · Micro-organisms · Metagenomics · Metabolomics · Symbiosis

1 Introduction

Energy continues to be a resource of great demand, globally. A considerable quantity of energy used worldwide is derived from fossil fuels which has two major consequences, the detrimental effect its use has on the environment and the lack of sustainability associated with its use. It is without a doubt, a dwindling resource. Thus, one of the greatest millennial challenge is to find other sources of energy that reduce our carbon footprint and should be derived from sustainable sources. Methane derived from organic degradation is considered an example of such an energy source. According to the World Nuclear Association (2018), methane has an energy density of 50–55 MJ/kg and it is the inflammable component of biogas, a consequence of the degradation of organic wastes in a process regarded as anaerobic digestion (AD). Biogas normally consists of active methane (CH_4), carbon dioxide (CO_2), very little amounts of hydrogen sulphide (H_2S) as well as water vapour. The term 'anaerobic' implies the absence of oxygen (Náthia-Neves et al. 2018), which from a biochemical perspective is associated with incomplete and inefficient break down of the organic substrate such as glucose in the formation of adenosine triphosphate (ATP) (Goldwag and Potts 1990; Zuddas 2019; Reece et al. 2011), and it is for this reason that significant potential energy resident in H^+ ions is still present in the by-products in particular methane making this compound a useful energy derivative from the AD process (Thangarasu and Anand 2019). The first anaerobic digester for methane production in biogas was developed in 1859 in Bombay, India (Van 2012) and the AD technology used for its production has employed various kinds of organic wastes (substrates) ranging from animal manures (Karaca and

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Öztürk, 2018), vegetative biomass (Cioabla et al. 2013), municipality solid wastes (Eriksson et al. 2011; Ag et al. 2007) wastewater and its derived sludge (Cabirol et al. 2014) as well as different kinds of industrial wastes i.e. agro-industrial wastewater (Real Olvera and Lopez-Lopez 2012), tapioca and tofu (Hadiyarto et al. 2018), pulp and paper (Priadi et al. 2014) and sugar industrial wastewater (Artsupho et al. 2016).

Biogas quantity and composition are determined by the type of substrate (Montgomery and Bochmann 2014; Loughrin and Lovanh 2019), physical and chemical operating conditions being maintained during the AD process (Majd et al. 2017). This review focuses on the AD processes as it is currently understood and manipulated for better methane yields by use of various strategies. It describes the microorganisms that have been identified in certain AD systems and facilities such as animal manures and wastewater treatment plants and highlights the knowledge gap that still exists on the functional roles played by these micro-organisms during AD. The review especially emphasises on the need for a comprehensive molecular understanding of the fundamental biological processes and chemical pathways followed in the AD of animal manures such as cow, poultry, pig and horse manures. This is a grey area that according to the authors' research, has been overlooked regardless of the common understanding that these manures are major substrates in biogas production (Maile and Muzenda 2014; Sibiya et al. 2017). The emphasis of this review is to advocate for the research on AD systems to be directed to a quantitative and qualitative understanding of the microbial communities in digesters and relationships existing among the micro-organisms and between the changing microbial population during degradation and digester function.

2 Background

Anaerobic digestion is a sustainable biological waste management method that can reuse vast amounts of nearly all kinds of organic materials and wastes ranging from agriculture to food industries and the associated municipal landfills and water treatment facilities to generate biogas and organic fertiliser under anaerobic conditions (Arvanitoyannis and Varzakas 2008; Wang, 2014a, b; Ileleji and Jones 2018). Higher yield of methane in biogas is achieved from waste with a high chemical oxygen demand (COD), well-digestible starch and very little or no toxic/inhibitory compounds (Gopal 2019). According to Gopal, AD can convert between 58 and 90% of COD under the above-mentioned circumstances to 0.24–0.4 m^3 CH_4/kg COD. The anaerobic digestion process emits less amounts of greenhouse gases compared to other waste treatment processes such as composting and is therefore an environmentally friendly process (Lin et al. 2019).

2.1 The anaerobic digestion (AD) process

Anaerobic digestion is a complex process which significantly depends on the interaction of microorganisms participating in a myriad of activities within both simultaneous and sequential pathways during the degradation of organic matter. Within a closed AD system are some substrate limiting tendencies and this leads to different forms of evolving close, long-term association between two or more organisms often synonymous with successional patterns seen in microbial ecological population uniquely defined by changes in substrate compositions and concentrations towards the conversion of organic matter to CH_4 and CO_2 (Finstein et al. 1980; Li et al. 2016; Majd et al. 2017; He et al. 2018; Shin et al. 2019). Nevertheless, the AD process as we understand it, thus far, has four syntrophic stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Divya, et al. 2015). Moreover, the rate of any one of these AD steps influences the overall rate of methane formation, thus, it is essential to identify the rate-limiting step in any given AD process since this may differ from one system to another depending largely on substrate biochemical structure. This assertion is supported by Ma et al. (2013) in their rate-limiting step determination studies where they supplemented metabolic intermediates from each digestion step during the AD of complex substrates. This same perspective is expressed by Song et al. (2020), in a study that elucidated functional genes participating in the metabolism of starch and sucrose pepper rhizosphere in bulk soil fertilised with chicken manure under plastic greenhouse cultivation. In this lab scale study, it was noted that the metabolite profiles which include amino acids, organic acids, carbohydrates, ketones, lipids, alcohols, etc., were strongly correlated with the structures of the identified bacterial communities. Therefore, taking these observations into consideration, we can infer that a clear understanding of the biochemical pathways would better inform the relevant AD process control measures to be taken with the varying substrates and composition being a major factor.

A good understanding of the food web, microbial abundance, interaction of microorganisms in the bioreactor, the presence and absence of inhibitory and promoting factors and microbial responses under certain conditions is fundamental in methane yield optimisation (Fanedl 2013).

2.1.1 Hydrolysis

Hydrolysis is a reaction of biomass polymers with water to produce monomers and hydrogen and is usually the rate determining step in AD processes associated with recalcitrant feedstock (Bajpai 2017). Its importance stems from the initiating reaction it provides, which is necessary for the breaking of strong complex chemical bonds often responsible for the recalcitrance to degradation of these compounds. With

organic substrates commonly used in the AD process, lignin is by far the most recalcitrant compound present. However, according to Prasad et al. (2017), it is possible to accelerate the hydrolytic phase of AD processes. In their experiments, 4% hydrochloric acid (HCl) was used to pre-treat recalcitrant sunflower stalks at 170 °C, over 90% of uronic acids and hemicelluloses were removed, increasing the methane production by 21–29%. This acidic treatment was however performed under laboratory conditions and certainly this approach wouldn't be feasible for large scale methane production. Alkaline pre-treatment using NaOH increased the methane yield from leaves, rice straw and wheat straw by 24%, 30%, 47% respectively while use of $Ca(OH)_2$ improved the methane yield from the organic fraction of municipality solid waste (OFMSW) by 172%. Hydrolysis is facilitated by hydrolytic enzymes (exoenzymes such as amylase, cellulosomes, lyposomes, xylanases, lipases and proteases) from a number of bacteria, protozoa, and fungi (Madigan et al. 2008; Singh et al. 2018). Proteolytic microbes secrete proteases capable of converting proteins to amino acids while cellulolytic and xylanolytic microbes secrete xylanases to breakdown complex cellulose and xylose into glucose and xylem and lipolytic microbes produce lipases that convert lipids (fats and oils) into long-chain fatty acids and glycerol (Kangle et al. 2012).

Different substrates usually have different hydrolytic bacteria. In some studies, crop substrates have been found to harbour *Actinobacteria*, *Cytophaga–Flavobacterium, Chloroflexi, Betaproteobacteria, Gammaproteobacteria, Alphaproteobacteria,* and *Firmicutes* (Cirne et al. 2007) while municipal sludge cultured *Porphyromonadaceae, Prevotellaceae* and *Ruminococcaceae* (Maspolim et al. 2015) and other substrates commonly have *Cellulomonas, Bacillus* and *Mycobacterium* (Cavinato 2009).

Gómez et al. (2018) and Mahmood et al. (2006) consider the hydrolysis stage to be the rate limiting step. Singh et al. (2018) further adds that the rate of hydrolysis depends on substrate pH, particle size, production of enzymes, diffusion, and adsorption onto the substrate particles during the digestion process. Pertinent to note is the observation by Maspolim et al. (2015) that the hydrolysis rate also depends on the substrate being hydrolysed with simple carbohydrates taking a few hours and lignocellulosic material taking several days. Venkiteshwaran et al. (2015) observed the phylogenetical diversity of hydrolytic bacteria, however, they noted *Firmicutes* and *Bacteroidetes,* constitute most of the known species growing more rapidly and demonstrating lower sensitivity to environmental factor disturbances such as shifts in pH and temperature compared to methanogens. Prasad et al. (2017) noted the presence of the bacteria of the genera *Lactobacillus, Bacteroides, Propionibacterium, Megasphaera, Sphingomonas, Sporobacterium, Bifidobacterium* as common to the hydrolysis step, these groups of bacteria include both obligate and facultative anaerobes. Most of the studies on hydrolysis done thus far, have however not elucidated the link between product formation i.e. metabolites formed at a particular stage of the AD process and the prevalent microbial profiles which would have provided greater insights and validation to existing biochemical pathways linked to biomethane production.

Plausibly, the rate determining step differs from substrate to substrate since the microbiome also differs as well as the anaerobic pathways. A deeper understanding of any identified or novel hydrolytic microorganisms and their symbiotic relationships during the intermediate steps within the AD of various substrates in specificity, would provide clarity as to the factors that interplay during the rate limiting step and selection of strategies to boost activities of these microorganisms towards optimising intermediate products that would go into the next stage of biodegradation. This perspective is similar to that proposed by Cirne et al. (2007), in their study that sought to identify the general bacterial groups involved during the hydrolysis stage of AD of different crop substrates in order to develop strategies for stimulating hydrolysis and ultimately increase methane production rate and yield from reactor-based digestion of organic substrates.

Hydrolytic microorganisms (primary fermentative bacteria) exhibit a symbiotic relationship with the acidogenic (secondary fermentative bacteria) in that the metabolite products of hydrolysis are what the acidogenesis microorganisms consume in their contribution to the formation of methane (Sikora et al. 2017). However, besides this metabolic cooperation providing trophic benefits, there exists a commensalistic symbiosis of hydrolytic microorganisms with some host eukaryotes that have anaerobic conditions for instance the rumen of ruminants, caeca of various monogastric vertebrates and termite hindgut (Wrede et al. 2012). The microorganisms which include unique species of bacteria, yeasts, protozoa, and fungi, found within these gastrointestinal tracts (GITs) are well-suited for the breakdown of the often-recalcitrant plant biomass; cellulose, hemi-cellulose and lignin (Dimijian 2000). Some microorganisms identified to be able to readily form symbiotic associations with eukaryotic hosts include the genus *Streptomyces* of the *Actinobacteria* phylum (Book et al. 2016) and a number of genera from the *Bacteroidetes* phylum (Bertucci et al. 2019). According to Book et al. the ability of *Streptomyces* to rapidly decompose cellulose is further enriched in symbiotic stains associated with diverse hosts that feed on plant biomass. Cellulolytic fungi and bacteria with the ability to efficiently degrade highly recalcitrant substrate have also been identified to be commonly associated with herbivorous eukaryotes. It is therefore important to take note of any sort of symbiotic association whether mutualistic, commensalistic or parasitic, between potentially useful communities of microorganisms and their natural environments with the intention to understand their naturally favoured environment. This knowledge can therefore be inferred to the

potential substrates the microorganisms are good at degrading, metabolites they can produce and the physical conditions under which they thrive most comfortably. With this information, a strategy to mimic their favourable conditions in AD processes for methane production can be developed.

