Inhibition of Metabolic Intermediates and Enhancement of Methane Production in Dry Anaerobic Digestion

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Abstract

Dry anaerobic digestion (AD) is advantageous over wet AD in treating high-solid organic wastes (e.g., livestock and food wastes). However, it often suffers from low methane production and metabolic intermediates inhibition (such as ammonia) due to the high solids content. In addition, there are still considerable knowledge gaps concerning the differential ammonia tolerance of acetoclastic and hydrogenotrophic methanogens (AMs and HMs). To evaluate the effects of the total solids content on the performance of co-AD of pig manure (PM) and food waste (FW), investigate the impact of different ammonia levels on methanogenesis, and enhance the methane production of dry AD of PM and FW, experiments were carried out in laboratory-scale digesters under mesophilic conditions.

The results showed the specific methane yield (SMY) had no significant difference with the increase of total solids (TS) contents from 5% to 15% (278.8-291.7 NmL/g VS_{added}), while it was reduced at a 20% TS content (259.8 NmL/g VS_{added}). The analysis on the microbial community structure clearly showed that in dry AD (20%TS), there was a general shifting from the acetoclastic pathway to the mixotrophic pathway and the hydrogenotrophic pathway.

The tolerance level of HMs to free ammonia (FAN, IC_{50}=1345 mg N/L) and NH_4^+ (IC_{50}=6050 mg N/L) was nearly 11 times and 3 times that of AMs (NH_3, IC_{50}=123 mg N/L; NH_4^+, IC_{50}=2133 mg N/L), respectively. The HMs were more adversely affected by NH_4^+ when the pH was ≤8.0. A low TAN (1.0-4.0 g N/L) could cause irreversible inhibition of the AMs due to significant cell death, whereas the activity of HMs could be fully or even over recovered from severe ammonia stress (FAN≤ 0.9 g N/L or TAN≤10 g N/L; pH ≤8.0).

The addition of biochars can mildly elevate the SMY in dry AD of PM and FW under mesophilic conditions by 7.9%, 9.4% and 12.0% for bamboo, rice husk and pecan shell derived biochar additions, respectively. Enhancing electron transfer might play an important part in dry AD process.

These results in this research could contribute to an in-depth understanding of wet and dry AD and distinguishing responses of acetoclastic and hydrogenotrophic methanogens to

ammonia exposure. Besides, these findings provide a basis for developing tailored operating strategies to reduce ammonia inhibition and offer an effective approach to improve the dry AD performance in practice.

Keywords: Acetoclastic methanogen, Ammonia inhibition, Biochar, dry co-digestion, hydrogenotrophic methanogen, methanogenesis, total solids

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Declarations

This thesis or any part thereof, has not been, or is not currently being submitted for any degree at any other university.

Zhongzhong Wang

The work reported herein is as a result of my own investigations, except where acknowledged and referenced.

Zhongzhong Wang

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List of Abbreviations

AD, anaerobic digestion

AM, acetoclastic methanogen.

ANOVA: analysis of variance

ASBR, anaerobic sequencing batch reactor

BB, bamboo derived biochar

C/N, carbon to nitrogen ratio

COD, chemical oxygen demand

e, exp (1) = 2.71828

FAN, free ammonia nitrogen

FW, food waste

FVFA, free volatile fatty acid

GC, gas chromatography

GHG, greenhouse gas

HM, hydrogenotrophic methanogens

HPLC, high performance liquid chromatography

IC_{50}, the inhibitor concentration when a 50% inhibition of SMA is reached

LCFAs, long-chain fatty acids

k, the hydrolysis rate constant

K_a, the dissociation constant of individual VFA

OUT, operational taxonomic unit

P_{1max}, the methane production potential (maximal SMY) from the readily biodegradable organic matters, mL/gVS_{added}

P_{2max}, the methane production potential (maximal SMY) from the poorly biodegradable organic matters, mL/gVS_{added}

P_{CH4}, the cumulative methane production (mL/gVS_{added}) at a certain time t (day)

PM, pig manure

P_{max}, the methane production potential (mL/gVS_{added})

PSB, pecan shell derived biochar

RDP, Ribosomal Database Project

RHB, rice husk-derived biochar

R_m, the maximum methane production rate [mL/(g VS_{added}·d)]

RS, raw sludge

SAMA, specific acetate methanogenic activity.

SAO-HM, syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis.

S/X, substrate/inoculum ratio

SCOD, soluble chemical oxygen demand

SHMA, specific hydrogen methanogenic activity

SMA, specific methanogenic activity

SMA_{max}, maximum specific methanogenic activity

SMY, specific methane yield

SS, suspended solids

STP, standard temperature and pressure

TAN, total ammonia nitrogen

TCOD, total chemical oxygen demand

TS, total solids

TVFA, total volatile fatty acid

VFA, volatile fatty acid

VS, volatile solids

VSS, volatile suspended solids

λ, the lag phase duration (day)

Chapter 1
Introduction

1.1 Background

Nowadays, the rapid consumption of fossil fuels and the rapid increase of greenhouse gas (GHG) emissions have brought about severe energy crises and environmental issues (such as climate change). The generation of carbon-neutral and renewable energy is a key challenge to mitigating climate change on a global scale. An increasing consensus now views exploiting renewable energy to gradually replace fossil fuels as a key strategy to address the energy and environmental issues (Burke & Stephens, 2018). Anaerobic digestion (AD) is a well-established biotechnology, which generates renewable energy in the form of biogas (hydrogen and methane) via the decomposition of various organic wastes (Yin et al., 2018; Zhang et al., 2018a), while simultaneously offering an alternative for the stabilization and minimization of organic wastes as well as nutrients recovery by digestate application (Capson-Tojo et al., 2018). Additionally, AD with closed operations has proven to be particularly effective for managing organic wastes, avoiding the risks of uncontrolled GHG emissions due to landfilling and open storage. Thus, it has been generally perceived as an eco-friendly, competitive, and promising biotechnology to surmount energy and environmental issues (Zhang et al., 2018a).

Wet AD systems are typically fed with substrates with a total solid (TS) content below 10%, which means the addition of a large amount of water is required in digesters when dealing with high-solid organic wastes. Furthermore, the generation of a large amount of digestate undermines the economic feasibility of AD systems, when being utilized as organic fertilizer. For example, the cost of transporting digestate could account for 30-70% of the total operation cost of AD systems (Dennehy et al., 2017a). Therefore, minimizing digestate generation is an effective approach to reduce the operational costs of AD. In this regard, dry AD, which is characterized by feeding substrates with high TS content (usually ≥15%) (Abbassi-Guendouz et al., 2012), is a good alternative for the treatment of pig manure. Moreover, dry AD is advantageous over wet AD in some other respects, such as a smaller reactor size, less energy requirement for heating, reduced water usage, and efficient inactivation of pathogens (Arelli et al., 2018; Jiang et al., 2018a).

The TS content of substrate has significant impacts on methane production. Abbassi-Guendouz et al. (2012) reported that the cumulative methane yield was reduced as the TS contents were increased from 10% to 25% in mesophilic digestion of cardboards. They identified mass transfer limitation at high TS contents being responsible for the low methane production. Similarly, Motte et al. (2013) observed a reduced methane production rate (volatile solid, VS-based) at different substrate/inoculum ratios (S/X; 28, 37.5 and 47 $g_{vs\text{-}S}/g_{vs\text{-}X}$) along with the increase of TS from 15% to 25% in AD of wheat straw. On the contrary, some studies have reported that biogas production was improved at higher TS contents. Arelli et al. (2018) noticed an improvement of 70-85% in biogas production by increasing the TS content from 25% to 30% during dry anaerobic co-digestion of FW and cattle manure. Duan et al. (2012) demonstrated that a methane yield and VS reduction similar to those of wet AD systems could be obtained in the dry AD of dewatered sludge (TS=20%) under mesophilic conditions. As a result, it is difficult to draw a clear conclusion on the influence of TS content on performance of the AD systems. Meanwhile, the AD performance in terms of methane production also depends on the substrates themselves which have different biochemical compositions (i.e., carbohydrate, protein, and lipid contents). So far, few studies have been carried out to investigate the effects of TS content on the performance in terms of methane production of anaerobic co-digestion of PM and FW. In addition, insightful analysis of the impacts of operation conditions (like TS content) on the methanogenic pathway is lacking, although lots of studies have investigated the microbial community structure in AD systems via high-throughput DNA sequencing (Arelli et al., 2018; Jiang et al., 2018a). Therefore, the effects of TS content on the methane production performance of co-digestion of PM and FW require further investigation.

Despite the main advantages, dry AD has also suffered from biological and technological drawbacks. It often encounters high levels of ammoniacal nitrogen or/and volatile fatty acids, which would negatively affect the activities of microbes involved in AD processes and imposes limitations on AD performance (Dai et al., 2016). It is generally acknowledged that methanogens are more susceptible to excessive ammonia than other groups of anaerobic microbes participating in AD processes (Capson-Tojo et al., 2020; Kalamaras et al., 2020). It has been observed that high levels of both FAN and NH_4^+ can inhibit the methanogenic archaea (Astals et al., 2018;

Kayhanian, 1999). Besides, high ammonia levels have been shown to trigger a shift in the methanogenesis of acetate in AD systems. Tian et al. (2018b) indicated that the syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis (SAO-HM) pathway was enhanced throughout the ammonia acclimation process (up to 10 g NH_4^+-N/L) in mesophilic reactors co-digesting cattle slurry and microalgae, as proofed by the increase of the relative abundance of *Clostridium ultunense* and *Methanoculleus*. Similarly, Hao et al. (2017) demonstrated that the biodegradation of acetate gradually shifted from acetoclastic methanogenesis to the SAO-HM pathway in mesophilic AD reactors when TAN was elevated from 0.14 to 7 g/L. According to previous studies, methanogenic archaea involved in the two methanogenic pathways exhibited different levels of sensitivity to ammonia (Ruiz-Sanchez et al., 2019). Numerous researchers suggest that hydrogenotrophic methanogens (HMs) might be more resistant to ammonia inhibition than acetoclastic methanogens (AMs). By testing the ammonia toxicity on the acetate- and hydrogen-using populations under thermophilic conditions, Angelidaki and Ahring (1993) found that the AMs were more sensitive to ammonia exposure than the HMs, with the specific growth rate of AMs and HMs halved at ammonia concentrations of 3.5 g N/L and 7.0 g N/L, respectively. Likewise, Wang et al. (2016a) also inferred that HMs was more robust to the ammonia inhibition than the AMs in the hydrogen enriched biogas production and upgrading processes. There are, however, conflicting findings in the literature in terms of the sensitivity of AMs to ammonia exposure. Some researchers have reported that some members of the AMs (such as *Methanosarcina* spp.) are quite robust to ammonia toxicity and can withstand TAN levels of up to 7000 mg/L (De Vrieze et al., 2012; Yan et al., 2019). Besides, studies systematically comparing the responses of AMs and HMs to ammonia exposure are rare so far, and it is still unclear how different responses to ammonia between AMs and HMs may be. Thus, the effect of ammonia on AMs and HMs still needs further investigation. A deep understanding of the responses of AMs and HMs to ammonia exposure would then help to determine the methanogenesis status in AD systems, as well as develop tailored operating strategies to alleviate ammonia inhibition.

To improve AD performance, the utilization of various additives (such as activated carbon, graphite) has drawn considerable interests from many researchers (Xie et al., 2020; Yin et al.,

2018). Generally, the additives can offer a better habitat for the microorganisms participating in AD processes due to their porous structure and biostability, thus increasing biomass density and promoting microbial metabolic activity (Sun et al., 2019). Besides, the adsorption of inhibitory compounds such as ammonia by additives can also improve AD performance (Jang et al., 2018). However, the practical application of these additives has been restricted as disposal of these additives after AD could cause environmental issues, such as secondary pollution and threats to environmental safety. Additionally, the high cost for production weakens the economic feasibility of their practical application. As a cost-effective material, carbon-rich biochar is a promising additive for enhancement of CH_4 production in AD process. Ma et al. (2020) indicated that the shortened lag phase by 44 % and the increment in maximum CH_4 production rate by 25% were achieved when supplementing 15 g/L rice husk biochar in sorghum AD. Besides, the application of biochar to AD is advantageous over other additives. Biochar is generally produced by pyrolysis process at relatively low temperature in comparison to activated carbon (Lehmann & Joseph, 2009), which substantially reduces its production cost. Meanwhile, a wide range of materials or wastes have proven to be suitable feedstock for biochar production, such as wood, agricultural and forest residues, even digestate from AD systems (Chiappero et al., 2020; Jang et al., 2018; Wang & Wang, 2019). Additionally, biochar blended in digestate after digestion could directly be used as soil amendments. Thus, eco-compatible and accessible biochar may serve as a good additive candidate for the promotion of CH_4 production in AD process. Nonetheless, the effect of biochar addition on dry AD has been rarely reported. Sun et al. (2019) studied the effect of cow manure-derived biochar addition on dry AD of beer lees and found that the biochar addition exerted a positive influence in promoting CH_4 production in dry AD systems. However, it is still unknown whether the use of biochar can enhance dry co-AD of PM and FW. As typical feedstocks for biochar production, bamboo, rice husk, and pecan shell derived biochars (BB, RHB, and PSB) were reported to be widely used as good adsorbents for wastewater treatment and soil amendments to improve soil fertility (Chiappero et al., 2020), but the application of them to improve AD performance is seldom explored.