2.1.2 Acidogenesis

The acidogenesis step involves the conversion, by partial oxidation, of the sugars, organic acids such as fatty acids and amino acids produced during hydrolysis to hydrogen, carbon dioxide and acetate and volatile fatty acids (VFAs) such as n-butyric and iso-butyric, propionic, alcohol (ethanol) and lactic acid (Rajendran et al. 2012; Divya et al. 2014). Acidogenic bacteria regenerate within 36 h (Meegoda et al. 2018) and according to Venkiteshwaran et al. (2015), acidogenesis is rapid, leading to VFAs accumulation and pH drop. This rapid increase in the flux of VFAs causes acid utilization inhibition due to organic overload and the accumulation of toxic by-products as a result of incomplete mixing, often associated with rapid temperature changes due to the temperature gradient created between the slurry already degrading in the digester and the freshly fed feedstock (Lawrence et al. 2014). This step is also relatively fast and the myriad of metabolites including lactate, propionate, butyrate, alcohol, butanediol, and acetate influence the product formation routes during the subsequent step of acetogenesis, however these alternate routes maybe dictated by flux and thermodynamics in relation to intermediate product concentrations within the system as well as enzyme concentration (Ferrara et al. 1984). An interesting symbiotic relationship exists between the microorganisms that produce hydrogen and those that produce the non-gaseous acidogenesis products; acetate, butyrate, lactate, propionate. A study by (Sikora et al. 2017), revealed that an increased number of the above-mentioned non-gaseous products decreases hydrogen production which in-turn impacts on the activities of the hydrogen-producing enzymes; pyruvate ferredoxin oxidoreductase and nicotinamide adenine dinucleotide ferredoxin oxidoreductase since the two enzymes are regulated by the concentration of hydrogen concentration. Another noteworthy symbiotic relationship during acidogenesis is the cross-feeding which exists between lactic acid bacteria and butyrate-producing bacteria where the butyrate-producing bacteria form more butyrate from lactate and acetate (Detman et al. 2019). This culminates in a higher yield of hydrogen which can potentially lead to H_2-inhibition if produced in excess.

Of the metabolites produced during acidogenesis, propionic acid and hydrogen have inhibitory effects to the formation of acetic acid during acetogenesis. Sträuber et al. (2012) established in their findings that the propionic acid concentration in an AD digester should generally be kept below 1.5 g/L in order to avoid propionic inhibition. It is however fortunate that the degradation of propionic acid during subsequent AD steps is largely promoted by the high activity of butyric acid-converting bacteria which makes the rate of butyric acid conversion higher than that of the other VFAs (Breure and van Andel 1984). Hydrogen on the other hand, when in excess is another inhibitory metabolite and therefore its excessive production and that of propionic acid do not favour methane production. From these findings, it would therefore be necessary to control the concentration of butyric acid-forming and converting microorganisms and enzymes respectively in a strategy to avoid process any negative process inhibition. Having outlined some important symbiotic associations of various acidogenic microbial groups, we can plausibly conclude that that the metabolite composition of the acidogenesis process affects the subsequent acetogenic and methanogenic performance and it is therefore apparent that acidogenesis is the product-determining step in AD processes upon which process control and optimization strategies should focus. These findings as agreed by Sträuber et al. (2012), are in sync with their recommendation to pursue more detailed analyses of acidogenesis steps in solid-state fermentation for the efficient utilisation of feedstocks that are more sustainable such as energy crops and straw.

2.1.3 Acetogenesis

Acetogenesis is the conversion of the products of acidogenesis into acetate and H_2 by acetogenic bacteria and obligate H_2-producing bacteria (Kangle et al. 2012; Guo et al. 2015a, b). The microorganisms involved include propionate decomposers such as *Syntrophobacter wolinii*, butyrate decomposers such as *Syntrophomonos wolfei*, and acid formers such as *Clostridium* spp., *Lactobacillus*, *Peptococcus anerobus*, and *Actinomyces* (Kangle et al. 2012). While H_2-producing acetogenic bacteria produce H_2, CO_2 and acetate from VFAs and alcohol, homoacetogenic bacteria produce acetate from CO_2 and H_2 (Sterling et al. 2001) but however, H_2-producing acetogenic bacteria produce most of the acetate (Angelidaki et al. 2000).

The H_2 gas formed in the acetogenesis step if in excess of certain critical partial pressures in various metabolic pathways and operating conditions of temperature and pH, can inhibit the action of acetogenic bacteria. The critical H_2-partial pressures depend on the redox potential of the catalysing enzyme, which is organism/ consortia specific. An example is the inhibition of the consumption of propionate and butyrate intermediates when the H_2-partial pressure goes above 10^{-4} atm during the AD process (Venkiteshwaran et al. 2015), although methane-producing bacteria (hydrogenotrophic) functioning as H_2-scavenging bacteria can consume and convert the H_2 into methane (Bajpai 2017; Meegoda et al. 2018). Other examples include the nicotinamide adenine dinucleotide (NADH): ferredoxin oxidoreductase/ hydrogenase and

ferredoxin: hydrogenase metabolic pathways, which have critical H_2-partial pressures of 8.4×10^{-4} atm and 0.024–4.5 atm respectively for different species of *Clostridium* (Clark et al. 2012). There is therefore a beneficial syntrophy between the acetogenic and hydrogenotrophic methanogens. Acetogenesis is a critical step to maintain rapid yet stable AD process operation since some of the VFAs, especially propionate, inhibit the subsequent step of methanogenesis at high concentrations (Shah et al. 2014).

Considering H_2 inhibition and the symbiosis of micro-organisms, it is not ultimately clear and exhaustive, regardless of the studies by Venkiteshwaran et al. (2015) and Clark et al. (2012), at what point H_2 accumulation begins to inhibit specific micro-organisms during the degradation of various different substrates. This knowledge is especially important given the previously discussed assertion that there's intimate correlation between micro-biome and AD process metabolome. It is therefore worthwhile to establish H_2 inhibition levels for different substrates through a profiling of the taxonomy and functional roles played by various micro-organisms during the AD of the substrates.

2.1.4 Methanogenesis

The methanogenesis step is the ultimate in the AD process and here acetic acid is converted to methane gas (Lemmer et al. 2013). This step has been divided into two main routes namely hydrogenotrophic route, where H_2 and CO_2 are converted into methane by H_2/CO_2 consumers and acetotrophic (acetoclastic) route, where acetate consumers form methane from acetate conversion (Smith et al. 2013; Costa et al. 2016; Majd et al. 2017). Kangle (2012) noted that the common methanogenic archaea include *Methanobacillus*, *Methanobacterium, Methanosarcina* and *Methanococcus*. Studies on the contribution of the 2 classes of methanogenic microorganisms revealed that they can compete for molecular hydrogen (Kushkevych 2017) but in most cases acetate conversion contributes (70%) of the methane while CO_2 reduction by H_2 and other electron donors gives the remaining 30% (Kangle et al. 2012). Shah et al. (2014) highlighted another methane formation route by a methane-producing bacteria group referred to as the methylotrophic bacteria which create methane from methyl compounds such as methylamines, methanol, and methylsulfides. This assertion was supported by Venkiteshwaran et al. (2015) and Boontian (2014).

Further studies have revealed yet another methane formation route named syntrophic acetate oxidation (SAO), which occurs at increased ammonia levels such as those experienced during protein-rich material degradation. This happens when the acetoclastic methanogens suffer ammonia inhibition thereby giving way to syntrophic acetate-oxidizing bacteria (SAOB) such as *Clostridium* sp. to oxidise acetate to formate, CO_2 and H_2 which are then utilised by hydrogenotrophic methanogens to generate methane (Müller et al. 2012; Westerholm et al. 2016). In their study, Venkiteshwaran et al. (2015) asserted that ammonia concentration of 3 g/L NH_3-N and elevated temperature might be inhibitory to acetoclastic methanogens and hinder biogas production. These studies have thus revealed that when inhibitors (VFAs and ammonia in particular) are present, the SAO route takes over from the acetotrophic route and becomes the main mechanism for acetate degradation (Karakashev et al. 2006). SAO-capable bacteria currently known are the thermophilic *Pseudothermotoga lettingae*, *Thermacetogenium phaeum*, the thermotolerant *Tepidanaerobacter acetatoxydans* and the mesophilic *Syntrophaceticus schinkii* and *Clostridium ultunense*. SAOB, however, grow very slowly with a doubling time of up to 28 days at 37 °C compared to the doubling time of acetoclastic methanogens, determined empirically to be about 8–36 h and 1–9 days for *Methanosarcina* and *Methanosaeta*, respectively and can only assume relevance in acetate degradation when the acetoclastic methanogens are suffocated by ammonia and VFAs (Westerholm et al. 2016).

Methanogenic archaea are highly sensitive to the environmental changes in temperature, oxygen, pH levels (Zeikus 1977). They are slow growing with generational times ranging from 5 to 16 days in comparison to the average of 1 to 2 day of other bacteria in AD processes. Although, *Methanococcus maripaludis* and some other hydrogenotrophic species have reportedly been observed to have a doubling time of only two hours (Meegoda et al. 2018). Knowledge of the microbial doubling time or multiplication rate is essential for effective AD system design, operation and troubleshooting, and as such, the abundance of micro-organisms participating at any stage of the AD process must be determined for various substrates in order to maximise on COD reduction and methane yield. This stated perspective is shared by Fanedl (2013) and this was surmised from his studies that observed how dynamic the AD process is in terms of substrate loading, inflow and generation of toxic compounds, substrate pre-treatments and variations in chemical and physical conditions (temperature, pH, VFAs, phenols, ammonia, heavy metals, etc.). Fanedl (2013) recommended the monitoring of the microbial community in order to diagnose the dynamic AD conditions and avoid decrease in efficiency, souring and economical loss in full-scale biogas digesters.

Regardless of the established understanding that methanogenic species constitute very sensitive microbial groups, *Methanosarcina* spp. has been observed in a recent research by Majd et al. (2017) and demonstrated to be relatively robust and capable of withstanding the inhibitory effects of humic acid and ammonia on methane production. In their studies, the authors highlighted that a free ammonia concentration as little as 150 mg/l may have high inhibitory effects on AD, but a gradual increase in digester bacteria concentration enhances

adaptation to ammonia concentrations as high as 5000 g/l total ammonia nitrogen. Similar findings were reported by Shah et al. (2014a, b), although in their studies the critical ammonia inhibition that continued to support the proliferation of *Methanosarcina* was 7000 mg/l total ammonia nitrogen. It is therefore important to identify and boost any beneficial microbial species such as *Methanosarcina* which are more tolerant to some specific inhibitors of the acetoclastic pathway, such as ammonia, methyl fluoride and fluoroacetate thereby achieving stable growth at high organic loading rates and low retention times in AD systems. Meegoda et al. (2018) suggested the increment of resistant microbial species so as to reduce chances of process inhibition at inhibitor concentrations that would otherwise be injurious to other methanogenic microorganisms. However, this is only possible when the correlation between taxonomy and functions of the micro-organisms present in an AD system are holistically understood.

Methanogens have a well-characterized syntrophic interaction (interspecific electron transfer) based on the transfer of hydrogen and formate with fermentative bacteria (syntrophs), which cooperatively produce volatile fatty acids (VFAs) such as butyrate, propionate and acetate for conversion into methane (Kouzuma et al. 2015). In this interaction, through the oxidation of propionate into acetate, syntrophic propionate-oxidizing bacteria acquire energy. The oxidation of propionate generates reducing equivalents which are used for the reduction of protons to produce H_2 which is then efficiently scavenged by hydrogenotrophic methanogens to produce CH_4 as a part of their energy metabolism and growth.

Another case of interspecies electron transfer (IET) is the interspecies hydrogen transfer (IHT) between syntrophs and any of the three major groups of H_2-consuming microorganisms (hydrogenotrophs) i.e.; methanogens, acetogens and sulphate-reducing bacteria (SRB) (Nakamura et al. 2010). Atypical example of a syntrophic association between fermentative bacteria and methanogens is that between *Ruminococcus albus* and Wolinella *succinogenes* in the rumen as studied by Nakamura et al. (2010). Such symbiotic association was supported by Imachi (2017) in his study on anaerobic digestion processes in rice paddy fields. In IHT processes such as this, the symbiotic relationship between the microorganism communities is such that the growth of the H_2 producer is possible only if the H_2 partial pressure is maintained below a certain threshold by the H_2 consumer and that the absence of H_2-consuming organisms thermodynamically restricts fermentation.

It should be noted that although environmental heterogeneity may enable distinct types of hydrogenotrophic reactions to proceed simultaneously, allowing SRB and methanogens to coexist, direct competition for H_2 may occur among the three groups of hydrogenotrophs. It has been reported that SRBs are dominant in sulphate-rich, H_2-limiting environments (Nakamura et al. 2010). This is due to their kinetic growth parameters which are favourable for H_2 more than those for methanogens and acetogens (Nakamura et al. 2010; Lin et al., 2019; Roland, 2010; Ishii et al. 2005). Competition for sulphate between different types of SRB may also explain partially the competitive interaction of hydrogenotrophic microbes in sulphate-limited environments.

Symbiotic interactions have also been shown to develop among certain species of syntrophs and methanogens organised in direct physical contact or close proximity in accordance with Fick's law (Kouzuma et al. 2015). The spatial organization of these syntrophs and methanogens is therefore considered to be crucial for efficient methanogenesis since it influences the IHT efficiency. Random cell-to-cell associations of these special species with other microbial species may cause the deterioration of methanogenic metabolism. To discriminate their syntrophic partners from other microbial species, these syntrophs and methanogens exploit specific interspecies cell-to-cell recognition systems such as flagellum interspecies recognition / entrapment and signalling mechanisms. This hypothesis was been demonstrated in a flagellum mediates symbiosis studies by Shimoyama et al. (2009) and Kato and Igarashi (2019), where a syntrophic consortium consisting of propionate-oxidizing bacterium, *Pelotomaculum thermopropionicum* and *the* hydrogenotrophic methanogen, *Methanothermobacter thermautotrophicus*, have major cell-surface components that differ markedly with respect to chemical structures (pseudomurein and glycoprotein, respectively) and adhere to each other via specific cell-surface components, such as cell-surface proteins or carbohydrate moieties, rather than major cell-surface structures. This protein-based interspecies communication between prokaryotes is one of very few symbiotic associations of its kind and more discoveries have the potential to improve AD efficiency.

Furthermore, some observed syntrophs are often found in close proximity to methanogens within microbial aggregates such as granules and biofilms and co-aggregation has been observed in defined co-cultures of syntrophs and methanogens that do not form aggregates in pure cultures (Kouzuma et al. 2015). *Pelotomaculum thermopropionicum* and *Methanothermobacter thermautotrophicus* were again observed to co-aggregate under syntrophic methanogenic conditions discovered by Ishii et al. (2006) in analysing partially aggregated syntrophic co-cultures with various substrates. The Ishii et al. studies also showed that the degree of co-aggregation, which is characterized by the abundance and size of aggregates is influenced by the available growth substrates (Ishii et al. 2005).

Recently, syntrophy in anaerobic microbiota has been discovered to proceed not only via the diffusion of electron carriers such as hydrogen and formate but also via electric current between electron-donating and accepting microbes, a process

referred to as direct IET. Various bacteria with outer membrane c-type cytochromes and electrically conductive pilus-like structures (nanowires), have been shown to be able to extracellularly transfer electrons and to generate current in microbial fuel cells. Electrically conductive substances which include carbon materials such as conductive magnetite particles and mineral particles can facilitate direct IET and syntrophic acetate catabolism. Kato and Igarashi (2019) demonstrated that magnetite nanoparticles facilitate IET from *G. sulfurreducens* to *T. denitrificans*, thereby promoting the oxidation of acetate coupled to nitrate reduction. In their community analyses study based on 16S rRNA genes, it was revealed that *Geobacter* and *Methanosarcina* species were predominant in the enrichment cultures supplemented with semi-conductive hematite and magnetite nanoparticles. This suggested that these minerals facilitated IET between the two microbial communities hence promoting syntrophic methanogenesis and growth. In another study, granulated activated carbon (GAC), was added to the co-cultures of *G. metallireducens* and *G. sulfurreducens* and it markedly accelerated syntrophic ethanol metabolism coupled to fumarate reduction, suggesting that GAC served as an electron conduit which facilitated IET via electric current (Kouzuma et al. 2015).

The methanogenesis stage is referred to as the terminating step of the AD process during methane production. The amount of methane retained in the anaerobic reactor (digester) is dependent on the balance between microbial methane production and oxidation rates by methane-producing archaea and methane-oxidizing bacteria, respectively (Imachi 2017). Methane-oxidizing bacteria (MOB) gain energy from the oxidation of methane (Oremland and Culbertson 1992; Kojima et al. 2014). Three groups of MOB are the bacterial classes *Gamma proteobacteria* (Type l), *Alpha proteobacteria* (Type ll), the phyla *Proteobacteria* and *Verrucomicrobia* (Type III) (Kojima et al. 2014; Mateos-Rivera et al. 2018). These aerobic methanotrophs oxidize CH_4 into CO_2 by converting it into methanol through use of the enzyme methane monooxygenase (MMO) (Mateos-Rivera et al. 2018).

Although numerous studies have been done on microbial symbiosis in anaerobic digestion processes, ranging from trophic metabolic cooperation, interspecific electron transfer, interspecific hydrogen transfer, direct interspecific electron transfer, appendage interspecies recognition / entrapment and signalling, co-aggregation and methane producing vs. methane oxidation, the examples given suggest that there still exist many more unidentified symbiosis mechanisms that occur within AD systems and have the potential to increase the efficiency and robustness of microbial interspecies interactions. More findings of this nature would provide novel opportunities for the design of more optimal microbial processes in the production of methane. Given the great diversity of microbes in nature, it is likely that there exists a vast collection of unique molecular mechanisms for facilitating interspecies interactions.

2.2 Effect of operational parameters on microbial profiles and symbiosis in AD processes

2.2.1 pH

According to Majd et al. (2017), good acidity for methane generating bacteria is between 6.5 and 7.8 while it is between 5.0 and 6.0 for acid-forming bacteria and thus for effective growth of sludge seeds and anaerobic microorganisms, optimum pH should be in the range of 6.5–7.5. The introduction of a chemical buffer solution as prescribed by Singh et al. (2018) may not be ideal in some AD systems since the AD process is micro-molecularly a symbiosis of a lot of microbial communities which simultaneously work best under different pH conditions. Addition of a buffer solution may thus maintain a neutral pH level at the expense of some microbial communities and their healthy symbiosis with other groups of microorganisms. Better control is therefore achieved through a holistic understanding of the simultaneous behaviour of co-existing microorganisms and the use of a phasic approach that timeously varies the pH with the changing trends in microbial population and the optimal pH requirements of the predominant organisms in these phases.

2.2.2 Temperature

AD can occur under 3 major temperature ranges: 1) Psychrophilic (0 to 20 °C), 2) Mesophilic (20 to 40 °C), and Thermophilic (40 to 60 °C) (Majd et al. 2017). The thermophilic AD bacteria are easily influenced by toxins and small changes in environmental conditions while mesophilic microbial consortia have high tolerance to environmental changes, better stability and are thus easy to maintain (Singh et al. 2018). Given this background, it is important to determine the operational temperature range of the micro-organisms predominant in each substrate or AD system in order to operate it within the most appropriate temperature range. A good example is the SAOB group which are relevant in a methanation route that becomes energetically favourable at the thermophilic temperature range since at higher temperatures the proportion of NH_3 and acetate concentration increase. This was the case in a study by Westerholm et al. (2016) in an industrial AD process where the SAO pathway became the main mechanism for acetate conversion at thermophilic conditions.

2.2.3 Organic loading rate (OLR)

The loading rate of organics into an AD system should be optimal since a high loading rate would result in an increased

abundance of acidogenic bacteria which decreases the pH and eliminates some methanogenic bacteria hence resulting in system failure (Singh et al. 2018). The critical question to then answer is how to effectively determine the optimal OLR for different substrates since they have different microbiome structures. It is therefore critical to understand the microbial profile of the substrate being digested in order to feed the AD system optimally without upsetting the unique microbial ecology and balance. Studies that have been done so far which include the work of Mel et al. (2015) on fruit and vegetable wastes, have only focused on maintaining a neutral pH as the basis of optimal OLR without necessarily understanding the microbial profile of the AD system.

Meegoda et al. (2018) hypothesized that increased diversification (acetoclastic microorganisms giving way to SAOB and hydrogenotrophic in a symbiotic relationship) of methanogenic microorganisms improves resistance to overloading and digester performance. Such increased diversification could indeed come in handy in reducing cases of AD system failure, however it can only be achieved through microbial trend analysis to allow insights into specific aspects during monitoring of these microbial profiles that reveal the point of diversification in order to reproduce this beneficial diversification needed to optimise yield.

2.2.4 Nutrient availability

Biogas potential is enhanced by the supplementation of anaerobic digesters with inorganic nutrients such as cobalt, nickel, iron and manganese to stimulate bacterial activity. However, Ishaq (2008), states that the existing research lacks information on the portion of the added nutrients that actually gets utilised (bioavailability) within the AD environment and this makes it virtually impossible to quantify any beneficial effect resulting from the supplementation. Profiling of the microbial activity in AD systems could increase our understanding of how bio-supplementation could be used to improve the efficiency of microbiological performance in anaerobic digesters for biogas production enhancement. A study of the changes in the microbial profile within the resident consortia that respond to increased concentrations of these metals especially if the study incorporates both up and down regulation trends during synthesis may provide a clearer in sight as to the benefits or lack thereof of their inclusion in the AD environment.

2.3 Methane yield

The AD of different animal manures yields different methane volumes since the biomethane potentials BMP is influenced by nutritional composition which in turn is dependent on nutrient uptake and seasonal variations of the fodder ingested by the animals (Prasad 2012; Esposito et al. 2012). This means therefore, that the autochthonous nature of bacteria in each manure will also vary (Ohemeng-Ntiamoah and Datta 2019). This variation can only be truly comprehended with studies that lend unique insights to the various substrates, an area which so far has only received little attention. A lab-scale study conducted in China examined the methane potential of chicken, pig and cattle manures digested under thermophilic conditions (55 °C). From the results the maximum methane potential of the manures were 292 ml/g VS, 272 ml/g VS and 266.4 ml/g VS, respectively (Fen et al. 2017). Methane yield from the AD of horse manure varies widely depending on feed and the bedding used. In another study the methane yield of pure horse faeces and straw mixture was found to be 222.33 ± 13.60 and 233.01 ± 31.32 ml/gVS, respectively (Nitsche et al. 2017). This is different from the findings by Dobre et al. (2014) where the methane yields were 520 ml/gVS, 260-280 ml/gVS, 480 ml/gVS and 200-300 ml/gVS for chicken, cow, pig and horse manure respectively**.** These variations in methane yields may point to a possible correlation between the participating consortia in the AD process and the product yield which was not taken into cognizance by the different researchers. It is a moot point that an emphasis needs to be put on determining the effect of these unique consortia with the different methane yields under uniform AD operational conditions.