1.2 Objectives

This study will concentrate on methanation performance of dry co-digestion of pig manure and food waste and clarification of metabolic intermediates inhibition on methanogenesis. It will be carried out by using laboratory-scale experiments combined with established physical, chemical, microbiological techniques, and advanced techniques such as high-throughput DNA sequencing, and mathematical modelling. The specific objectives of this research are:

(1) to investigate the impacts of the TS content on the performance of anaerobic co-digestion of PM and FW, and the effects of TS content on the microbial (bacterial and archaeal) community structure and metabolic characteristics, in particular methanogenic pathways.

(2) to explore the inhibitory effects of different ammonia levels on specific acetoclastic and hydrogenotrophic methanogenic activities in mesophilic mixed cultures, the recoverability of HMs or AMs after short-term effects of elevated TAN concentrations, and the possible mechanisms of ammonia-induced inhibition of AMs and HMs.

(3) to assess the effects of commonly used biochars (bamboo, rice husk, and pecan shell) on methane production performance of dry AD under mesophilic conditions, and possible mechanisms of enhanced methane production with the addition of biochar.

1.3 Procedures

To address the research objectives mentioned above, the research plan is shown in Figure 1-1.

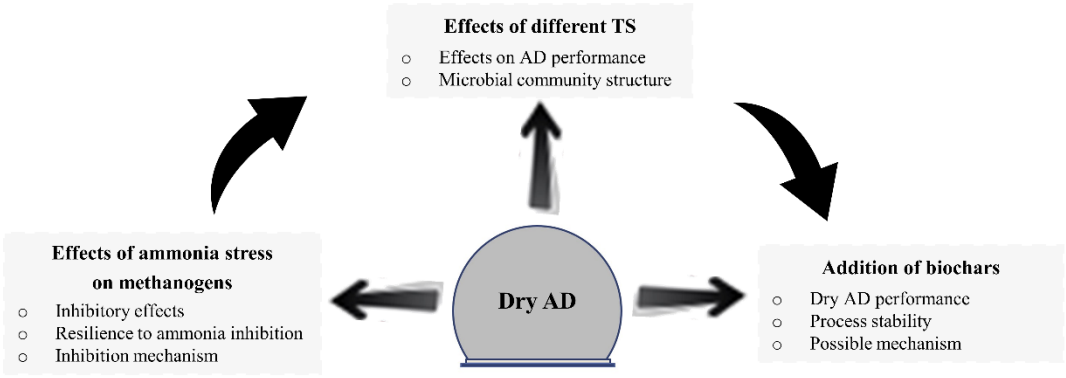


Figure 1-1. An overview of the research plan structure

The PhD research is mainly based on laboratory-scale studies.

The batch assays for examining impacts of TS content on anaerobic co-digestion of pig manure and food waste and investigating effects of biochar addition on dry anaerobic co-digestion of pig manure and food waste under mesophilic conditions, were conducted using 2-L glass digesters sealed with rubber stoppers. The blending ratio between PM and FW used in this study was 1:1 on VS basis and the S/X ratio was also 1:1 (VS basis). In the first batch experiments, the mixture was diluted with tap water to obtain the target TS contents and then fed into the digesters after fully mixing. Each TS condition was conducted in triplicate. In the second batch experiments, the mixture of substrates and inoculum was well prepared and then diluted to a TS content of 15% with tap water. Four experimental groups were established, consisting of three test groups amended with different biochars (bamboo-derived biochar, rice husk-derived biochar, and pecan shell-derived biochar) and one control group without any biochar addition. All these lab-scale digesters were subsequently placed into a lab incubator with a constant temperature of 37.0 ± 1.0 ℃. The digesters were shaken manually once every day.

Regarding the study on distinguishing responses of acetoclastic and hydrogenotrophic methanogens to ammonia stress in mesophilic mixed cultures, batch inhibition experiments were performed using 160 mL serum bottles as bioreactors. All the experiments were conducted in triplicates for each condition. In the batch inhibition experiments with acetoclastic methanogens, a certain amount of sludge pellet was resuspended to form a total of 50 ml of mixed liquor and then placed into 160-ml serum bottles. In the batch inhibition experiments with hydrogenotrophic methanogens, aliquots of sludge pellet were dispensed into 160-ml serum bottles, and finally a total of 30 ml of mixed liquor was obtained for each serum bottle. Within each bottle, the initial volatile suspended solid (VSS) concentration was around 2.5 g/L. Afterwards, all serum bottles were made anaerobic by flushing with pure nitrogen gas and then immediately closed with butyl rubber stoppers and aluminum crimp seals. Then all bioreactors were transferred to an orbital platform shaker at 150 rpm, inside an air bath incubator at 37.0±1.0 ℃.

The description of these experimental systems is detailed in individual chapters.

1.4 Structure of thesis

This dissertation has 6 chapters:

Chapter 1 is the introduction. The background of the research, main objectives and research procedures are presented.

Chapter 2 reviews the literature relating to dry anaerobic digestion of organic wastes. The following topics are included in this review: characteristics of dry anaerobic digestion, operating conditions for dry AD process, inhibition of high ammonia/volatile fatty acids concentration, and methods to enhance dry anaerobic digestion.

Chapter 3 studies the impacts of total solids content on anaerobic co-digestion of pig manure and food waste with insights into shifting of the methanogenic pathway.

Chapter 4 studies impact of ammonia conditions on activities of acetoclastic and hydrogenotrophic methanogens in mesophilic mixed cultures.

Chapter 5 studies stimulatory effects of biochar addition on dry anaerobic co-digestion of pig manure and food waste under mesophilic conditions.

Finally, Chapter 6 presents the conclusions drawn from all the studies described in Chapters 3-5. Recommendations are also made for further research.

1.5 Publications

Published:

Chapter 3: **Zhongzhong Wang**, Yan Jiang, Shun Wang, Yizhen Zhang, Yuansheng Hu, Zhenhu Hu, Guangxue Wu, Xinmin Zhan. Impact of total solids content on anaerobic co-digestion of pig manure and food waste: Insights into shifting of the methanogenic pathway. *Waste Management* 2020, 114, 96-106. (Cited by 55 times on google scholar)

Chapter 4: **Zhongzhong Wang**, Shun Wang, Yuansheng Hu, Bang Du, Jizhong Meng, Guangxue Wu, He Liu, Xinmin Zhan. Distinguishing responses of acetoclastic and hydrogenotrophic methanogens to ammonia stress in mesophilic mixed cultures. *Water Research* 2022, 119029.

Chapter 5: **Zhongzhong Wang**, Shun Wang, Sihuang Xie, Yan Jiang, Jizhong Meng, Guangxue Wu, Yuansheng Hu, Guangxue Wu, Xinmin Zhan. Stimulatory effects of biochar addition on dry

anaerobic co-digestion of pig manure and food waste under mesophilic conditions. *Environmental Science and Pollution Research* 2021, 1-12.

Manuscripts under review：

Chapter 2: **Zhongzhong Wang**, Yuansheng Hu, Shun Wang, Guangxue Wu, Xinmin Zhan. A critical review on dry anaerobic digestion of organic waste: characteristics, operational conditions, and improvement strategies. *Renewable & Sustainable Energy Review* (Under review)

Chapter 2
Literature review

2.1 Introduction

Currently, AD is extensively applied to treat a variety of organic solid wastes, such as agricultural residues, municipal solid wastes, and industrial wastes (Yin et al., 2020). Anaerobic digestion is generally driven by four sequential stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis, which are correspondingly functioned by hydrolytic bacteria, acidogenic bacteria; acetogenic bacteria and methanogenic archaea (Kothari et al., 2014). Generally, conventional AD is used for treating organic materials with low total solids (TS) contents (2-6%) (Liu et al., 2016), which leads to a large amount of digestate with a high-water content after digestion. The post-treatment or disposal of digestate has been a major challenge for AD processes (Wang et al., 2020c). Using digestate directly as organic fertilizer for farmland, Dennehy et al. (2017a) estimated that transportation costs accounted for approximately 30-70% of the total AD operation costs.

Water, as the medium for nutrient transport and necessity for microbials growth, has played a pivotal part in the controlling of AD process (Kothari et al., 2014). Additionally, moisture content in the substrate significantly affects AD performance in terms of methane production (Wang et al., 2020c). Nowadays, it is widely accepted that AD processes are classified into three categories based on the TS content in the raw biomass, including the wet AD (<10% TS), semi-dry AD (10% < TS < 15%) and dry AD (≥15% TS) (Arelli et al., 2018; Elsamadony et al., 2015; Garcia-Bernet et al., 2011b; Karthikeyan & Visvanathan, 2012). Compared to wet AD, which has been extensively used in treating organic wastes for decades, dry AD has become increasingly attractive in recent years (Abbassi-Guendouz et al., 2013; Liu et al., 2016). Dry AD can offer several benefits, including higher volumetric methane production, smaller reactor volumes, reduced energy consumption for heating, less digested residual, and low moisture in digestate that is easier to handle (Angelonidi & Smith, 2015). Minimal material handling issues during pre-processing and post-processing is appealing in dry AD (Arelli et al., 2018). It has been reported that dry AD can be designed at higher organic loading rates (OLRs) even at TS content of up to 40% (Cho et al., 2013). Therefore, dry AD is a more economical and promising technology for the management of organic wastes.

Despite its main advantages, dry AD also has biological and technological shortcomings due to the excessive solids content and the small amount of water in digester (Abbassi-Guendouz et al., 2012; Angelonidi & Smith, 2015). As a result of high TS conditions, dry AD systems have difficulty in mixing and homogenizing, which negatively affects on methane production by increasing the diffusive transport resistance of soluble compounds (substrates or intermediates) (Abbassi-Guendouz et al., 2012; Xu et al., 2014a). In addition, compared with wet AD, the high organic loading in dry AD would result in the build-up of inhibitors (such as ammonia and volatile fatty acids (VFAs)), which would negatively affect AD performance in terms of methane production. In a previous study on the effects of TS content on anaerobic co-digestion of pig manure and food waste, the authors found that 20% TS decreased the specific methane yield (SMY) by 11% compared to 10% TS (Wang et al., 2020c).

Anaerobic process stages and relevant biochemical reactions in dry AD, as well as the microbials inside the digesters, are similar with those in wet AD. However, they have many differences in terms of technical operations, reactor design, and process performance resulting from the high TS content. Therefore, the focus of the present review is critically assessing the previous scientific literatures on dry AD, mainly including specific characteristics of dry AD, operational conditions, and strategies for improving the performance of dry AD. Additionally, this chapter aims to emphasize the knowledge gaps that need to be addressed in order to develop a suitable and feasible dry AD process.

2.2 Fundamental aspects of AD

Microbiologically driven AD is a complex biochemical process depending on a consortium of microorganisms that perform different metabolic pathways (Li et al., 2011a). The microorganisms involved in AD can basically be divided into four groups according to their different functions: hydrolytic bacteria, acidogenic bacteria, acetogenic bacteria, and methanogenic archaea, which are correspondingly responsible for hydrolysis, acidogenesis, acetogenesis, and methanogenesis, respectively (Zhan et al., 2018). Main steps and metabolic pathways in dry AD are illustrated in Figure 2-1 and Table 2-1.