2.4 Approaches used to improve methane yield

A major research conducted by Divya et al. (2015), with a focus on improving methane yield and enhancing the efficiency of AD processes provides a good framework for research areas around the process, they proposed a strategy that hinges on four aspects: 1) Biomass utilisation, 2) Microbial treatment, 3) Enzyme addition and 4) Process optimisation. Pertinent in this research was the aspect of biomass utilisation which discussed on the biological availability of substrates used to produce biogas and their physico-chemical properties. It includes research on co-digestion of several different substrates and substrate pre-treatment. The premise of co-digestion of substrates is to balance the concentration of nutrients such as nitrogen and carbon to avoid ammonia inhibition inside AD reactors (Gelegenis and Georgakakis 2007; Avs and Tufaner 2016; Prapinagsorn et al. 2017). Previously, Ishaq (2008) had explained the supplementation of nutrients in AD processes while Gómez et al. (2011) had described how the addition of readily degradable substances such as cheese whey, residual glycerine and food wastes to digesters increased biogas production rates.

To further buttress the need to balance the carbon/nitrogen ratio (C/N), several other studies have worked on various aspects of this. In a study by Tanimu et al. (2014), substrate comprising meat, fruits and vegetable wastes was co-digested to increase the (C/N) ratio from 17 to 26 and 30. The observed methane yields of 0.352 L/gVS, 0.447 L/gVS

and 0.679 L/gVS respectively were achieved. It should be noted that variations of C/N ratio with its concomitant effect on the pH of a slurry especially if it tilts towards an increase in the carbon content (C > N) will give rise to the formation of more carbon dioxide which lowers the pH to the detriment of methanogens, while a higher value of nitrogen (low C < N) will enhance the production of ammonia gas, which increases the pH to the detriment of the same microorganisms with a heightened inhibition when the pH reaches a value ≥8.5 (Dioha et al. 2013; Orhorhoro et al. 2016). In addition, Wang (2014) notes that the increase in C/N ratio reduces the chances of ammonia inhibition often typical in AD systems operated under thermophilic conditions and those degrading substrates that have a high concentration of ammonia above that necessary for microbial growth. Substrates with high C/N ratio on the other hand, meet the protein requirements and hence promote the growth of methanogen populations at the expense of carbon content degradation, and this results in low biogas production. The optimal C/N ratios for various substrates have thus been studied by some researchers such as Rodriguez et al. (2017) who suggested a C/N ratio of 30 for AD of palm wastes, Rao and Singh (2004) who reported an optimum C/N ratio of 25 for municipal solid wastes, Yasin and Wasim (2011) who recommended an optimal C/N ratio of 30 for buffalo dung and Guarino & Carotenuto (2016), who recommended that the ratio should generally be between 20 and 30 for efficient biogas production. The results demonstrated that hydraulic retention time and biogas yields are affected by C/N ratio of substrates.

When the substrate C/N has been adjusted to an optimal range, the other parameter of concern is the organic loading rate (OLR). Studies conducted on the effect of OLR revealed that increasing the OLR considerably results in the accumulation of volatile fatty acids (VFAs) and this condition is harmful to methanogenic archaea (Eslami et al. 2018). In a study on the effect of OLR on AD of vegetable, fruit waste and cow dung, Mel et al. (2015) recommended an OLR of 50,000 mgCOD/L/day as design criteria for pilot biogas production, beyond which continued increase in the OLR, will cause reduction in the degradation of the chemical oxygen demand (COD) as well as the decline in the biogas yield due to a decrease in methanogenic archaea. Rattanapan et al. (2019) co-digested canteen food waste and domestic waste water and asserted that co-digestion is more efficient with the control of VFAs than the use of determined optimal OLR for different substrates.

The process optimisation aspect focuses on bringing about improved digester designs by giving attention to nearly all of the critical operating parameters in a more holistic approach and these include; temperature, pH, organic loading rate (OLR), hydraulic retention time (HRT), carbon to nitrogen ratio (C/N), etc. In their studies on the effect of pH on methane production from water hyacinth and food waste using pH range of 5–7 and 5–9 respectively, Nurfitri Astuti et al. (2013) and Jayaraj et al. (2014), agree that a neutral pH of 7 produces the highest methane since the methane-forming bacteria work best under neutral pH conditions. Ghatak and Mahanta (2014) observed that the highest methane yield under mesophilic and thermophilic temperature ranges is produced at 35 °C and 55 °C respectively and that it is least at 45 °C. These observations suggest that temperature effect on AD processes is informed by the type and abundance of microorganisms present in the system and not merely the physical and chemical reactions that are in direct proportion to temperature. This is supported by the observation made when Tian et al. (2018) studied changes in microbial communities and their utilised metabolic pathways in the anaerobic digestion of pig manure by designing an experiment that looked at temperature increments within the range of 9 – 55 °C. The set-up had six batch biogas reactors with varied temperatures of incubation of 9, 15, 21, 35, 45 and 55 °C. Their findings showed similarities in both microbial communities and metabolic pathways within the range of 15 – 35 °C. A lowered temperature of 9 °C facilitated the promoted hydrogenotrophic metabolism. However, increased temperatures of 45 °C allowed for greater bacteria diversity within the phases of hydrolysis, acidogenesis and acetogenesis. In addition, an increased temperature of up to 55 °C reduced the presence of previously dominating bacteria but cause the promotion of acetotrophic-type metabolism, it also meant the system at this point tended to evolved from mesophilic range bacteria to thermophilic range bacteria that was markedly observed once the temperature of 45 °C was reached. This study profoundly lends insights to how optimisation can be achieved to promote specific type bacteria if a selected metabolite is desired in a higher flux within the AD system.

Other operational parameters approaches that have been explored towards optimising and improving AD process include the reduction of the OLR in order to minimise the proliferation of biosurfactant-producing and filamentous bacteria; also, the increasing of the AD stirring rate to disrupt the growth of *Actinomyces* and other filamentous bacteria which trigger digester foaming. Foaming is considered a major problem in AD processes and results in financial losses. Other issues associated with foaming include clogged pumps and piping systems and overflowing sludge, the latter is considered an environmental nuisance. Pertinent to note is that pioneering countries such as Denmark, Germany and the United States of America where the AD process is perceived to be advanced typically report at least 80% foaming on an annual basis (He et al. 2017) with the concomitant economic challenges associated. According to He et al. (2017), foaming results from a combination of the proliferation of acid-producing bacteria such as *Petrimona* and *Fastidiosipila,* and ammonia producers such as *Proteiniphilum, Gelria* and *Aminobacterium*, accumulated surface-active materials such

as NH_4^+ and VFAs and transformative products generated in the different biochemical pathways within the AD process that creates an enabling environment for increased biomass of these detrimental organisms. As such understanding the associated optimised conditions that provokes a shift in the ecological dynamics that promotes the proliferation of these implicated microorganisms, within the degrading consortia will allow pre-emptive steps to be taken to ameliorate the foaming attributed to their microbial activities. It must also be highlighted that causes and effects as it relates to foaming is considered multi-factorial as described in a study by Moen (2012), where it was explained that foaming in digesters is caused by various factors which include: 1. Process control-based procedures such as the blend of primary sludge to secondary sludge, excessive filaments in the secondary sludge, feed fluctuations, 2. Operational causes such as inadequate or inconsistent mixing, excessive or fine bubble mixing, rapid change in internal pressure, insufficient volume, temperature fluctuations, rapid change in liquid level, and 3. Digester internal causes such as surfactants and microorganisms competing for food (acid formers vs. methane formers). There is therefore a need to gain a thorough comprehension and elucidation of foaming mechanisms in AD systems which sets a theoretical basis for further research on effective suppression strategy upon detection of early signs of foaming. He et al. (2017) however noted that the cause of foaming in AD systems that use substances other than sewage sludge as substrate is unclear. These findings necessitate the identification, quantification and functional role determination of bacterial species during AD processing of various substrates as they are major role players in the chemical process of conversion.

There is an undeniable link between substrate composition and foaming. In order to minimise the chances of foaming in digesters, the pre-treatment of certain substrates has been adopted. Pre-treating AD substrates reduces the accumulation of foam promoting surface-active substances by improving the bioavailability of surfactants such as oils, greases and proteins (Roland 2010) for efficient breakdown during hydrolysis. This in-turn improves the conversion of VFAs and biosurfactants such as glycolipids, phospholipids hydroxylated and cross-linked fatty acids, lipoproteins, proteins and polysaccharide-lipid complexes produced by the metabolic activity of microorganisms during acidogenesis for efficient acetogenesis (Ganidi et al. 2009; Moeller and Görsch 2015). The pre-treatment of substrates also reduces the quantity of particulate matter in form of lignin and cellulose which is known to contribute to foaming (Montgomery 2014). In an attempt to optimise nutrient availability to microorganisms, substrate pre-treatment studies have explored the use of thermal and microwave treatment (Sapci et al. 1998; Beszédes et al. 2009; Sapci et al. 2011; Chuchat and Skolpap 2015; Rodriguez et al. 2017), alkaline treatment (Gregor 2012; Marques and Gil 2013; Arisutha et al. 2016; Fen et al. 2017; González et al. 2019) and acid treatment (Borowski et al. 2013; Skovsgaard and Jacobsen 2015; Shayegh 2016; Oginni et al. 2017; Prasad et al. 2017; Yu et al. 2017). Some research has focused on ultrasound methods (Ek 2005; Farooq et al. 2009), high pressure treatment (Lemmer et al. 2017), lysis chemical oxidation and electro oxidation (Feki et al. 2015) as well as the combination of some of these processes to enhance hydrolysis of the organic material (Tyagi and Lo 2011). The coupling of biological processes such as the dark fermentation process, a short hydraulic retention time reaction which produces hydrogen and short chain acids with AD as a first stage has also been used to improve the biodegradable matter availability and ultimately the efficiency of biogas production (Gómez et al. 2011; Akinbomi et al. 2015). These pre-treatment approaches were however successful to varying degrees.

The use of microbial pre-treatment and digestion is based on the premise of our understanding of microbial growth kinetics as well as the eminent role of biomass in generating adequate enzyme quantities to facilitate catalysis. One strategy that is currently being exploited is the genetic modification of microorganisms that have demonstrated the ability to produce unique arrays of enzymes or those that have been identified as high-titre volumes producers of these enzymes. This approach focuses on the comprehension of enzyme activity, stability and optimisation towards increasing product yield. Although, there has been considerable attention on increasing our understanding of co-digestion of various substrates and the physico-chemical properties that are complimentary as well as the optimisation of operational process parameters (Venkiteshwaran et al. 2015), the same cannot be said for the bio catalysis necessary for the AD processes using a variety of animal manures as feed for the AD processes. Moreover, there are few, if any studies that have investigated the interplay of microorganisms that are uniquely autochthonous to the different manure compositions and how co-digestion changes the ecological interspecific interactions of the varied consortia. A biomolecular approach and management of the AD process offers the much desired process design insights and solution to major challenges, such as low methane yields, given that previous works have suggested a fundamental relationship between the stability and efficiency of the AD technology and the complex communities of microorganisms and their functionalities in digesters (Aguiar-Pulido et al. 2016).

While the microbes involved in the digestion of various AD substrates such as cow, pig, poultry and horse manures are to a certain extent well characterized in terms of taxonomic identification, the specific functional roles played such as the anaerobic pathways catalysed by the micro-organisms have been understudied (Cai et al. 2016). It is plausible that an insight into the consortia interactions of microorganisms

particularly the determining factors which include interspecies interactions; syntrophy, competition and predation and the metabolic products generated as a consequence (Vanwonterghem et al. 2014), within the AD digestion process may offer a solution to some AD challenges or at least provide a strategy for pre-emptive measures.

This review however suggests that regardless of the effort exerted in improving AD process efficiency using the described approaches, the metagenomic profiling approach which can reveal the underlying biological processes in AD systems has not been given enough attention yet it is the findings from this approach that will inform the best AD operation, control, troubleshooting and maintenance strategies. This perspective is supported by Meegoda et al. (2018) that a complete understanding of the intricate biological processes occurring in AD systems especially in the batch mode, is yet to be realised regardless of the optimisation innovations so far proposed and accumulated. Majd et al. (2017) also shared the same thinking in his findings and advocated for more research to focus on the microbiome of the AD system from the commencement to the termination of an incubation cycle of the process for the best prediction and basis for process improvement strategy. Furthermore, his study opined that all the discussed factors or biogas yield determinants must seek to create a friendly environment for the microorganisms performing the conversion.

Effective AD process optimisation and control is a direct result of a comprehensive understanding of the function of microorganisms involved and their connectivity with the AD environment (Luo et al. 2016; Zhang, et al. 2019). Currently, very little knowledge and information explaining the effect of the combination of various substrates on the AD microbiome is available since each anaerobic digester has a unique microbial community. Knowledge of the composition of microbial consortia in digesters processing various input raw material combinations is therefore essential for effective management of AD processes (Kushkevych et al. 2018). According to Vanwonterghem (2014), microbial functional redundancy is central to stable digester performance since it ensures the availability of a backup pool of microorganisms capable of performing the same ecological and symbiotic functions. Few studies have however given attention to the relationship between microbial community dynamics and functional stability in AD systems and thus the interaction of microbial communities and its link to performance of the digester remain a grey area poorly understood.

3 Determination of microbial functional roles, symbiosis and impact on AD processes

Understanding the role of the symbiosis of microorganisms in AD processes is essential for the efficient running of the process as highlighted earlier on. Multi-omics studies which include metagenomics, transcriptomics and metabolomics are currently being used in other applications such as the health sector to give a holistic understanding of the functions of complex consortia of microorganisms facilitating certain reactions and highlighting specific metabolites produced or utilised by individual species within the consortia, thereby enabling the development of personalized medicines and efficient treatment of disease condition. This paper reviews the application of metagenomics and metabolomics in some AD processes and how the approach can be useful in elucidating the symbiosis of microorganisms in the AD of animal manures for better methane yield systems control and management.

3.1 Metagenomics

Metagenomics enables the rapid identification of microbes present in natural environments both at the genus and species levels. Primarily, the elucidation of these microbial communities and consortia begin with the extraction of entire pooled genomes (deoxyribonucleic acid (DNA) and sometimes ribonucleic acid (RNA) (Nazir 2016). The usefulness of this application hinges on its vast potential as a resource for the identification of novel genes, proteins and enzymes which can be employed in several industrial and environmental processes to identify microbial communities. Most importantly, metagenomics has the added advantage of exposing genomes of otherwise non-cultivable and often obscure strains of bacteria leading to the identification and devising of strategies for optimisation of growth conditions or improvising of cloning techniques to increase such populations or genes. Moreover, it has provided the opportunity to scientists in population-dynamics studies to present a realistic perspective of the actual microbial diversity in many environments (Kunin et al. 2008) as compared to the classical microbiological isolation techniques. One important tool utilised, towards the discovery of novel enzyme, their function and activities, is the development of metagenomics libraries that serve as points of reference and facilitate genetic tracking for all biotechnological applications. These libraries guarantee our understanding of the ecology and evolution of microbial ecosystems, thus providing a framework for better experiment strategy planning as it relates to application of consortia microbial interactions in industrial processes (Nazir 2016; Kunin et al. 2008).

Studies done by Sierra-García et al. (2014) in describing novel functional metabolic pathways involved in aromatic compounds biodegradation in a metagenomic library emphasised the need to broaden our perspective and current knowledge of microbial diversity as such knowledge will benefit our understanding of the degradation processes. It is inevitable that such understanding will improve our bioremediation and industrial strategies. For example, research done

using the shot gun sequencing technique for the characterisation of both the viral and bacterial composition of a vermicomposting unit containing cattle manure (80%) and food waste (20%) demonstrated not only the microbial composition, but also provided insights that can be used to determine measures necessary for the safe handling of manure (Blomström et al. 2016).

The improvement of the efficiency of processes such as anaerobic digestion employed in the production of methane in biogas can also benefit from an in-depth understanding of the microbial consortia involved and their interactions during the degradation of the milieu of substrates often employed. This is best achieved by carrying out metagenomic studies through sequencing-based approaches to identify, at a genomic level, much more microbial species diversity within a given microbial community than what the limited culture-based approaches would give. It is fair but critical to state that the AD process and its complexities does not benefit from a simple microbial characterisation using the traditional microbiological technique of plating bacterial colonies, since very few organisms even within the cultivable range can be represented on plates due to the fastidious growth requirements of most strict anaerobic microorganisms and also understanding that all the steps involved are achieved within interspecific microbial consortia and not necessarily driven by one species. This assertion is supported by authors such as Campanaro et al. (2016), who also reiterate that any solution to the limitation presented by the classical plating techniques must "go beyond a simple identification of the microbial species and unveil their functional roles in the biogas production system".

Several authors have alluded to the limited and poor understanding of the AD technology (Skiadas 1999; Yu et al. 2013; Meegoda et al. 2018; Van 2020). However, it is possible to infer that this may be attributed to scarce correlative studies done on the relationships between microbial communities and their roles and functions in the sequential degradation process synonymous with biogas production.

Although, the last decades have witnessed significant progress in identifying key microbial role players that influence AD of various feedstocks such as blackwater (Gao et al. 2019) and lignocellulosic biomass such as grass (Joyce et al. 2018), the effect of operational parameter disturbances on AD microbiome (Šafarič et al. 2018; Westerholm and Schnürer 2019; Shaw et al. 2019), and the effect of microbial community ratio (Ma, et al. 2013), it is unarguable that more research is needed to reveal the crucial relationships between the microbial population (community structure) and how their function determines such qualities as, methane production rates and overall digester resilience to changing environmental conditions. Venkiteshwaran et al. (2015) provides knowledgeable insights into several promising areas of research to improve AD technology and it includes an understanding of community population dynamics and roles played by individual members towards biogas production. They reiterate the need for an interdisciplinary approach to the elucidation and improvement of AD technology as opposed to a unidimensional study that focused on engineering solutions, with the understanding that the involvement of a variety of scientists will lead to the development of better models and designs that will improve the AD system and maximise yield.

Metagenomics currently utilises next-generation sequencing (NGS) technique developed as an improvement of Sanger's method of sequencing. In Sanger sequencing, in vitro DNA replication utilises a discriminatory fusion of chain-terminating dideoxynucleotides facilitated by DNA polymerase using a variety of primers. Its cost is a significant limitation particularly with large-scale projects involving the sequencing of entire genome or metagenome as found in microbial communities. Nevertheless, the Sanger technique remains a credible sequencing approach for monocultures and for the validation of NGS derived characterisation (Obenrader 2003; Bisht and Panda 2013; Hakeem et al. 2016) which is suitable for phylogenetic identification based on the sequencing of cloned full length 16S rRNA gene amplicons using dideoxynucleotides for chain termination. Using capillary gel electrophoresis, the mix of the randomly terminated DNA fragments is separated and since the terminators are fluorescently labelled, they each emit different wavelengths making it possible for a laser to read the sequence. The results are presented in a chromatogram and compared to databases. In metagenomics, total community DNA can be sequenced using various DNA templates in parallel with commercial technologies such as Eppendorf (Eppendorf 2019); Illumina ® (Illumina, San Diego, CA, USA) which for years has dominated high-end NGS technologies and has launched a remarkably small sequencing platform such as the one-cubic-foot iSeq 100 sequencer in 2017 thus making it possible to perform NGS in virtually any lab; also Thermo Fisher Scientific Inc. (US) which began offering the Ion AmpliSeq technology for researchers using Illumina's NGS platforms in 2018; and Pacific Biosciences of California Inc. (US), BGI (China) which launched a plan in 2018, aimed at data-mining species through sequencing in order to deliver digital data on all plants and animals and ultimately elucidate the laws of life hidden within the metadata. The technology has seen improvements especially with PerkinElmer (US), Agilent Technologies (US) which improved the accuracy and sensitivity of NGS detection using molecular and sample barcoding patent portfolios of Population Genetics Technologies; QIAGEN N. V. (Germanyich targets developing cell-free DNA assays focused on pre-natal screening for GeneReader in collaboration with Natera, Macrogen Inc. (South Korea), Oxford Nanopore Technologies Ltd. (UK) and Pacific Biosciences (PacBio) (California) supplying Sequel® system sequencers (MarketWatch 2019; Philippidis 2018), which facilitates the determination of the association between a given microbial

pattern and physical conditions in a biogas reactor at the time of sampling and provide inferred explanation as to whether the state of microbial community is a cause or effect of given conditions in the bioreactor.

In summary, the metagenomic approach is based on isolating DNA from a sample, randomly fragmenting it and inserting the fragments into appropriate vectors then sequencing all the DNA fragments. A metagenomic library is then constructed by performing DNA cloning and transformation of suitable host. For construction of metagenomic libraries, vectors (plasmids, fosmids, cosmids, or BAC vectors, depending on the length of the insert) are commonly cloned into host cells. With the library in place, clones are then screened. For investigation of microbial diversity through the analysis of conserved rRNA gene sequences, sequence-based screenings which are based on nucleotide sequences are used. Ultimately, the metagenomic output is collected and shared in public databases.

According to Illumina (2016), the concepts behind the traditional Sanger sequencing and NGS technologies are similar since in both methods, DNA polymerase adds fluorescent nucleotides one after the other onto a growing DNA template strand and a fluorescent tag is used to identify each incorporated nucleotide. The fundamental difference between the two, however, is the sequencing volume. The Sanger method only sequences a single DNA fragment at a time while the NGS methods is massively parallel such that it can simultaneously sequence millions of fragments per run, a high-throughput process which translates into the sequencing of hundreds to thousands of genes at a time. A greater discovery capacity to detect novel or rare variants with deep sequencing is also offered by NGS.

Illumina outlines the advantages of NGS to include higher sensitivity to detect low-frequency variants, comprehensive genomic coverage, faster turnaround time for high sample volumes, higher throughput with sample multiplexing, lower limit of detection and the ability to sequence hundreds to thousands of genes or gene regions simultaneously. The main and possibly only disadvantage of NGS is that it is expensive. The iSeq 100 sequencing system is however making the use of NGS more cost effective (Illumina 2017).

3.2 Metabolomics

Metabolomics can be useful as a diagnostic tool and an indicator of the phase of degradation of a given substrate. Since the substrate characteristics determine the predominant microorganisms in an AD process and sometimes, substrates may not be autochthonous sources of AD-relevant microorganisms, with some even containing contaminating microorganisms that could have impact on the overall chemical reactions taking place within a given system; therefore a microbial profile of the substrate before degradation and during the actual digestion using inoculum from known sources of biogas producers is crucial. Moreover, a comprehensive correlation of microbial species catalysing specific AD pathways to produce known intermediate products (metabolites) in the AD of a given substrate would lead to better control of the whole process and higher methane yields. This knowledge would also serve to predict process inhibition potential that often comes as a result of organic overloading and presence of inhibitory compounds formed at various stages. Mchardy et al. (2013) lend credence to this argument for the inclusion of metabolomics studies in biogas process optimisation, with their findings, that demonstrated a notable correlation between certain metabolites and structure of the microbial community. It can further be surmised that a focus on metabolomics studies can enhance knowledge of the role of the microorganisms in the transformation of abiotic factors such as nutrients, inhibitors build-up, and pollutants that may affect the stability of the AD environment. Aguiar-Pulido et al. (2016) also provide evidence that the microbiome in a digestion process strongly influences the critical biogeochemical cycles, and the development of predictive biomarkers for AD environmental stressors can best be done through the study of the system metabolome.