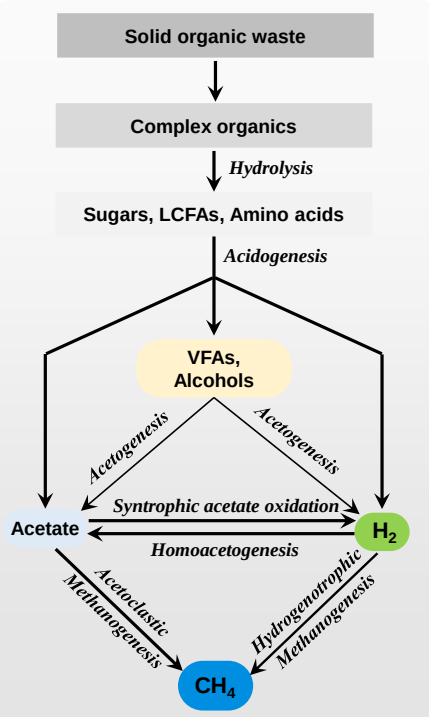


Figure 2-1. Main steps and metabolic pathways in dry AD (Adapted from Kothari et al. (2014))

The biodegradable organic matter in feedstock primarily consists of carbohydrates, proteins, and lipids. In general, microorganisms are not capable of using these polymers directly due to the large size of individual molecules, which cannot penetrate the cell wall of the microorganisms and have access to intercellular matrix. Hence, at the beginning of the AD process, via extracellular enzymes excreted by the hydrolytic bacteria, hydrolysis realizes the breakdown of complex organic polymers (particulate or dissolved) into smaller soluble molecules, such as sugars, amino acids, and long-chain fatty acids (LCFAs) (Montero et al., 2008). The hydrolysis is mainly performed by heterotrophic acidogenic bacteria, which are both hydrolytic enzyme producers and hydrolysis product consumers (Li et al., 2019). Generally, hydrolysis is regarded as the limiting step of AD when using solid organic matter as substrates (Pavlostathis & Giraldo-Gomez, 1991).

The reduced compounds (such as sugars, amino acids, LCFAs) formed in the hydrolysis step are then further transformed by acidogenic bacteria (or acidogens) to volatile fatty acids (VFAs) as well as a small amount of other minor products such as CO_2 and H_2. These

bacteria grow rapidly, and the minimum doubling time is approximately 30 minutes (Mosey, 1983). The acetogensis, functioned by acetogenic bacteria (or acetogens), is responsible for consuming the non-acetic acid VFAs generated in the preceding stage and producing acetate, carbon dioxide, and/or hydrogen which are precursors for methane production. Basically, there are two types of microorganisms involved in acetogenesis: (1) syntrophic acetogens, which are capable of metabolizing non-acetic acid VFAs, alcohols, and fatty acids into acetate (Amani et al., 2010). Since all acetogenic reactions are thermodynamically unfavourable and easily inhibited by the accumulation of their metabolites, especially H_2, these organisms form syntrophic associations with methanogens. (2) non-syntrophic homoacetogens, which produce acetate by using H_2 and CO_2. In addition, homoacetogens are very versatile anaerobes, capable of converting a variety of substrates into acetate (Diekert & Wohlfarth, 1994). These bacteria grow slowly with a doubling time of 1.5 to 4 days (Mosey, 1983). The final stage of AD is methanogenesis, where a variety of methanogenic archaea utilize acetate, CO_2, and H_2 to produce methane. According to the substrates used, methane can be generated in the following three ways: (1) acetotrophic pathway, in which methane are produced from acetate; (2) hydrogenotrophic pathway, in which methane is produced by reducing CO_2 with using H_2 or formate as electron donors, and (3) methylotrophic pathway, in which methane is produced form methyl groups (like methanol, methylamines and methyl sulfides). The methanogens grow slowly with a doubling time of 2 to 4 days (Mosey, 1983). The methanogenic reactions of the three pathways can be described with the following equations (Bueno-López et al., 2020).

$$CH_3COO^- + \mathrm{H}^+ \rightarrow CH_4 + CO_2,\ \triangle G_0' = -36\ \mathrm{kJ/mol}\ CH_4 \quad (2\text{-}1)$$

$$CO_2 + 4\mathrm{H}_2 \rightarrow CH_4 + H_2O,\ \triangle G_0' = -130\ \mathrm{kJ/mol}\ CH_4 \quad (2\text{-}2)$$

$$4CH_3\mathrm{OH} \rightarrow 3CH_4 + CO_2 + 2H_2O,\ \triangle G_0' = -106\ \mathrm{kJ/mol}\ CH_4 \quad (2\text{-}3)$$

All functional microorganisms responsible for different processes have different optimal pH ranges for their enzyme activities, such as a pH below 5 for hydrolysis and acidogenesis, a pH between 6.8 and 7.6 for acetogenesis, and 6.5 to 7.2 for methanogenesis. It is therefore recommended that anaerobic digesters operated at a pH range between 6.8 and 7.4, which allowed active microorganisms to perform a good degree of metabolism. For dry AD process, it possesses the identical steps in biogas production mechanism as wet anaerobic digestion

process. It has a poor start-up performance during hydrolysis because of a low water amount present (Kothari et al., 2014).

Table 2-1. Common microorganisms involved in main processes in AD systems.

Processes	Functions	Microorganisms	Optimal pH	Doubling time	Reference
Hydrolysis	Decomposing complex organic polymers into smaller soluble molecules	Acidogenic bacteria, e.g., *Butyrivibrio, Propionibacterium*, *Clostridium*, *Bacteroides*, *Ruminococcus*, *Acetivibrio*, *Bifidobacterium*, *Eubacterium*,	< 5	30 minutes to 3 days	(Mosey 1983; Amin et al., 2021; Okoro-Shekwaga et al., 2019)
Acidogenesis	Converting the organic monomers into volatile fatty acids, H_2 and CO_2.	*Peptostreptococcus*, *Peptococcus*, *Selenomonas*, *Lactobacillus*, *Streptococcus*.			
Acetogenesis	Transforming non-acetic acid VFAs, alcohols and fatty acids to H_2, CO_2 and acetate.	Syntrophic acetogens, e.g., *Syntrophomonas, Syntrophobacter*, *Syntrophus*, *Syntrophococcus, and Desulfovibrio.*	6.8–7.6	1.5 to 4 days	(Mosey, 1983; Okoro-Shekwaga et al., 2019)
	Using H_2 and CO_2 to produce acetate.	Non-syntrophic monoacetates, e.g., *Acetobacterium*, *Acetoanaerobium*, *Acetogenium*, *Butyribacterium*, *Clostridium*, *Eubacterium*, and *Pelobacter*.	6.8–7.6	1.5 to 4 days	(Mosey, 1983; Okoro-Shekwaga et al., 2019)
Methanogenesis	Utilizing acetate, CO_2, and H_2 to produce methane.	Methanogenic archaea, e.g., *Methanothrix*, *Methanosarcina*, *Methanobacterium*, *Methanobrevibacter*, *Methanococcus*, *Methanomicrobium*, *Methanogenium*, *Methanospirillum*, *Methanoplanus*.	6.5–7.2	2 to 4 days	(Mosey, 1983; Okoro-Shekwaga et al., 2019)

2.3 Characteristics of dry anaerobic digestion

2.3.1 Feedstock

The feedstock is defined as any organic materials fed into AD systems to biologically produce biomethane. Conventionally, a large variety of organic wastes can serve as AD feedstock, such as food waste, livestock manure, sewage sludge, agricultural waste. etc. Generally, the commonly used feedstock in AD should be characterized by: (1) high biodegradability, which means it has a high proportion of biodegradable organic material that is readily biodegradable under anaerobic conditions; (2) balanced nutrient composition in macro and micro elements, which is in favour of anaerobic microbial growth. Dry AD operated under high solid conditions prefers to treat these organic wastes featured with relatively low moisture (e.g., municipal solid waste, agricultural residues), while wet AD is more suitable for feedstocks like wastewater or animal slurry with high moisture contents (Kothari et al., 2014). The dry AD system is designed primarily to treat solid organic waste from four main sources, including, agricultural residues, industrial waste, municipal solid waste, and energy crops and plants, as shown in Figure 2-2. In practical applications, the selection of a particular substrate for dry AD systems should take the following factors into consideration: (1) local availability of raw material; (2) physicochemical characteristics, in particular the biomethane production potential; and (3) economic aspects in terms of operational parameters.

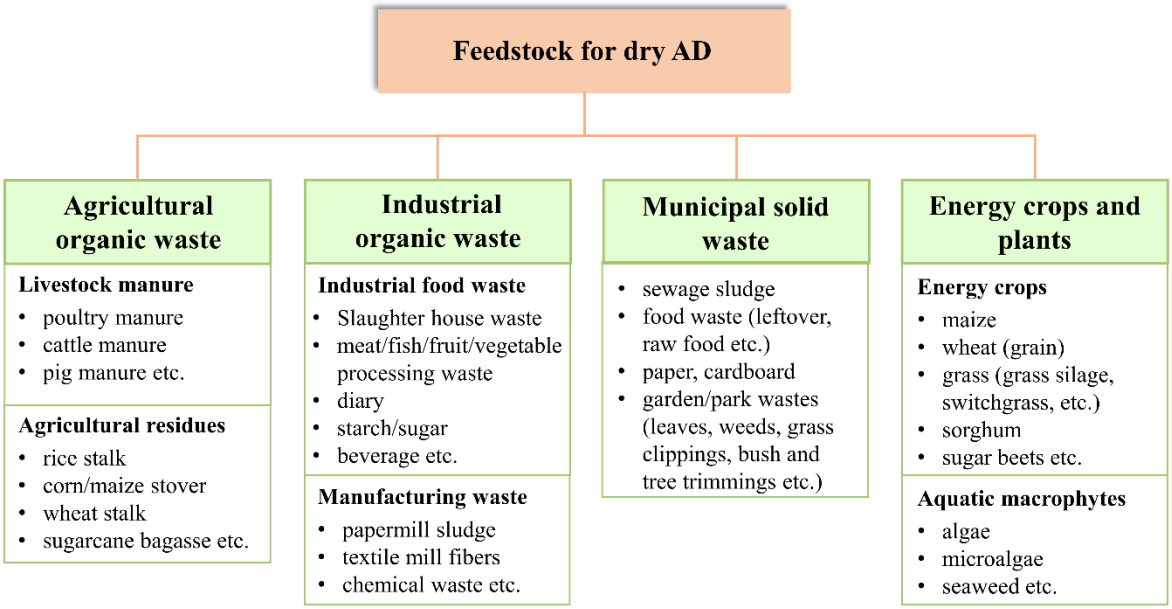


Figure 2-2. Various feedstocks for dry AD

2.3.2 High TS content

In comparison with wet AD, dry AD is typically characterized by a high TS content, usually 15%-40%, which allows it to deal with a larger amount of organic waste per unit volume of the digester, thereby decreasing the size of the digester and reducing capital costs (Angelonidi & Smith, 2015; Jiang et al., 2018b). The high TS content results in mass transfer limitation between microbes and substrates and/or metabolites, which inevitably reduces the accessibility of nutrients to the microbial biomass and negatively affects their metabolism, ultimately leading to lower VS degradation as well as biogas production. The TS content of substrates as one of the most important operational factors for dry AD process has significant impacts on methane production. Numerous studies have examined the effects of TS content on AD performance in terms of methane production when using different organic wastes as substrates, as shown in Figure 2-3. The results of most studies show that when the TS is above 15%-20%, an increase in TS leads to a reduction in methane production, with exception of Yi et al. (2014), who obtained better performance in terms of VS reduction and methane yield in AD of food waste with increasing TS from 15% to 20%. Abbassi-Guendouz et al. (2012) performed batch experiments with TS contents ranging from 10% to 35% to assess the impact of the TS content on the performance of AD fed cardboard as the substrate.

They observed a slight decrease in methane production when TS contents increased from 10% to 25%, with a sharp decline at TS contents of 30-35%. Hence, the authors indicated that 30% TS may be reckoned as a threshold for causing an inhibitory effect on dry AD. Moreover, they concluded that TS contents affected the global AD performance through reducing the microbial hydrolysis rate at 10-25% TS and physical restriction associated with mass transfer at TS above 30% according to experimental observations and ADM1 simulations.