3.3 A review of metagenomic characterisation applied to AD systems

In a study by Guo (2015) on methane production from AD sludge derived from a municipal wastewater treatment plant, the microbial community structure was characterised using metagenomic sequencing. In this study, using Illumina HiSeq 2000 platform over 3.0 gigabases of metagenomic sequence, data was generated and an MG-RAST server used for taxonomic analysis indicated that the dominant microorganisms were bacteria (approximately 93%) while archaea constituted approximately 5.6% and Eukaryota approximately 1.1% of the microbial population under study. Some specific micro-organisms facilitating anaerobic digestion pathways were determined. Their observation for the hydrolysis stage of the AD process found the dominance of the *Halothermothrix* genus and other *Halanaerobiales* while the *Clostridia* class and the *Bacteroidaceae* family dominated during the acidogenesis stage and again, *Clostridium*, as well as *Eubacterium, Treponema, Moorella and Thermoanaerobacter* facilitated the acetogenesis step. Methanogenesis was facilitated dominantly by acetoclastic methanogens; *Methanosaeta* which only uses acetate for methane production and *Methanosarcina* which uses methanol, methylamine, acetate, carbon dioxide and hydrogen for methane production. Other hydrogenotrophic methanogens such as *Methanospirillum, Methanoculleus* and *Methanoregula* and methylotrophic methanogens such as

Methanohalophilus, Methanococcoides and *Methanolobus* were also detected in lower abundances.

The determination of the functional roles played by the various micro-organisms identified was done by identifying and annotating the functional enzyme-encoding genes for the hydrogenotrophic, acetoclastic and methylotrophic pathways of methanogenesis in the anaerobic digester with reference to a database of methanogenesis genes extracted from the Kyoto Encyclopedia of Genes and Genomes (KEGG). The pathways also show the metabolic cooperation (symbiosis) of various microorganism in the breakdown of organic compounds leading to the formation of methane gas. According to this analysis, the hydrogenotrophic pathway involved the conversion of formate, an anion of formic acid to CO_2 by a precursor glutathione-independent formaldehyde dehydrogenase (FdhA), while the enzyme hydrogenase subunit A (EchA) catalyses the interconversion of H_2 to protons and electrons resulting in the formation of formyl methanofuran ($C_{31}H_{44}N_6O_{16}P$) through the agency of another enzyme, formylmethanofuran dehydrogenase subunit A (FmdA) which formylates methanofurans (chemical compounds found in methanogenic archaea) using CO_2 and H_2. This enzyme is found in methanogenic and sulphate-reducing archaea and the synthesis of formyl methanofuran is crucial for the energy metabolism of archaea. Methanogenic archaea derive the energy for autotrophic growth from the reduction of CO_2 with molecular hydrogen as the electron donor. The enzyme formyl methanofuran-tetrahydromethanopterin N-formyltransferase then catalyses the formation of 5-formyl-tetrahydromethanopterin ($C_{31}H_{45}N_6O_{17}P$). An enzyme, methenyltetrahydromethanopterin cyclohydrolase catalyses the reversible chemical reaction of 5,10-methenyl-5,6,7,8-tetrahydromethanopterin ($C_{31}H_{44}N_6O_{16}P$) and H_2O to give 5-formyl-5,6,7,8 tetrahydromethanopterin ($C_{31}H_{45}N_6O_{17}P$) as shown in Fig. 1.

The compound 5-formyl-5,6,7,8 tetrahydromethanopterin subsequently forms 5,10-methenyl tetrahydromethanopterin ($C_{31}H_{44}N_6O_{16}P$) through the facilitation of a coenzyme F_{420} ($C_{19}H_{22}N_3O_{12}P$) which gets reduced to 1,5-dihydrocoenzyme F_{420} ($C_{29}H_{38}N_5O_{18}P$) during the reaction as shown in Fig. 2.

The 5,10-methyl tetrahydromethanopterin is then transferred to coenzyme M which facilitates methyl-transfer reactions during methanogen metabolism, in a reaction catalysed by tetrahydromethanopterin S-methyltransferase (MtrA) to form methyl-CoM which is eventually reduced to CH_4 through the catalytic activity of the methyl-coenzyme M reductase alpha subunit (McrA) in the ultimate step as shown in Fig. 3.

During acetoclastic methanogenesis, acetate is converted to acetyl-CoA, where acetate kinase (AckA,)-phosphotransacetylase (PTA), a low-affinity system is utilised by *Methanosarcina* to activate acetate to acetyl-CoA, while adenosine monophosphate (AMP)-forming acetyl-CoA synthetase (ACSS), with high affinity is utilised by *Methanosaeta*. Acetyl- CoA is then converted to a methyl group which in turn converts to methane in a reaction catalysed by acetyl-CoA decarbonylase/synthase complex subunit beta (CdhC), MtrA and McrA respectively Fig. 4.

For methylotrophic methanogenesis, the enzyme methyl-Co (III) methanol-specific corrinoid protein reacts with coenzyme M methyltransferase (MtaA) to give methyl-CoM ($C_3H_8O_3S_2$) according to Fig. 5, which is subsequently reduced to CH_4 by McrA.

The 3 routes by which methanogenesis can proceed described above are complex routes which require further studies to: (1) evaluate rate-limiting steps within the various metabolic pathways; (2) quantify the contribution of each derived intermediate product to the final methane yield and (3) manipulate the chemical reactions for improved methane yield.

Another study was carried out on the AD of municipal sludge and wastewater by Cai et al. (2016) to examine the metagenomes of an industrial wastewater digester in Guangzhou, China and a municipal sludge digester in Shek Wu Hui, Hong Kong. The taxonomic and functional characterisation of the AD of municipal sludge and industrial wastewater done is shown in Fig. 6. In this study, DNA extraction and Illumina sequencing on wastewater samples collected from full-scale wastewater treatment facilities was done. Taxonomic and functional annotation of metagenomes was done using the MG-RAST version 3.0 tool. The top 5 microorganisms active in the catalysis of major intermediate products; formate, acetate, butyrate, lactate, ethanol, propionate and other minor intermediate products and eventually methane are clearly highlighted in red for municipal sludge and blue for industrial wastewater on the schematic representation. In

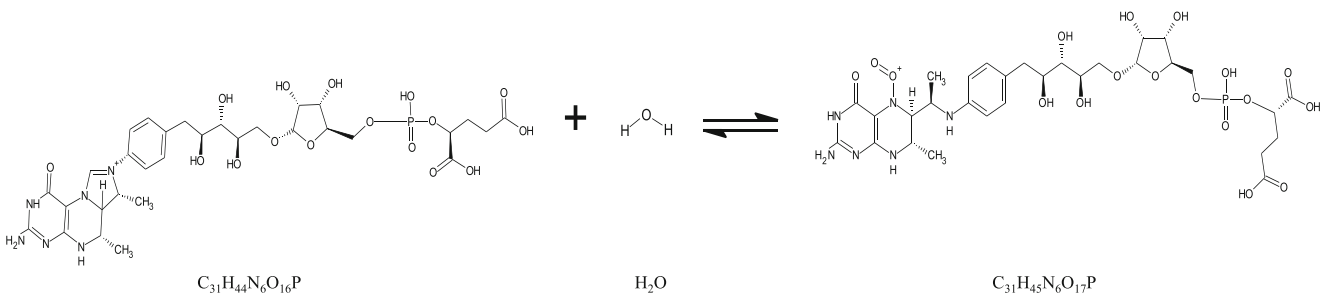

Fig. 1 Chemical reaction of 5,10-methenyl-5,6,7,8-tetrahydromethanopterin and water to form5-formyl-tetrahydromethanopterin

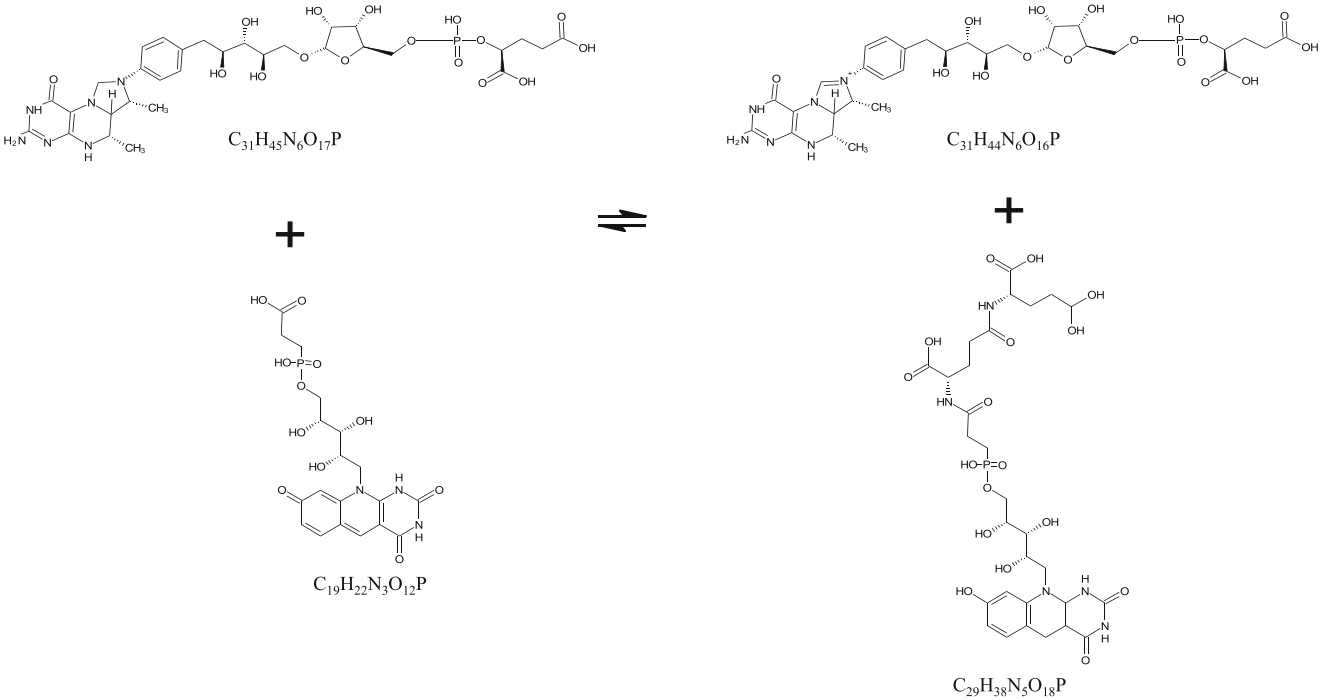

Fig. 2 Chemical reaction of 5,10-methylenetetrahydromethanopterin to form 5,10-methenyl tetrahydromethanopterin

this study the functional roles played by specific consortia of micro-organisms was elucidated, hence shedding light on the relationship between microbiome and metabolome of AD degradation of the wastes in question. Similar research must be done for various animal manures with the targeted aim of revealing and fine-tuning the metabolic pathways for improved final product (methane) formation.