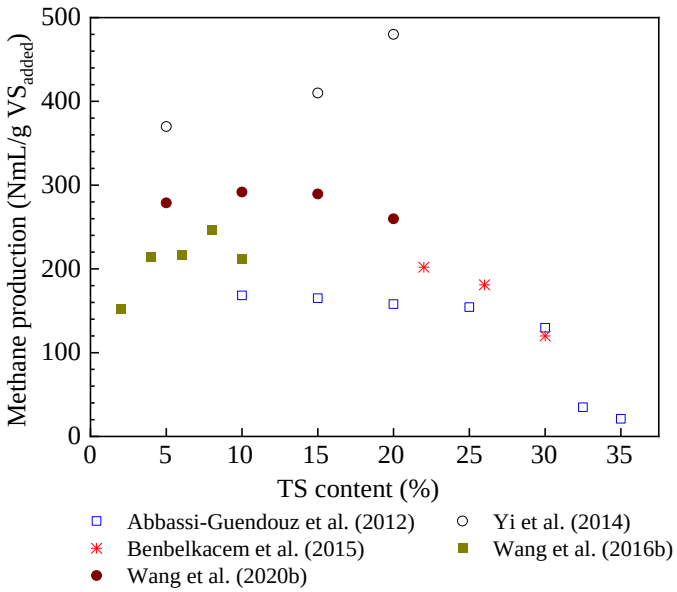


Figure 2-3. Effects of TS content on methane production in AD when using cardboard (Abbassi-Guendouz et al., 2012), food waste (Benbelkacem et al., 2015; Yi et al., 2014), sewage sludge (Wang et al., 2016b), and pig manure and food waste (Wang et al., 2020c), as substrates.

2.3.3 Rheological behavior and mass transfer limitations

Dry AD processes are often carried out under high-solid conditions (typically 15 to 40% TS), so the medium has the appearance of a paste containing granular particles. It looks like that there is no or less free-flowing water in such dry AD systems. Garcia-Bernet et al. (2011a) analyzed the water distribution in digestates collected from two industrial dry AD plants and found that for 20%-TS digestates around 50% of the water was free water, and the same fraction was bound water. As a result, the pasty media in dry AD systems inevitably present

significant differences over the water-based media in wet digestion, in the aspects of rheological behaviors and mass transfer (Bollon et al., 2011).

Generally, the rheology of Newtonian fluids (such as water) behaves in a viscoelastic manner and its rheological property can be described by the apparent viscosity (referred as the ratio between shear stress and shear rate) (Dai et al., 2014). However, anaerobically digested solid waste, such as sludge, is usually considered as a non-Newtonian fluid because the shear stress is not linearly correlated to the shear rate (Di Capua et al., 2020; Tang & Zhang, 2014). Therefore, its rheological characterization cannot simply be determined by the viscosity. Previous studies employed a plastic model to characterize rheological behaviors of different types of digested sludge. Garcia-Bernet et al. (2011b) measured the yield stress by using the slump test and demonstrated that the yield stress increased with the TS content based on an exponential correlation model. Similarly, Battistoni (1997) found that there was an exponential correlation between the yield stress and total VS content in the OFMSW sector. Clearly, the digestate in dry AD systems is characterized by high yield stress levels, with the yield stress increasing exponentially with TS content, making it more difficult for the digesters to get mixed and homogenized.

Studies have shown that adequate mixing can effectively boost biogas production in dry AD systems by increasing the contact between biodegradable organic matter (including substrates and intermediate metabolites) and microorganisms (Liu et al., 2019; Singh et al., 2019; Yu et al., 2011). However, mechanical mixing is an energy-intensive process that usually accounts for a large share of energy consumption in full-scale AD plants. Lemmer et al. (2013) reported that approximately 51% of the total electric energy consumption was for the mixing process in a biogas plant. Energy consumption for mixing in dry AD systems is greatly affected by the high TS content since the yield stress increases exponentially with TS content. Therefore, more research should be conducted on the development of mixing strategies for dry AD and optimization of the mixing process.

Currently, most industrial dry AD systems with TS higher than 15% are usually not mixed, or sometimes intermittently mixed (Bollon et al., 2013), whereas the low TS and the low-medium viscosity in wet AD systems allow to use a stirring system for continuous operation

(Hernandez-Shek et al., 2021). Under non-agitated conditions, convective mass transfer is almost negligible, while the diffusive mass transfer dominates and plays a critical role in controlling the mobility of soluble substrates or metabolites. The diffusion of soluble molecules within the digesting medium is also highly affected by the TS content in dry AD systems (Cazier et al., 2022). After experimentally determining diffusion coefficients, Bollon et al. (2013) found that the effective diffusion coefficient decreased sharply along with the increase of TS content. For example, the diffusion coefficient of iodide ion (I^-) at 25% TS was 55–185 times smaller than that in water. An insufficient diffusion rate of soluble compounds in dry AD systems with high TS contents, which adversely affects the mass transfer in dry AD.

2.3.4 Ammonia and VFA accumulation

Ammonia nitrogen is derived from the breakdown of nitrogenous organic matter in the substrate in dry AD, such as proteins, amino acids and urea. Ammonia nitrogen at low concentrations (50-200 mg/L) can act as a preferred nitrogen source for the growth of microorganisms and also raise the buffering capacity of the AD system (Agyeman et al., 2021). Nevertheless, exceeding ammonia-tolerant limits would negatively affect the activities of microbes involved in AD processes and ultimately compromise AD performance (Dai et al., 2016; Rajagopal et al., 2013). According to Chen et al. (2008), ammonia concentrations ranging from 1.7 to 14 g N/L depending on the experimental conditions could result in a 50% reduction in cumulative methane production. Due to high TS content, TAN levels are typically higher in dry AD compared with wet AD, which makes dry AD often prone to ammonia inhibition (Shapovalov et al., 2020). Wang et al. (2020c) conducted the batch AD reactors fed with pig manure and food waste as co-substrates to examine the effects of TS contents on AD performance, and the results showed a higher TAN concentration of 5293 mg N/L was observed in dry AD with a TS of 20% as compared to wet AD (5%TS) with 1094 mg N/L TAN. Moreover, the direct consequence resulting from ammonia inhibition is the build-up of VFAs and instant pH drop, which aggravates the deterioration of dry AD process. Jiang et al. (2018a) conducted batch dry AD reactors co-digesting food waste and pig manure under mesophilic conditions and demonstrated that methane production was largely inhibited

by high VFA concentration, with the threshold VFA inhibition concentrations ranging from 16.5 to 18.0 g/L.

In aqueous solutions, ammoniacal nitrogen exists in two forms, free ammonia nitrogen (FAN or NH_3) and ammonium nitrogen (NH_4^+), which are in a pH-dependent equilibrium (Wu et al., 2009), as shown in the Eq. (2-4). The proportion of FAN to total ammonia nitrogen (TAN) is dependent on pH and temperature, demonstrated as Eq. (2-5). Figure 2-4 shows the variation of the proportion of FAN at different conditions of pH and temperature. Clearly, FAN concentration increases with pH and specially with temperature. To date, FAN is widely considered to be more accountable for the ammonia inhibition of AD processes compared with NH_4^+ (Müller et al., 2006; Rajagopal et al., 2013). Hence, thermophilic dry AD is more prone to ammonia inhibition due to a higher FAN concentration at thermophilic conditions. Moreover, the ammonia inhibition is intensified at high pH levels owing to the high proportion of FAN to TAN.

$$NH_3(aq.) + H_2O(l.) \leftrightarrow NH_4^+(aq) + OH^+(aq.) \quad (2\text{-}4)$$

$$\frac{TAN}{FAN} = \left(1 + \frac{10^{-p\mathrm{H}}}{10^{-\left(0.09018 + \frac{2729.92}{T}\right)}}\right) \quad (2\text{-}5)$$

where, FAN and TAN are the free (NH_3) and total (NH_3 + NH_4^+) ammonia concentrations, respectively, mg/L; and T is the temperature in Kelvin, K.

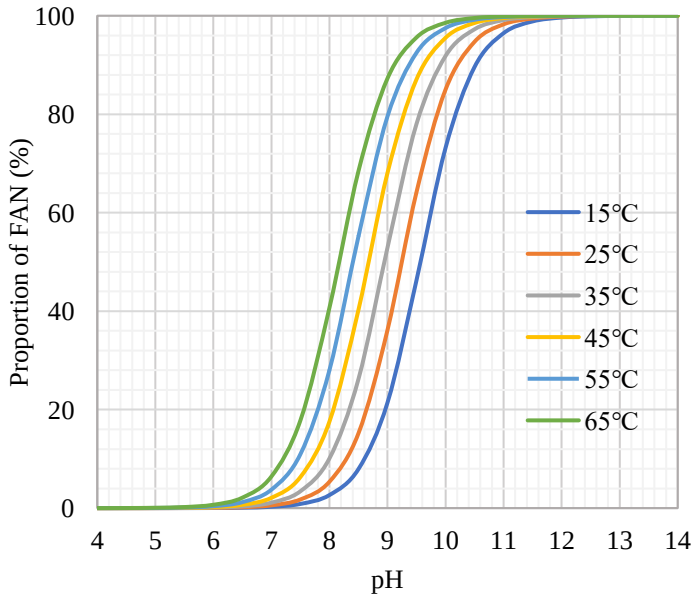


Figure 2-4. Variation of the proportion of FAN at different conditions of pH and temperature

It is generally acknowledged that methanogens (especially acetate-utilizing methanogens) are more susceptible to ammonia tress than other groups of anaerobic microbes participating in AD processes (Capson-Tojo et al., 2020; Kalamaras et al., 2020). It has been observed that high levels of both FAN and NH_4^+ can inhibit the methanogenic archaea (Astals et al., 2018; Kayhanian, 1999). The inhibitory effects of ammonia levels on methanogenic archaeal are discussed in Section 2.3.5. To decrease the effect of ammonia-induced inhibition, several strategies have been used in practice, such as reducing OLR (or increasing retention time), co-digesting two or more substrates to balance the C/N ratio (Zhang et al., 2012). Another approach is to inoculate with anaerobic sludge that has been acclimated to high ammonia concentrations and has a higher resistance to ammonia exposure. Yan et al. (2019) conducted two mesophilic continuous stirred tank reactors (CSTRs) used OFMSW as the sole substrate, along with gradually increasing ammonia concentration from 1.1 to 9.5 g NH_4^+-N/L. The results proved that CSTRs were successfully acclimatized up to 8.5 g NH_4^+-N/L, with their methane yields fluctuating below 10%, compared to the methane yields without ammonia addition.

2.3.5 Microbial communities

Microbial communities play a key role on the performance and stability of dry AD. As described in Section 2.2, there are four groups of microbial communities involved in the AD process: hydrolytic bacteria, acidogenic bacteria, acetogenic bacteria, and methanogenic archaea, which are the performers of hydrolysis, acidogenesis, acetogenesis, and methanogenesis, respectively. Generally, the preferred conditions for AD process are neutral pH (6.8-7.2), constant temperature (mesophilic 30-40 ℃, or thermophilic 50-60 ℃), and a relatively stable feeding rate (Kothari et al., 2014). The succession of microorganism communities takes place among the four functional groups of microbes, and if operating conditions are not maintained near optimal levels, imbalances across the functional groups occur. Due to high solids content, dry AD is usually prone to high ammonia and VFA concentrations (Dai et al., 2016; Rocamora et al., 2020), which inevitably affects the activity of microorganisms. Consequently, microbial communities in dry AD are more likely to experience greater environmental stress compared with wet AD.

Methanogenic archaea, the executors of methanogenesis, are widely reported to be more susceptible to high-stress environments than other groups of anaerobic microbes participating in AD processes (Abbassi-Guendouz et al., 2013). According to the substrates they can use, methanogenic archaea are generally classified as acetoclastic methanogens, hydrogenotrophic methanogens, and methylotrophic methanogens, while use acetic acid, H_2 and CO_2, and C1-methyl, respectively (Cai et al., 2021). Many previous studies have shown that the TS content has significant effects on the archaeal community. Liu et al. (2016) investigated the evolution of microbial communities along with the increase in TS (10-19%) in sewage sludge AD. They observed an increase in the relative abundance of hydrogenotrophic methanogens from 6.8% at TS 10% to 22.3% at TS 19%, while that of acetoclastic methanogens *Methanosarcina* decreased from 82.2% to 56.3%, although acetoclastic methanogenesis, primarily conducted by the genus *Methanosarcina*, was found to be the predominant pathway for methane production. The authors asserted that the high TS conditions undermined the competitive advantage of acetoclastic methanogens, which allowed more hydrogenotrophic methanogens to grow. Additionally, *Methanosarcina* species are versatile and capable of both the hydrogenotrophic and the acetoclastic methanogenesis pathways for growth and methane production, which makes them more resistant to the severe conditions such as high levels of ammonia and VFA in dry AD. In batch high-solid AD (TS from 5% to 20%) using food

waste as the sole substrate in mesophilic conditions, Yi et al. (2014) found that *Methanosarcina* was the predominant methanogen throughout the whole digestion, with its relative abundance increasing as the TS content increased. Zhou et al. (2019) explored the metabolic pathways of methanogenesis by analysis the microbial community structures in dry and wet AD sludge. The results showed the hydrogenotrophic methanogenesis was the predominant metabolic pathway in dry AD, with relative abundances of 48.0% and 15.3% for *Methanocorpusculum* and *Methanobrevibacter*, respectively, while the acetoclastic methanogens *Methanosaeta* (77.0%) dominated in wet AD. Schnürer et al. (1999) indicated that most methanogens were able to utilize hydrogenotrophic methanogenesis pathway under stressful conditions, such as high levels of ammonia, VFAs, sodium, heavy metals or sulfide. Therefore, it can be inferred that hydrogenotrophic methanogens play an important role in methanogenesis under high-solid conditions in dry AD systems.