3.4 A review of the metagenomic characterisation of various animal manures

The determination of microbial community species that have been carried out and recorded in literature so far for AD of animal manures are largely limited to phylogenetic analyses. Ngoc et al. (2014) identifies some microbial species in pig

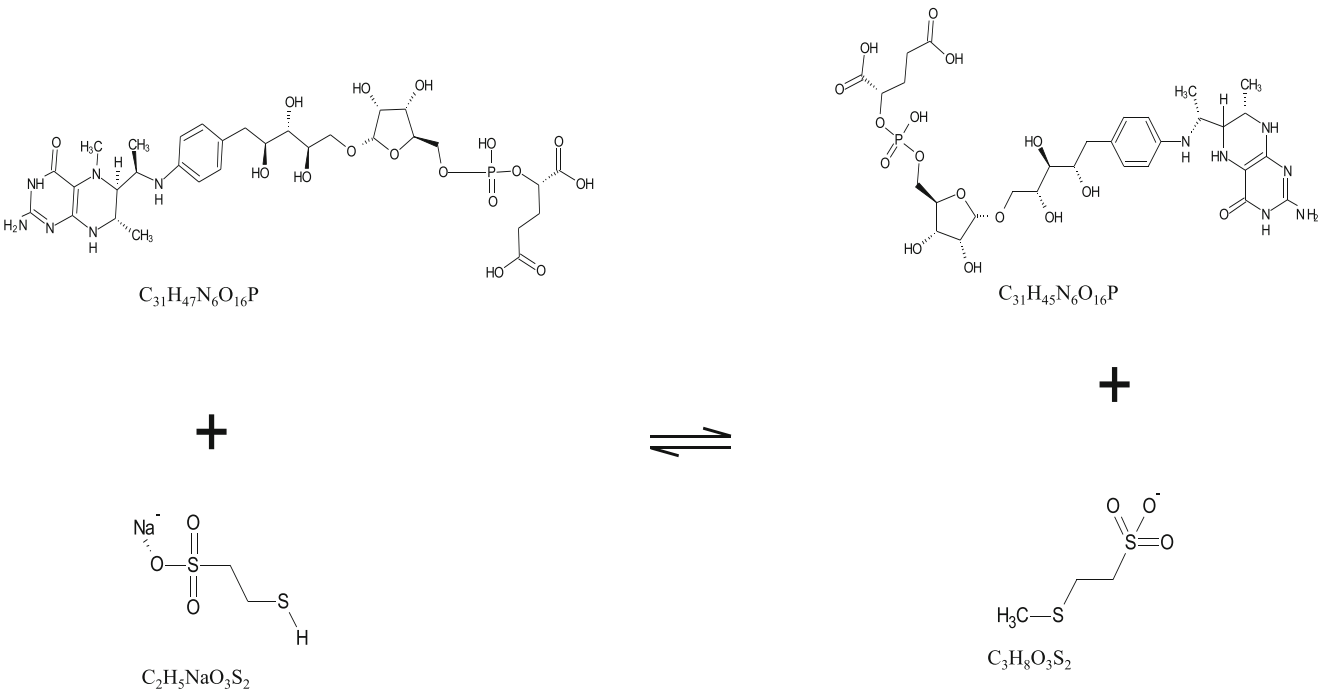

Fig. 3 Formation of methyl-CoM during hydrogenotrophic methanogenesis

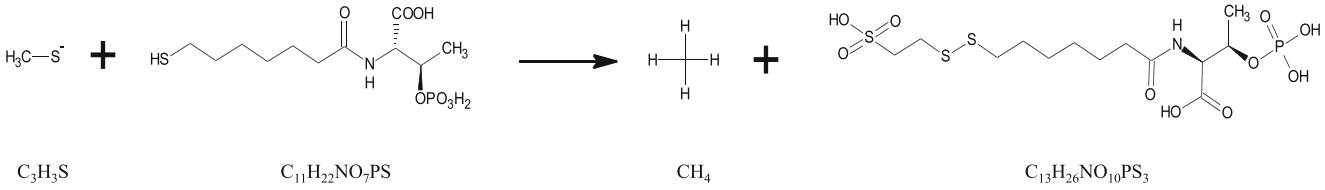

Fig. 4 Formation of methane during acetoclastic methanogenesis

manure using pyrosequencing techniques while Shepherd et al. (2011) and Beckers et al. (2017) identify those in chicken and horse manure respectively using the same techniques. Campanaro et al. (2016) and Cai et al. (2016) determined the functional roles in the AD of cow manure and industrial wastewater. Presently, to the authors' knowledge from literature, the functional metagenomic analyses of various animal manures commonly used as feed for AD processes have however not been done. The following aspects of this review highlight the metagenomic analyses done with respect to cow, pig, poultry and horse manures in recent times.

3.4.1 Pig manure metagenomic characterisation

In a study done by Zhu et al. (2011), major groups of methanogens were detected using statistical analysis of mcrA library; the AD process was achieved in a biogas reactor under mesophilic conditions of 39 °C, using pig manure as feed stock. A comparison of the cDNA extracts and sequences with the methyl coenzyme reductase subunit A gene (mcrA) clone library with 123 clones revealed that about 5.7% clones belonged to unclassified genera, however, *Euryarchaeota,* 2.4% to *Methanosarcinales,* 34.2% to *Methanomicrobiales* and 57.7% clones were affiliated to *Methanobacteriales*, noteworthy is that, the functional roles of these identified microorganisms were not determined. In another survey of the microbiome in a thermophilic anaerobic digester done by Ngoc et al. (2014) using pig manure in which they employed the clone library, denaturing gradient gel electrophoresis (DGGE) and pyrosequencing techniques and other approaches, it was revealed that members of the *Bacteroidetes, Firmicutes* and *Proteobacteria* dominant bacterial phyla and *Actinobacter, Armatimonadetes, Synergistetes, Chloroflexi, Spirochaetes, Thermotogae, Nitrospira* and *Planc-tomycetes* constituted very low abundances. Most archaeal 16S rRNA sequences could be assigned to *Methanobacteriales.* (Ngoc et al. 2014). These studies however did not also establish the functional genomics of the identified microbial species nor did they establish a link between metabolite production and any of these microorganisms.

3.4.2 Horse manure metagenomic characterisation

Shepherd et al. (2011) used pyrosequencing of 16S rRNA gene amplicons to investigate the diversity of the bacterial community in the manure of horses fed with cool-season grass hay. *Cyanobacteria* and *Actinobacteria* phyla were found in minute abundances while *Bacteroidetes* constituted 3.7%, *Proteobacteria* (3.8%), *Verrucomicrobia* (4.1%) and *Firmicutes* (43.7% of total bacterial sequences). Unclassified sequences represented about 38.1% of the total bacterial sequences. In another study, *Bacteroidetes* were found to be the most abundant phylum (34.3%) followed by *Verrucomicrobia* (32.5%), and *Firmicutes* (14.9%) (Beckers et al. 2017). The study also found that when comparing manure microbiome composition with time, *Planococcaceae, Bacillaceae, Enterococcaceae, Moraxellaceae and Enterobacteriaceae* were found to be more abundant after about 12 h while *Moraxellaceae* and *Planococcaceae* were not detected in fresh horse manure.

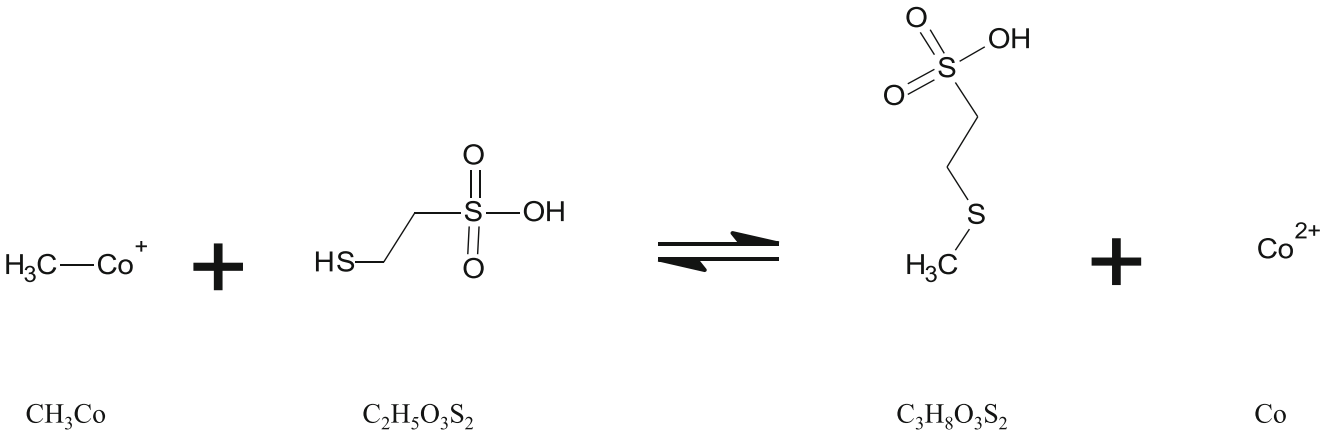

Fig. 5 Formation of methyl-CoM during methylotrophic methanogenesis

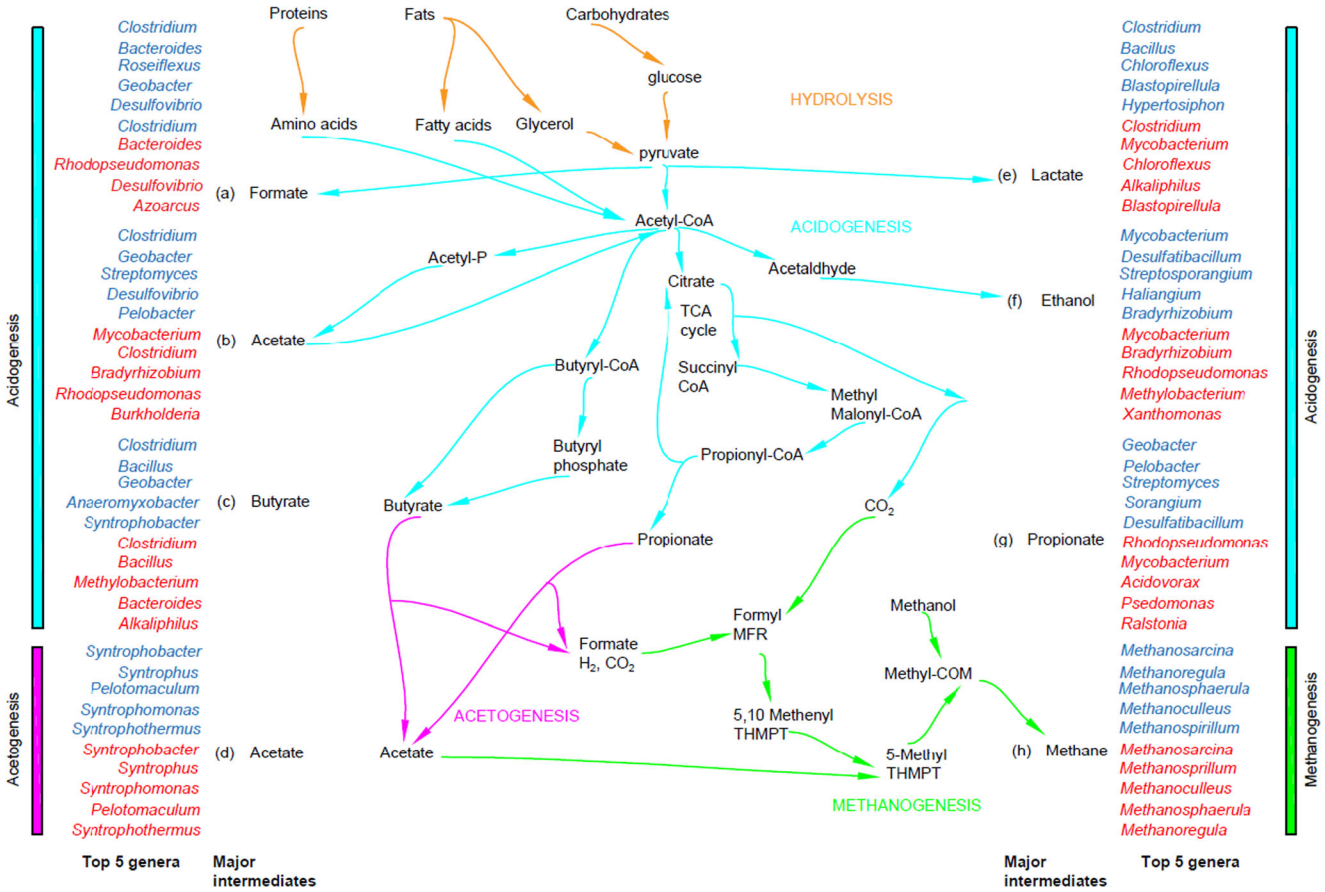

Fig. 6 Phylogenetic and Functional characterisation of the anaerobic digestion of Municipal sludge and Industrial wastewater: Adapted from Cai et al. (2016)

Comamonadaceae, Paenibacillaceae, Rhodocyclaceae, Flavobacteriaceae, Pseudomonadaceae, Weeksellaceae, Xanthomonadaceae, and *Sphingobacteriaceae* were also found to be more abundant after about 12 h but were not detected in all aged samples. At present there is no study that characterises these organisms based on their functional roles nor offers explanation for the disappearance of certain species with the obvious changes in the composition of the manure (substrate).