The mechanism that mostly explains the change in the methanogenic pathway during dry AD claims that methanogens possess different abilities to resist varying digestion conditions due to their differences in adaptability to specific environmental conditions (Abbassi-Guendouz et al., 2013). For example, the consensus from different studies is that hydrogenotrophic methanogens are much more resistant to ammonia inhibition than acetoclastic methanogens (Capson-Tojo et al., 2020). High ammonia levels have been shown to trigger a shift in the methanogenesis in AD systems. In dry co-AD (TS=20%) of pig manure and food waste Wang et al. (2020c) found that a manifest shifting from the acetoclastic pathway to the hydrogenotrophic pathway occurred, with a high ammonia concentration of 5293 mg N/L. Tian et al. (2018b) found that the syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis (SAO-HM) pathway was enhanced throughout the ammonia acclimation process (up to 10 g NH_4^+-N/L) in mesophilic reactors co-digesting cattle slurry and microalgae, as proofed by the increase of the relative abundance of *Clostridium ultunense* and *Methanoculleus*. Similarly, Hao et al. (2017) demonstrated that the biodegradation of acetate gradually shifted from acetoclastic methanogenesis to the SAO-HM pathway in mesophilic AD reactors when TAN was elevated from 0.14 to 7 g/L.

2.4 Operational conditions affecting process stability of dry AD

The performance of dry AD process is affected by several different factors, including temperature, pH, carbon to nitrogen (C/N) ratio, OLR, and Inoculation.

2.4.1 Temperature

Temperature has been thought as one of the most crucial parameters for the operation of AD process, because it can affect the diversity and community structure of microbials, the kinetics and thermodynamical balance of the biochemical reactions, and the conversion pathways of the substrates and their metabolites. Generally, AD can be classified into three types depending on the temperature in operation, including psychrophilic digestion (below 20 ℃), mesophilic digestion (20-45 ℃), and thermophilic digestion (above 45 ℃) (Borja et al., 2002). Owing to its low reaction rate and methane production, the psychrophilic digestion has not been extensively studied and practically applied compared to the other two. Currently, mesophilic digestion is most commonly used in practical applications because of its higher stability and lower operating costs (Basinas et al., 2021), while thermophilic digestion is typically applied in most of the large-scale centralized biogas digesters (Kothari et al., 2014).

In comparison to mesophilic digestion, thermophilic digestion possesses a number of advantages, including a superior growth rate and activity of microbes that lead to a shorter retention time, higher removal rate of organic compounds and methane production, and better performance on pathogen inactivation (Jiang et al., 2020; Momayez et al., 2019; Rocamora et al., 2020). Fernández-Rodríguez et al. (2013) studied the kinetics of mesophilic and thermophilic digestion of OFMSW at a 20% TS and found that compared to the mesophilic conditions, the maximum specific growth rate of microorganisms was enhanced by 27-60% under thermophilic conditions. Besides, it has been widely reported that the performance of dry AD was improved under higher temperatures. Sun et al. (2019) observed a 21% increase in the maximum cumulative methane yield in dry AD of beer lees at 55 ℃ in comparison to 35 ℃. Likewise, according to Nguyen et al. (2017), in semi-continuous dry AD of food waste, the average reduction rate of VS and the biogas production under thermophilic conditions were higher than under mesophilic conditions by 6.88% and 16.4%, respectively. Regardless of these benefits, thermophilic dry AD process still has several drawbacks in terms of technical and economic aspects, such as low stability and reliability that requires

close oversight and precise operational controls, and higher energy demand for heating (Matheri et al., 2018; Paritosh et al., 2021a). Thermophilic digestion is more prone to inhibition and instability due to the higher hydrolysis rate which can result in the build-up of LCFAs and a decrease in pH level (Labatut et al., 2014). Shi et al. (2013) compared the performance of dry AD of corn stover at mesophilic and thermophilic temperatures. They observed a sharp pH drop from day 6 to day 12 in the thermophilic reactors, with the VFAs concentration being 5 times higher than in the mesophilic reactors. Moreover, it is reported that thermophilic digestion is highly sensitive to ammonia, because FAN levels increase with temperature (Rajagopal et al., 2013).

In general, dry thermophilic digestion gains higher efficiency compared with mesophilic digestion in terms of methane production and biodegradation of organic compounds, but it requires more precise operational controls to maintain the process stability. In the practical application of dry AD, it is, therefore, important to trade off efficiency of methane production and operational costs and energy consumption.

2.4.2 pH level

Anaerobic digestion is a complex biochemical process mediated by a consortium of microorganisms, making it highly sensitive to pH conditions. Even though pH is one of the most important parameters for dry AD operation, the pH effect on the dry AD process has rarely been reported. Because of the similarity of the bioprocesses and anaerobic bacteria, the pH effect on dry digestion can be inferred from the results of wet digestion. It is widely reported that the optimal pH for an efficient wet AD process is in the range of 6.8-7.2, although methane production still occurs in a pH range between 5.5 and 8.5 (Dague, 1968; Ward et al., 2008; Yi et al., 2014). Nevertheless, the optimal pH value varies greatly for different stages (hydrolysis, acidogenesis, acetogenesis, and methanogenesis) because the enzymatic activities of all functional microorganisms at each stage are principally determined by different optimal pH levels (Chandra et al., 2012). According to Yu and Fang (2002) and Kim et al. (2003), the optimal pH for hydrolysis and acidogenesis is found in the range of 5.5-6.5. Lay et al. (1997) indicated that methanogenesis only proceeds at a high rate when the pH is maintained around 7.0. This is one of the most important reasons for researchers to

develop a two-stage AD process where the hydrolysis/acidification and acetogenesis/methanogenesis processes proceeds in two separate reactors.

In single-stage AD systems, the pH is generally dependent on VFA concentration and alkalinity. Accordingly, the pH of the reactor varies with the reaction time because the VFA and ammonia concentrations fluctuate during digestion. In the initial stage of the batch AD reactor, the pH is prone to decline because of the rapid kinetics of hydrolysis/acidification process and the buildup of organic acids. Meanwhile, the reduction in pH is partially offset by the increase in ammonia produced by the degradation of proteins. Subsequently, the pH begins to increase when the methanogenic archaeal gradually converts VFAs into methane (Chandra et al., 2012). When the pH falls below 6.5 during the hydrolysis/acidification process, it may negatively impact methanogenic archaeal activities, or even inhibit them completely (Wang et al., 2014). Therefore, excessive acidification should be avoided in AD process. Acidification of digesters is a common phenomenon in practice. Chandra et al. (2012) claimed that the pH of the digester can be maintained within an acceptable range by regulating an OLR appropriately. Additionally, improved buffering capacity may provide an alternative option for maintaining a constant pH for the AD process and preventing excessive acidification. Normally, the buffering capacity of an anaerobic digester is evaluated by measuring the alkalinity, which is a result of the equilibrium that forms between carbon dioxide and bicarbonate ions, and that effectively prevents significant and rapid pH fluctuations, and it is therefore principally determined by the bicarbonate concentration in the digester (Ward et al., 2008). There are several methods to modify the alkalinity of the digester, including co-digesting with a substrate with high alkalinity such as livestock manure, adding alkaline reagents such as sodium bicarbonate ($NaHCO_3$) or sodium hydroxide (NaOH), modifying the I/S ratio or decreasing the OLR (Guwy et al., 1997; Rocamora et al., 2020; Ward et al., 2008).

Apart from the issue of AD process instability caused by low pH levels (such as acidification), high pH levels could potentially aggravate the ammonia inhibition in AD systems. According to Kadam and Boone (1996), the methanogenesis was completely inhibited at a TAN concentration above 5 g/L when the pH was 7.5, comparatively only at 1.6 g/L when pH was 8.5, because FAN was greatly higher at high pH levels. It is therefore

necessary to monitor the pH of the system regularly to ensure the successful operation of anaerobic reactors. Meanwhile, the relationship between pH, alkalinity and other operational parameters should be taken into account when running a dry AD process. For example, when operating a dry AD digester, it is acceptable to raise the OLR as long as the alkalinity is sufficient to buffer a peak of VFA production with the pH being within a proper range; otherwise, some measures should be taken, such as lowering the OLR, adding alkaline reagents like bicarbonate or elevating the ratio of the substrate with high buffer capacity fed into the digester.

2.4.3 C/N ratio

Carbon and nitrogen are two essential nutrients for microorganism growth, and the C/N ratio is one of the most critical parameters affecting the performance of the dry AD process. An inappropriate C/N ratio can lead to a high release of TAN and/or a build-up of VFAs in the digester, both of which potentially inhibit the AD process (Yan et al., 2015). The literature generally recommends an operating C/N ratio between 20 to 30, with 25 being the optimal C/N ratio for the growth of anaerobic bacteria in an AD system (Bouallagui et al., 2009; Shah et al., 2015). Nevertheless, the optimal C/N ratio depends on the type of feedstock used in the AD process. Yen and Brune (2007) found that the optimal C/N ratio for co-digestion of algal sludge and wastepaper was within the range of 20-25. Zhang et al. (2013) indicated that the C/N ratio of 15.8 was optimal when co-digesting food waste with cattle manure under mesophilic conditions. The C/N ratio represents an indicator of organic waste input into a dry anaerobic digester and varies greatly with feedstock type. Table 2-2 shows typical feedstocks as characterized by lower or higher C/N ratios. A better C/N balance can be achieved by combining substrates with low and high C/N ratios in appropriate proportions. This is one of the main reasons to perform AD by co-digesting in practice to improve the process stability. To enhance the performance of co-AD of dairy and chicken manure, Wang et al. (2012) used wheat straw to elevate the C/N ratio. The high FAN concentration of 223 mg/L was found at a C:N ratio of 15 and the FAN concentration distinctly decreased with the addition of wheat straw, with the FAN concentrations of 9.1, 7.5 and 2.2 mg/L at C/N ratios of 25, 30 and 35 respectively, ultimately achieving a stable performance.

Table 2-2. Typical feedstocks as characterized by lower or higher values of C/N ratio (Hagos et al., 2017; Siddique & Wahid, 2018)

Substrates with a low C/N ratio	C/N ratio	Substrates with a higher C/N ratio	C/N ratio
Livestock waste		*Agricultural waste*	
Pig manure	6-14	Rice straw	51-67
Cattle manure	15-26	Wheat straw	50-150
Poultry manure	5-15	Corn stover	50-56
Sheep manure	20-34	Sugar cane waste	139-151
Goat manure	10-17	Sawdust	200-500
Cow dung	16-25		
Industrial waste		*Energy crops and plants*	
Slaughterhouse waste	22-37	Algae	75-100
Municipal solid waste		Seaweed	70-79
Food wastes	2-18	Potatoes	30-60
Sewage sludge	10-20		
Grass/grass trimmings	12-16		

2.4.4 Organic loading rate and retention time

Organic loading rate (OLR) generally refers to the amount of organic waste fed into the digester through continuous or semi-continuous processes per unit volume of the digester per day (usually expressed in kg VS/m^3/d or kg COD/m^3/d) (Pera et al., 2021), which represents the capacity of AD systems for treating organic wastes. Compared to wet digestion, dry AD often operates with a higher OLR due to the high TS content of substrates (Karthikeyan & Visvanathan, 2013). According to Duan et al. (2012), the dry AD system can operate at a 4-6 times higher OLR compared with conventional wet AD systems while achieving comparable methane yields and VS reduction. The OLR values for dry AD are strongly dependent upon the characteristics of the feedstock materials (Srivastava, 2020), and vary greatly when using different feedstocks, such as 3.5-8.5 kg VS/m^3/d for corn silage (Veluchamy et al., 2019), 7-10 kg VS/m^3/d for OFMSW (Zeshan et al., 2012), and 4.0-8.5 kg VS/m^3/d for swine manure (Hu et al., 2019). Operating at a proper OLR is vital for dry AD process efficiency and stability, since a high OLR that exceeds the digester's degradation capacity will result in a

reduction in methane production and disturbance of the process (Ganesh et al., 2013). High loads easily result in the accumulation of VFA, and pH drop during the acidogenesis process, which impairs methanogenic activities, and causes lower effectiveness of the process or even collapse (Zamri et al., 2021). The effects of varying OLR on methane production have been studied extensively to determine optimal operational conditions. Using corn silage as feedstock, Veluchamy et al. (2019) investigated the effects of increasing OLR on methane production performance in a plug flow reactor through a semi-continuous process. The authors found that the VFAs concentration increased along with OLR ranging from 3.5-8.5 kg VS/m^3/d, while the AD system remained stable up to 6.5 kg VS/m^3/d, but became extremely unstable at 8.5 kg VS/m^3/d. Additionally, the highest maximum methane yield (410 mL/g VS_{added}) was obtained at 6.5 kg VS/m^3/d OLR, while methane yield decreased by 12.2% at 8.5 kg VS/m^3/d OLR.

The retention time (RT) is defined as the average time that the organic substrate remains inside the digester. In continuous dry AD processes, The RT determines the contact time between microorganisms and the substrate (Pera et al., 2021). An effective RT was determined by the substrate composition and OLR, and the decrease in RT usually results in the build-up of VFAs, while a longer RT reduces AD effectiveness. The RT required for AD digestion is affected by operational conditions such as temperature. The RT for mesophilic dry AD typically ranges from 30 to 40 days (Srivastava, 2020), while it is usually lower for thermophilic digestion (Kothari et al., 2014). Additionally, the RT and OLR are coupled in continuous dry AD processes, where the increase in the OLR is accompanied by a decrease in the RT. The relationship between OLR and RT can be written as Eq. (2-6).

$$OLR = \frac{C_{Sub}}{RT} = \frac{daily\ flow \times VS\ concentration}{liquid\ volume} \quad (2\text{-}6)$$

Where, C_{Sub} is the substrate concentration expressed as total added mass (VS or COD) per digester volume unit. The OLR of the digester is inversely proportional to the RT in continuous dry AD processes. It is necessary to achieve a good balance between OLR and RT to optimize the fixed volume of the industrial anaerobic digester. Clearly, the OLR and RT are critical parameters for dry AD processes, and they should be carefully selected to

optimize plant productivity and maximize renewable energy production, simultaneously avoiding process instability.

2.4.5 Inoculation

Inoculation is another key operational factor affecting process stability of dry AD. It is an essential requirement for speeding up the biochemical reactions inside the dry anaerobic digester (Chen et al., 2008; Forster-Carneiro et al., 2007), thereby reducing the digestion time of the batch AD process (Sukhesh & Rao, 2018). Several materials, including activated sludge, manure, wet AD digestate, and dry AD digestate are often used as seed sludge for inoculation of the dry AD reactor (Forster-Carneiro et al., 2007; Xu et al., 2016). In particular, using digestate from a wet or dry AD often offers better performance since it contains more active methanogenic populations that has been enriched in another digester or previous batch of digestion, and are better adapted to AD conditions (Xu et al., 2016). Due to the methanogenesis generally being the rate-limiting step in wet AD, the inoculation investigations primarily focus on the methanogenic populations in the inoculum, whereas the hydrolysis stage also plays a vital role in dry AD systems and sufficient hydrolytic microbes present in the inoculum are critical for the start-up of the digester (Xu et al., 2013).

A proper amount of inoculum is an essential condition for achieving stable and efficient AD performance. The low inoculum content probably results in the accumulation of VFAs, subsequently inhibits the methanogenic activities (Zhou et al., 2017). The I/S ratio (usually on VS basis) is taken as the main parameter for the inoculation of the batch dry AD, but there is no an optimal ratio accepted for any cases, since it is greatly dependent on several factors, such as types of AD systems, operating conditions (such as temperature), and substrate characteristics (Li et al., 2011b; Rocamora et al., 2020). Many studies have been conducted to optimize the I/S ratio to enhance the dry AD performance. Jiang et al. (2018a) carried out the batch dry AD (20%TS) using food waste and pig manure as substrates at different I/S ratios under mesophilic conditions. The results showed that all the reactors with an I/S ratio of 1:4 obtained lower methane production and longer lag phase times compared to those with a ratio of 1:1, regardless of the ratio of food waste to pig manure ratios. Moreover, these reactors digesting food waste alone were found being completely inhibited, because more readily

degradable organic matter in the substrates led to the rapid hydrolysis and acidogenesis, and thereby resulted in a rapid accumulation of VFAs. Meng et al. (2018a) conducted the large-scale batch test using pig urine and rice straw as substrates at I/S ratios of 2/1 and 1/3 under thermophilic conditions to explore the effects of inoculum rates on anaerobic digestibility. The authors indicated that the highest reduction rate and cumulative methane yield (358 mL/ gVS) were achieved at a I/S ratio of 2/1.

2.5 Strategies for enhancing dry anaerobic digestion

2.5.1 Pre-treatment

Pre-treatment is usually applied to feedstock prior to dry AD in order to improve methane production and process stability. The commonly used pretreatment strategies involve mechanical treatment, chemical treatment, biological treatment, and thermal treatment. The main purpose of the pretreatment is to increase the biodegradability of feedstock (typically lignocellulosic biomass) before it is fed into a dry anaerobic digester. In this regard, the dry AD process is compatible with most of the pretreatments used in wet AD. Table 2-3 illuminates the performance of various pre-treatments used in recent studies for improving methane production in dry anaerobic digestion.

2.5.1.1 Mechanical treatment

The solid-state substrates used in dry AD usually span a wide range of particle sizes, e.g., agricultural waste, green waste, OFMSW, which is partially determined by physical characteristics of the substrates and the collection process (Zhang & Banks, 2013). A number of studies have revealed that a large particle size could result in a low methane production efficiency (Jain et al., 2015). Thus, to improve the dry AD performance, substrates are usually mechanically pretreated before digestion to obtain desired particle sizes in order to improve the mixing conditions between the substrates and inoculum. The process increases the surface area available for microbial contact and the bulk density, as well as altering the flow properties of the substrate, ultimately enhancing the AD process (Amin et al., 2017). Wang et al. (2019) reported an increase of up to 26.4% in the methane production in dry AD

of stored corn stover when the particle size was reduced from 12.7 mm to 1.0 mm (Table 2-3). Many types of mechanical treatment methods are used for the particle size reduction, including mechanical shredding, sonication, liquid shear, high pressure homogenizer, etc. (Coarita Fernandez et al., 2020; Elliott & Mahmood, 2012). Mechanical shredding is the most commonly used in the industrial applications due to its easy-operating and low maintenance requirements, with the hammer and knife mills being the most popular types (Kratky & Jirout, 2011).

The mechanical treatment for the particle size reduction of the substrate is an intensive energy process. The finer milling is bound to require larger amounts of energy, which leads to an increase in the overall operational cost of the dry AD process. The particle size of substrates has a significant effect on bioconversion of organic matter to methane. To date, only few studies have studied particle size reduction in dry AD, and the benefits of this approach on the enhancement of process performance have been contradictory. Motte et al. (2014) investigated the effect of substrate particle size on the performance of dry AD of organic wheat straw and found that particle size reduction significantly affected the performances of the reaction owning to increased substrate bio-accessibility. Zhang and Banks (2013) examined the effect of particle size distributions on the AD of OFMSW and found that the particle size did not affect the specific biogas yield but did affect the rate of reaction. Furthermore, the authors noted that the finer shredded and rotary cut material (with a finer particle size) resulted in the acidification of the dry digesters at an OLR of 6 kg VS/m^3/d and ultimately led to process failure. Likewise in wet digestion, many studies have shown that the preferred particle size can potentially increase the methane yield and/or the digestion rate (Dumas et al., 2015; Izumi et al., 2010). Similarly, excessive reduction of the particle size of substrate can also lead to the accumulation of VFAs, lowering the performance of AD (Dumas et al., 2015). Therefore, it is important to proceed with the proper optimization and design of substrate size reduction equipment in consideration of process efficiency and economical aspects.

2.5.1.2 Chemical treatment

Chemical treatment involves the use of chemical reagents, such as acids, alkalis, or oxidants to decompose or extract organic compounds present in the feedstock, thereby enhancing the biodegradability of the organic fractions (Paudel et al., 2017). This method is intended to be used for the pretreatment of lignocellulosic biomass with high lignin contents (such as agricultural wastes) before digestion (Paritosh et al., 2021b). However, it is not suitable when the feedstock contains a large fraction of readily biodegradable organic matter, since it accelerates the degradation of organic matter and leads to the build-up of VFAs that will adversely affect methanogenesis. Lignocellulosic biomass is mainly composed of three complex-structured compounds: cellulose (25-40%), hemicellulose (20-30%) and lignin (15-25%), and it is recalcitrant to biodegradation and become very difficult to break down during the hydrolysis stage of dry AD (Paudel et al., 2017). Chemical treatment methods have been extensively studied to enhance AD performance using lignocellulosic biomass as the substrate, including alkali, acid, and alkali hydrogen peroxide (Fu et al., 2018; Janke et al., 2017). Dilute acids (<4% w/w) are commonly used in acid pretreatment since strong acid can cause some negative effects such as the production of undesirable byproducts that suppress the AD process, as well as the excessive degradation of the complex substrates (Paudel et al., 2017). Zhang et al. (2011) showed that the acid pretreatment of cassava residue with 3% H_2SO_4 for around 20 min at 158℃ resulted in a maximum methane yield of 248 mL/g VS, which was 57% higher than that of the un-treated group (158 mL/g VS). In dry AD process, alkaline pretreatment is widely used to enhance the biodegradability of lignocellulosic substrates and improve the dry AD performance as it has shown several positive effects, including enhanced lignin disruption, decrease of crystallinity, and increased surface area (Table 2-3). Moreover, alkali creates a good condition for dry AD process by preventing a drop in pH during acidogenesis and substantially enhancing anaerobic microbes' accessibility via enlarging the substrate surface area (Li et al., 2012; Xu et al., 2020). According to previous studies, NaOH has been shown to be the most effective one for improving lignin removal and methane production among various alkaline solutions (such as NaOH, KOH, $Ca(OH)_2$, and NH_3) (Fu et al., 2018). Xu et al. (2020) used the black liquor of 12 g NaOH/L alkalinity to pretreat the corn stover for 24 h, and they observed that the removal of hemicellulose in corn stover reached 22.09% and the methane production was increased by 59.1% compared with the unpretreated. Likewise, Lomwongsopon and Aramrueang (2022)

found that the COD solubilization of cassava pulp was improved using NaOH pretreatment, as well as the disruption of hemicellulos and lignin.

Oxidative treatments using oxidizing agents (like hydrogen peroxide, potassium permanganate) possess potential to improve the hydrolysis rate and methane production in dry AD of lignocellulosic materials. Millati et al. (2020) claimed that the oxidizing agents possessed a strong oxidizing capability to realize the degradation of lignins and the increase of the soluble fractions. Song and Zhang (2015) found that the lignin and hemicellulose contents of wheat straw were significantly reduced by 5.4-21.9% and 12.5-45.2%, respectively, after oxidative pretreatment at 25 °C for 7 days by using 1-4% H_2O_2. Similarly, Wang et al. (2020b) observed that, $KMnO_4$ pretreatment for 8 h led to remarkable improvement in dairy manure degradation, with cellulose and lignin reductions of approximately 49% and 48%, respectively, at a dosage of 100 mg/g-TS for $KMnO_4$.

2.5.1.3 Biological treatment

In comparison to other methods, biological pretreatment has been identified as an economical, environmentally friendly, and efficient method for improving the AD performance due to its lower energy and chemical consumption, and moderate reaction conditions (such as temperature, pressure and pH) (Ali et al., 2020). Biological pretreatment involves the use of bacteria, fungi, and enzymes to degrade the biomass prior to the AD process (Hosseini Koupaie et al., 2019). As shown in Table 2-3, several biological pretreatment methods, like fungal pretreatment, microbial pretreatment, enzymatic pretreatment, and microaerobic pretreatment, are widely used to improve the biodegradability of substrates and methane production for dry AD.