3.4.3 Cow dung metagenomic characterisation

Campanaro et al. (2016) carried out a study which conducted high-throughput sequencing to determine the involvement of microorganisms in AD metabolic pathways. In the study, 8 lab-scale continuously stirred tank reactors (CSTR) fed with 1.9–2.9 gVS/L reactor-day of cow manure and operated under thermophilic conditions of 54 ± 1 °C for 15 days were analysed.

Genomic DNA was extracted using the RNA PowerSoil® DNA Elution Accessory Kit and the DNA quantity and quality determined by using the NanoDrop technology. The TruSeq DNA PCR-free Kit v2 and Nextera DNA Library Preparation Kits were used to prepare libraries for individual samples from the reactors and pooled samples. These samples were then sequenced using the Illumina HiSeq 2500 and the de-novo metagenome assembly performed that used both paired-end reads followed by gene finding on the scaffolds obtained from the assembly that employed Prodigal, run in metagenomic mode. Using the MG-RAST metagenomics analysis server and standard parameters, the taxonomic and functional analysis of the metagenome assembly was then done.

Bacterial communities identified included the *Syntrophomonadaceae* (Fi07, Fi08, Fi09), *Alcaligenaceae* (Pr05, Pr10), *Gammaproteobacteria* (Pr01, Pr02), *Clostridia* (Fi12, Fi62, Fi68), *Clostridiales sp.* (Fi61), *Clostridia sp.* (Fi12, Fi13, Fi38, Fi46, Fi62), *Tepidianaerobacter sp.* (Fi34), *Peptococcaceae sp.* (Fi18) and *Thermanaerovibrio acidaminovorans* (Sy02, Sy03, Sy05, Sy06). Archeal communities identified included the *Methanoculleus genus* (Eu01, Eu02), *Methanosarcinales* (Eu04) and *Euryarchaeota* (Eu03). The functional roles of the microorganisms are shown in Fig. 7.

3.4.4 Chicken manure metagenomic characterisation

In most studies reviewed that was relevant to this paper, only two major studies have focused on chicken manure. An

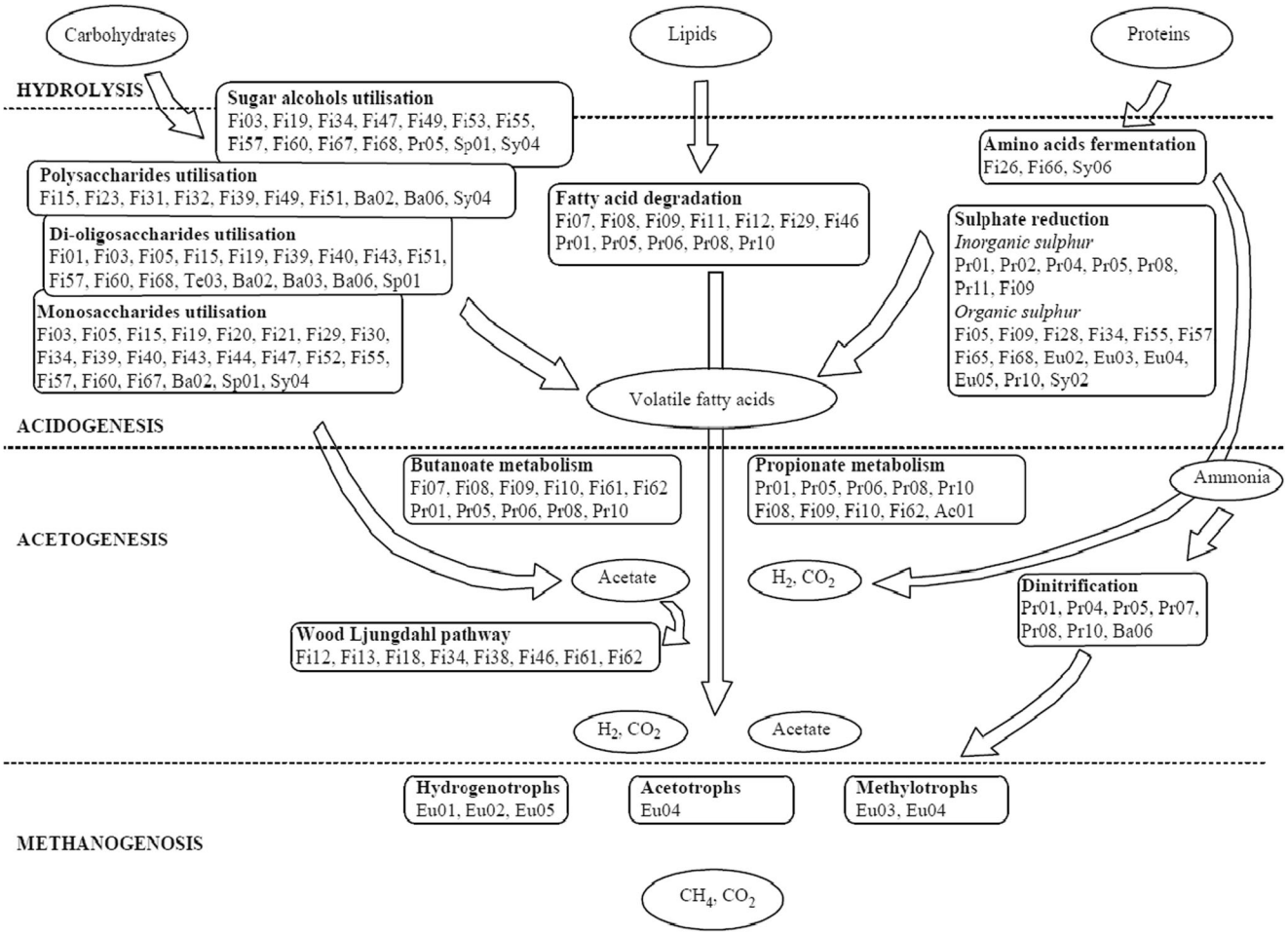

Fig. 7 Phylogenetic and Functional characterisation of the anaerobic digestion of cow dung: Adapted from Campanaro et al. (2016)

analysis of 471 non-redundant chicken metagenomic sequence data was carried out and the obtained results showed that the clone sequences were entirely similar to prokaryotic genes and that over 60% of these could not be assigned to functional roles previously characterized (Beckers et al. 2017). Secondly, a naive-analysis of all the available 16S rRNA gene sequences of poultry gut origin archived in the public databases that was performed found that *Bacteroidetes, Firmicutes*, and *Proteobacteria* were the largest phyla in both chicken and turkey, accounting for >90% of all the sequences (Lu et al. 2007). A detailed function-based metagenomics of the anaerobic treatment of poultry manure is yet to be done.

3.5 Summary on limitations of the taxonomic approach of characterisation of microorganisms in the AD process

Against the background that there are many factors affecting the microbial community structures and activities in AD process during the production of biogas, the taxonomic approach which has so far been employed by some researchers in an attempt to elucidate this process and its challenges has proven inadequate because of the apparent limitation associated with studies that primarily focus taxonomic identification. Merely identifying organisms within a population although insightful does not offer adequate correlation to the metabolic activities that interplay during the life cycle within any biological systems. In the AD process, factors such as substrate overload and changes in composition or concentration, generation of toxic and inhibitory compounds such as ammonia, VFAs and phenolic compounds during substrate degradation, substrate pre-treatment or variations in micro-environmental physical conditions can rapidly disturb the microbial community structure. Moreover, pH variations and temperature changes actively impact the activity of certain microbial groups and the community structure thus negatively influencing the path and rate of carbon flow during methanogenesis. As such, it will be necessary to carry out studies with a focus on providing causal links between these factors and the changing microbiome during the various stages of degradation as well as investigating unique dynamics that are often associated with systems up- and down-regulations cycles.

While the taxonomic approach sheds light on the phylogeny, abundance and multiplication rate of the micro-organisms

present in an AD system, it does not address the question on required optimal microbial abundances, OLR of the substrate, retention time to exhaust COD, co-digestion ratios or nutrient supplementation rate; which can only be explained when microbial population studies is paired with the determination metabolite transformation to provide a credible correlation between microbiome and metabolome conditions. Our sparse understanding is especially revealed in terms of our current inadequate knowledge base around the food web and microbial interactions within the AD bioreactor. The implication to current research is that until we thoroughly grasp this microbial interplay with aspects such as the rate-limiting step in the AD process, it will continue to be a stumbling block to our development of strategies that improve overall rates of reactions. Moreover, a perspective view of challenges such as hydrogen, ammonia and humic inhibition, demonstrates that the taxonomic approach alone does not elucidate the critical levels before which an AD process can withstand inhibition leading to system failure. For example, determining the best bioaugmentation strategy for the ammonia inhibition- stimulated symbiotic shift from the Acetotrophic route of methane formation to the syntrophic acetate oxidation route is rather difficult, if the perspective of investigation is one-sided. Especially, if considered with the challenge presented with bioaugmentation strategies which is critically the need to avoid substrate overload that shifts nutrient concentration and could promote the proliferation of undesired microorganisms at specific stages of AD causing a shift in the flux of metabolites and possibly the production of inhibitory compounds that may hinder the progression of the other steps within the process. In addition, without an analysis of the metabolites such as sulphur present in the environment around the micro-organisms, or the determination of the presence of sulphur-reducing microorganisms, it would also be difficult to establish strategies to suppress these elements that adversely affects the formation of methane.

3.6 Conclusion: Prospects - combining metagenomics and metabolomics for the optimisation of AD processes

This review has presented insights that sustainable control and optimisation of biogas production processes with the intention to maximise yield can be achieved by monitoring not only the physicochemical parameters but more importantly, the structure and metabolic activity of microbial communities facilitating organic waste degradation and biogas production. Of great importance is some knowledge of the functions performed by the different microbial communities and the interspecific interactions as well as the interaction mechanisms they use, whether beneficial or detrimental to methane production. This understanding would help in the development of AD process design and control strategies for maximum methane production. An approach that integrates taxonomy, metagenomics, transcriptomics and metabolomics studies will make it possible to reveal the microbial identity and abundance, biochemical pathways, symbiotic associations of the microorganisms and interaction mechanisms thereof, involved in the formation of methane. Presumably, if such a comprehensive understanding of intricate biological processes can be gained for the digestion of various substrates, it would see the development of incredibly more effective AD process control and optimisation strategies and solutions than have been achieved by many conventional approaches. It is quite encouraging that strides in such a direction are being made as slowly but surely, more research is being done utilising the discussed tools.

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