In fungal pretreatment, the white-rot fungi are the most popular to be used due to its effectiveness in breaking down polysaccharide-lignin bonds and removing lignin. The degradation of lignocellulosic materials by fungal pretreatment is mainly attributed to the highly active oxidative ligninolytic enzymes produced by the fungi. Tišma et al. (2018) applied the white-rot fungus *Trametes versicolor* for corn silage pretreatment in solid-state conditions prior to digestion at pilot-scale and found that 70% of the lignin content was

degraded after a seven-day pretreatment and the pH stability and biogas productivity were enhanced. Chaitanoo et al. (2021) found that implementing a long-time fungal pretreatment for broiler litter negatively affected the microbial activity owing to the moisture loss during this process, and they suggested that the optimal time was 7 days. Planinić et al. (2016) suggested that, to achieve a good fungal treatment effectiveness, more attention should be given to the I/S ratio, feedstock particle size, oxygen concentration, moisture content, and reaction time when designing the process of fungal pretreatment. Additionally, fungal pretreatment can be combined with another pretreatment method to enhance the degradation of lignocellulosic materials and methane production (Millati et al., 2020). For example, Alexandropoulou et al. (2017) performed a combination of fungal and alkaline treatment in pretreating the willow sawdust substrate prior to digestion, and the results showed that higher removal efficiencies of the cellulose and hemicellulose were achieved as well as higher BMP compared with the respective alkaline and fungal pretreatment alone.

Unlike the fungal treatment, the enzymatic treatment employs specific enzymes such as manganese peroxidase, versatile peroxidase, lignin peroxidase, and laccase to pretreat substrates directly, as a result the reaction time for the process is relatively short (Hosseini Koupaie et al., 2019). Additionally, the process can be carried out by either pretreating substrate with enzymes prior to digestion or by directly adding specific enzymes into the digester during dry AD (Millati et al., 2020). Many studies have proved that the enzymatic treatment using specific enzymes could effectively enhance the degradation of lignocellulosic materials. Schroyen et al. (2015) examined the effects of enzymatic pretreatment using laccase (2 U/g) and versatile peroxidase (1.5 U/g) on willow and corn stover, and they found that the release of total phenolic compounds was significantly increased after pretreatment which indicated the occurrence of lignin degradation by the enzymes. Similarly, Frigon et al. (2012) observed that the methane production was improved by 29% and 42% when harvested switchgrass was pretreated using lignin peroxidase (1 U/mL) and manganese peroxidase (2 U/mL) for 8 h, respectively. Yet, the enzymatic pretreatment is still facing a challenge related to the economic feasibility at an industrial scale because of the high enzyme cost.

In addition to fungus and enzymes, adapted microbial consortia can also be utilized in biological pretreatment process. Using adapted microbial consortiums has been identified as a

more cost-effective microbial pretreatment compared with pure enzymatic pretreatment. Microbial consortiums have been widely reported to improve biomass degradation performance over single-organism pretreatments, since lignocellulose degradation in natural habitats is dependent upon the interaction of multiple microorganisms (Raut et al., 2021). Additionally, microbial consortiums are more adaptable to complex substrate mixtures because more abundant degrading enzyme systems are present. The use of microbial consortia has been reported to improve the biodegradability of lignocellulosic substrates and in turn, to enhance the methane production of AD processes (Abraham et al., 2020). Raut et al. (2021) pre-treated the lignocellulosic co-substrates of barley straw and hay straw using barley straw-adapted microbial consortia at 25-27 ℃ under aerobic conditions before AD and found that the methane yield (15.2 mL/g TS) was 40-times higher than the untreated. Yan et al. (2022) applied domesticated paddy soil microbes (DPSM) to pre-treat the co-substrates of rice straw and pig manure prior to AD. The results showed that the DPSM treatment effectively promoted hydrolytic acidification of the co-substrates at 20% TS and shortened the methane production time by 43.4%.

2.5.1.4 Thermal treatment

An additional method for accelerating hydrolysis and boosting methane production from organic biomass is thermal treatment, which has been extensively used in conventional wet AD in treating various substrates (Ariunbaatar et al., 2014), such as sewage sludge (Dhar et al., 2012), food waste (Ariunbaatar et al., 2015). During the thermal treatment process, the temperature is gradually increased to a target temperature (usually 60-270 ℃) and then maintained for a desired period (varying from minutes to hours) (Kumar Biswal et al., 2020). With the thermal treatment process, the polymeric compounds within a substrate are effectively degraded and released into the liquild phase, and the biodigestibility of the substrate is improved due to the breakdown of chemical bonds (Kor-Bicakci & Eskicioglu, 2019). Besides improved biodigestibility, thermal pretreatment can also eliminate the pathogens from organic wastes (Kor-Bicakci & Eskicioglu, 2019). Based on its characteristics and mechanism, thermal pretreatment is potentially suitable for the enhancement of dry AD. Some studies have shown its effectiveness in improving methane production in a dry AD. For example, Hu et al. (2019) adopted the thermal pre-treatment at

70℃ for 3 days in order to enhance dry AD of swine manure and found that methane yield (416 mL CH_4/g VS) was significantly increased by 390% compared with the unpretreated.

For the thermal pretreatment process, its efficiency is primarily determined by two factors (temperature and duration), and the optimal conditions is dependent on the type and composition of feedstocks (Paudel et al., 2017). It has been reported that thermal treatment at high temperatures (≥ 100 °C) promotes solubilization of substrates, as it effectively breaks down the polymeric compounds and cell membranes, thus resulting in the release of complex substances (e.g., proteins, polysaccharides, and lipids) into the soluble phase (Tyagi et al., 2018). Hence, the high-temperature thermal treatment has been studied in dry AD of various substrates, such as dewatered sludge (Kim et al., 2022; Wu et al., 2021), food waste and cattle manure (Arelli et al., 2018), and sunflower stalks (Hesami et al., 2015), as shown in Table 2-3. From an economic standpoint, the high temperature thermal treatment requires more energy consumption, which will offset the benefits of the increased biogas production and hinder its practical implementation. Moreover, a high temperature treatment may cause the generation of refractory compounds or toxic intermediates, which might result in the reduced biogas production (Kor-Bicakci & Eskicioglu, 2019). Thus, the low-temperature thermal treatment (< 100 °C) has attracted considerable research interest. To improve biogas production in high solid AD of swine manure, Wu et al. (2017b) used a low-temperature thermal treatment process (70 ℃ for 3 days). The results showed that proteins, cellulose, and hemicellulose were significantly degraded during the thermal treatment, and the methane yield in high-solid swine AD was increased by 39.5%. Kim et al. (2021) operated a lab-scale continuous high-solid anaerobic digester with dewatered sludge as the substrate to evaluate the effect of low thermal pretreatment (at 60 ◦C for 3 h) on methane production performance of high-solid AD. The results indicated that, compared to the control group without pretreatment, the low-thermal pretreatment resulted in higher net energy production, improved sludge treatment efficiency, as well as improved digestion stability.

Table 2-3. Performance of various pre-treatments used in recent studies for improving methane production in dry anaerobic digestion [a]

Methods	Substrate	Pretreatment conditions	Effects of pretreatment	Methane production (mL/g VS) [b]	Mechanisms	Reference
Mechanical treatment						
Grinding/chopping	Corn stover	Using a Wiley Mill	1, 12.7 mm particle size	207 (+10.1%) [c]	Increased surface area, reduced degree of cellulose crystallinity and reduced degree of cellulose polymerization	(Wang et al., 2019)
	Wheat straw	Using a cutting mill	2.0, 1.0, 0.5, 0.25, 0.12 mm particle size	Increased biodegradation rate		(Dumas et al., 2015)
Chemical treatment						
Alkali	Corn stover	12 g NaOH/L alkalinity (black liquor from the paper industry); 24 h	22.1% HC removal	260.5 (+ 59.1%)	Degradation of HC, increased surface area, reduced crystallinity.	(Xu et al., 2020)
	Cassava pulp	0.5%-2% NaOH; 30 ℃	Enhanced COD solubilization, increased HC and lignin removal	324 (+ 33.0%)	Dissolved lignin and hemicellulos, increased surface area, reduced crystallinity.	(Lomwongsopon & Aramrueang, 2022)
	Pearl millet straw	2% KOH	39.4 % lignin disruption	140 (+ 65.2%)	Enhanced lignin disruption	(Paritosh et al., 2020)
Oxidant	Dairy manure	50-200 mg $KMnO_4$ /g-TS; 2-48 h	Enhanced HC, cellulose and lignin removal	12.38 NmL/g VS/d (+ 27%)	Decomposition of LC	(Wang et al., 2020b)
Biological treatment						

Fungal	Broiler litter (around 57% rice husk)	Inoculated with 10% (v/w) of fungal spore suspension (*Trichoderma longibrachiatum*); 50% moisture content; 7 days; 30℃	+5.4 % cellulose removal, +8.0 % HC removal, +1.5 % lignin removal	438 (2.0-fold higher)	Disruption of outer layer of lignocellulosic structure	(Chaitanoo et al., 2021)
	Corn silage	Inoculated with white-rot fungus *Trametes versicolor*;7 days; 27 °C	70% lignin removal	236 (+41.3%)	Degradation of lignin	(Tišma et al., 2018)
Enzyme	Willow Corn Stover	Laccase and Versatile peroxidase; 6 h–24 h; 30 ℃	Improved lignin degradation	(+33%) (+15%)	Degradation of lignin	(Schroyen et al., 2015)
Microbial consortia	Barley straw and hay (BSH)	Enriched microbial consortia to the BSH co-substrate ratio of 1:7; 40 days at 25–27 ℃; the oxygen level maintained 2-5 mg/L	Effective degradation of BSH residues by the enriched microbial consortia	15.2 mL/g TS (almost 40 times more)	Increased digestibility and surface area of the lignocellulosic material	(Raut et al., 2021)
	Rice straw and pig manure	35% domesticated paddy soil microbe's inoculum used, aerobic conditions, 7 days at 37 ℃	Increased hydrolysis rate of cellulose, hemicellulose, and lignin	Shortening methane production time by 43.4% (from 30 to 17 days)	Improved biodegradability	(Yan et al., 2022)
Thermal treatment						

High-thermal pretreatment	Dewatered sludge	140 °C for 3 h	Increased COD solubilization	195 (+81%)	Enhanced hydrolysis	(Kim et al., 2022)
	Dewatered sludge	160 ◦C; 210 min		(+400%) MAD (+67%) TAD		(Wu et al., 2021)
	Food waste and cattle manure	121 °C; 103.4 kPa; 30 min	Declined in TS concentration, increased VS content	320-430 mL/g VS $_{reded}$	-	(Arelli et al., 2018)
	Sunflower stalks	180 ◦C; 60 min	Enhance lignin removal	234 (around 87%)	Degradation of lignin, cellulose crystallinity reduction	(Hesami et al., 2015)
Low-thermal pretreatment	Swine manure	70 ◦C; 3 days	Enhanced HC, cellulose and protein degradation	282 (+39.5%)	Increased biodegradable organics	(Wu et al., 2017b)
	Dewatered sludge	60 °C; 3 h	-	138.5	-	(Kim et al., 2021)

Notes: a. Barley straw and hay (BSH); Hemicellulose (HC); Lignocellulose (LC); Mesophilic anaerobic digestion (MAD); Thermophilic anaerobic digestion (TAD).

b. Data shown in the bracket represents the methane yield improvement.

c. Compared to the AD reactor fed with 12.7 mm covered corn stover.

2.5.2 Co-digestion

Co-digestion is an AD process that allows more than one type of substrates to be fed into the same digester for biogas production. It can provide more balanced nutrients for the growth of anaerobic microbes by using multiple substrates, avoiding the deficiencies caused by feeding a single substrate, thereby improving methane production performance of the AD process (Angelidaki & Ellegaard, 2003). The utilization of co-digestion in the wet AD process has been shown to have several benefits, including: (1) improved balance of nutrients; (2) synergistic effects on microbes; (3) increasing the loading rate of biodegradable organic matter; (4) dilution of inhibitors/toxic compounds; and (5) higher methane production (Dai et al., 2013; Karki et al., 2021; Kothari et al., 2014; Zhang et al., 2022). For example, co-digesting food waste with other substrates, like sewage sludge, pig manure, or yard waste, has been shown to effectively eliminate the common issues of VFA accumulation and acidification, as well as significantly improving methane production and AD process stability (Begum et al., 2021; Jiang et al., 2018a). Though the operational TS conditions and substrate fluidity are dramatically distinguished from those in wet AD systems, dry AD systems may still benefit from co-digesting with two or more substrates. Thus, co-digestion is one of the most important strategies for intensifying dry AD systems in which the bioconversion of organic matter may be enhanced.

Typical types of organic wastes used for co-digestion to enhance biogas production in dry AD systems are illustrated in Table 2-4. Food waste with a high content of biodegradable organic matter is typically regarded as one of the attractive substrate candidates for the dry AD process. Nevertheless, due to its high biodegradability and relatively low C/N ratio, mono-digestion of food waste often encounters the accumulation of VFAs, and acidification accompanied by sudden pH drops, or even process failure, which is more pronounced in dry AD process (Jiang et al., 2018a). Thus, co-digesting food waste with other co-substrates is a common measure to optimize dry AD performance. Many studies have proven that a series of substrates with high C/N ratio are suitable for co-digesting with food waste in dry AD systems, including pig manure (Jiang et al., 2018a), yard waste (Panigrahi et al., 2020), green waste (Chen et al., 2014b), cardboard (Begum et al., 2021), and sewage sludge (Arelli et al., 2021; Dai et al., 2013). Chen et al. (2014b) indicated that co-digestion of food waste with

green waste at a ratio of 40:60 had synergistic effects on methane production, with increased methane yield by 18.7% compared with the weighted methane yield from food waste and yard waste. Dai et al. (2013) carried out long-term experiments in semi-continuous reactors to evaluate the system stability and methane production performance of dry co-AD of dewatered sewage sludge and food waste, and the results demonstrated that the system stability was greatly enhanced, and the digester could perform well under high OLRs conditions (4.6-18.5 g VS/L/d). Apparently, the organic loading rate used in dry anaerobic co-digestion was higher compared with mono-digestion, which would result in better efficiency of AD systems in treating waste and producing biogas.

In dry anaerobic co-digestion, the mixing ratio of the organic substrates is an important parameter that has significant effects on methane production (Fagbohungbe et al., 2015). By adjusting the organic substrate mixing ratios, it is possible to maintain a balance between the microbial population, nutrient levels, and organic loading. Studies have emphasized the importance of optimizing the ratio of organic substrates in a dry AD system. Li et al. (2016) examined the effects of feedstock mixing ratios on methane production and system stability of digesters when tomato residues were co-digested with dairy manure and corn stover at 20% TS under mesophilic temperature. The results showed that the highest methane yield (415.4 mL/g VSS) and VS reduction (46%) were achieved at the mixing ratio of 33:54:13, and the inhibition of VFAs to methane production was observed with a proportion of tomato residues exceeding 40%. Ziaee et al. (2021) reported that the best performance of the co-digestion of OFMSW and sawdust was achieved at the mixing ratio of 2:1, with the methane production of 300 mL/g VS. Jiang et al. (2018a) indicated that raising the food waste/pig manure ratio for dry AD may result in a rapid accumulation of high VFAs concentration and a longer lag phase due to an increase in readily biodegradable organic matter content. They also suggested that a mixing ratio of 50:50 could be more suitable for the operation of dry AD under mesophilic temperature, with a SMY of 263 mL/g VS_{added}. Therefore, it is necessary to conduct experimental trials for the determination of the most appropriate mixing ratio of multiple substrates during dry co-digestion in practical applications.

Table 2-4. Typical types of organic wastes used for co-digestion to enhance biogas production in dry AD systems

Co-substrates	Mixing ratio [a]	TS (%)	C/N ratio	Operational Conditions	VS reduction (%)	Methane yield (mL/g VS $_{added}$)	Remarks	References
FW + PM	0:100, 25:75, 50:50, 75:25, 100:0	20	-	Batch, mesophilic, no mechanical mixing system	40-71	200-304	Acidification and no methane production were observed in mono-digestion of FW; optimal FW/PM ratio is 50:50 at the I/S ratio of 1:1.	(Jiang et al., 2018a)
FW + yard waste	1:3 (TS base)	>15	25	Batch, mesophilic	-	335	Maximum methane yield was observed at the I/S ratio of 2.0.	(Panigrahi et al., 2020)
FW + green waste	100:0, 80:20, 60:40, 40:60, 20:80, 0:100	15	14.4-16.9	Batch, mesophilic	-	164-326	+ (4.0%-18.7%) in methane yield compared with the weighted yield from FW and green waste	(Chen et al., 2014b)
FW + cardboard	80:20, 60:40, 50:50 (v/v %)	19-24	-	Semi-continuous, mesophilic, SRT=40 d, OLR=4.7-5.7 g VS/L/d	45-65	130-240	The lower methane yield for digesters with cardboard content >40%.	(Begum et al., 2021)
FW + sewage sludge	1:1, 1:2, 1:3 (volume-based)	>15	13.0-26.8	Batch, mesophilic/thermophilic	76 (M) 88 (T)	350 (M), 420 (T), mL/g VS $_{reduced}$	The mixing ratio of 1:3 and 1:2 is optimum for mesophilic and thermophilic conditions, respectively	(Arelli et al., 2021)
DSS + FW	1:0, 2.4:1, 0.9:1, 0.4:1, 0:1	7.0-17.4	6.8-14.8	Semi-continuous, mesophilic, SRT=8-	27-86	157-465	Improved system stability and greatly enhanced volumetric biogas production	(Dai et al., 2013)

				30 d, OLR=4.0-21.8 g VS/L/d			were achieved in dry AD of DSS by co-digesting with FW	
Cattle manure + sorghum-vinegar residues	1:1	20	15.5-20.6	Batch, mesophilic	32	169	+ 58.2% in methane yield compared with the mono digestion of cattle manure	(Zhang et al., 2022)
Sheep manure + sorghum-vinegar residues	1:1	20	20.6-22.4	Batch, mesophilic	22	103	+45.5% in methane yield compared with the mono digestion of cattle manure	(Zhang et al., 2022)
PM + sorghum-vinegar residues	1:1	20	11.1-20.6	Batch, mesophilic	34	160	+10.1% in methane yield compared with the mono digestion of cattle manure	(Zhang et al., 2022)
Rice straw + pig urine	1: 25.9	21	20-23	Batch, thermophilic	83	354	With the I/S ratio of 1:2	Meng et al. (2018a)
PM+ corn stover + cucumber residues	5:1:4, 5:2:3, 5:3:2, 4:1:5, 4:2:4, 4:3:3, 4:4:2, 3:1:6, 3:2:5, 3:3:4, 3:4:3 (wet base)	22	13.8-15.6	Batch, mesophilic	-	305.4 (highest)	The optimal mixture ratio was 5:2:3 with the highest methane yield	(Wang et al., 2018b)
DSS + rice straw	-	20	18:1, 23:1, 26:1, 29:1	Batch, mesophilic	43-60	361-520 (biogas)	Low C/N ratios resulted in a higher biogas production rate, but a lower specific biogas yield	(Chu et al., 2015)

OFMSW + sawdust	1:1, 2:1, 4:1, 1:0	20	20-35	Batch, mesophilic	-	170-300 mL/g $VS_{reduced}$	The optimum OFMSW/sawdust ratio was 2:1 with the maximum methane production	(Ziaee et al., 2021)
MSW + sewage sludge	60:40	15 & 20	21.5	Batch, mesophilic	30 & 24	227.4 & 209	The optimum MSW/sewage sludge ratio was 60:40	Ahmadi-Pirlou et al. (2017)
Grape marc + cheese whey	3:1	28.5	102 (COD/N)	Batch, thermophilic	62 (COD reduction)	363	No overloading phenomenon was observed under high TS conditions	(Kassongo et al., 2020)
Tomato residues + dairy manure + corn stover	100:0:0, 0:100:0, 0:0:100, 33:13:54, 33:27:40, 33:40:27, 33:54:13, 13:33:54, 27:33:40, 40:33:27, 54:33:13 (wet base)	20%	11.7-28.7	Batch, mesophilic	46 (highest)	415.4 (highest)	The optimum ratio was 33:54:13. Inhibition of VFAs occurred with adding more than 40% tomato residues.	(Li et al., 2016)
SPW+ hay	100:0, 75:25, 50:50, 25:75, 0:100	17.6	10.8	Batch, mesophilic	-	258 (highest)	The optimum mixing ratio was 75:25, with 148% and 50% increase in the methane yield compared with that from SPW and from hay	(Zhu et al., 2014)
SMS+ yard trimmings	1:1	20	74.6	Batch, mesophilic	-	194	16 and 2 times higher than methane yields from SMS and yard trimmings.	(Lin et al., 2014)
SMS + wheat straw	1:1	20	71.9	Batch, mesophilic	-	269	22 times higher than methane yield from SMS	(Lin et al., 2014)

Notes: a. on volatile solids base. VS, volatile solid. OFMSW, organic fraction of municipal solid waste. MSW, municipal solid waste. OLR, organic loading rate. FW, food waste. PM, pig manure. DSS, dewatered sewage sludge. I/S, inoculum to substrate. COD, chemical oxygen demand. VFA, volatile fatty acid. SPW, soybean processing waste. SMS, spent mushroom substrate.

2.5.3 Conductive material supplementation

The use of conductive materials (e.g., activated carbon, biochar, magnetite, etc.) in wet/liquid AD to intensify the process performance is a research hotspot and has been extensively studied in the past decades (Park et al., 2018; Yin & Wu, 2019). In many studies, researchers have demonstrated that the addition of conductive materials in wet/liquid AD can promote electron transfer between electron-donating bacteria and electron-accepting methanogenic archaea by means of direct interspecies electron transfer (DIET), and thus enhance AD performance in terms of reduced lag phase, elevated methane production rates and yield, and tolerance to the shock of OLRs and inhibitory conditions (Park et al., 2018; Wang et al., 2018c; Yin et al., 2018). Furthermore, adding conductive materials to improve AD performance has also been studied in full-scale AD systems (Barua & Dhar, 2017). Similar problems may exist in dry AD systems, such as electron transfer limitation and substrate inhibition, and these can potentially be addressed by the supplementation of conductive materials (Pan et al., 2020).

Table 2-5 summarizes different conductive materials used in recent studies for improving methane production in dry AD. In general, all conductive materials have showed enhanced methane production compared to control digesters (without conductive materials), however, the intensity of improvement varies greatly with operational conditions, such as dosage, substrate, reactor type, and operating temperature. Sun et al. (2019) carried out dry AD of beer lees with supplementing10 g/L cow manure-derived biochar, which resulted in increased cumulative methane yield by 82.6% and 46.8% under mesophilic and thermophilic conditions, respectively. Wang et al. (2021b) found that adding 15 g/L of pecan shell-derived biochar to mesophilic dry co-AD of pig manure and food waste increased methane production by 12.0%, suggesting that enhanced electron transfer due to the addition of biochar might have played a role in the improvement of methane production. Xiao et al. (2019) observed that the addition of granular activated carbon (GAC) increased methane production of dry AD of swine manure under mesophilic conditions, as well as shortened the lag phase. Similar results of enhanced methane production with the addition of powdered activated carbon (PAC) were also obtained in dry AD of organic fraction of municipal solid waste (OFMSW) (Dastyar et al., 2021) and sewage sludge (Pan et al., 2020).

Currently, the conductive materials used in dry AD systems can be classified into two categories based on their nature: (1) carbon-based materials, such as biochar and activated carbon; and (2) metallic materials, such as limonite and zero-valent iron (ZVI). Different mechanisms for improving methane production performance of dry AD systems when using different types of conductive materials have been proposed in previous studies. An overview of possible mechanisms for enhanced methane production in dry AD with conductive material supplementation is shown in Figure 2-5. Due to their electrical conductivity, conductive materials are generally considered to act as electricity conduits, which facilitates electron transfer between syntrophic bacteria and methanogenic archaea via DIET (Park et al., 2018). Xu et al. (2019) assessed the effects of limonite on the dry AD of rice straw and cow manure, suggesting that limonite particles served as electron conduits in DIET to facilitate methane production. Apart from facilitating DIET, carbon-based materials (typically like biochar) can also offer a more favorable habitat for microbes owing to their physicochemical properties (porosity, high specific surface area, and functional groups) and biostability, and thus promote microbial metabolic activity and biofilm formation (Sun et al., 2019; Wang et al., 2021b). Moreover, some carbon-based materials have strong adsorption properties, which can mitigate adverse effects on anaerobic microbial habitats by adsorbing inhibitory substrates or intermediate metabolites (e.g., VFAs, ammonia) (Fagbohungbe et al., 2017). ZVI, different from other materials, has attracted more attention from researchers in recent years, because it not only has the potential to facilitate DIET, but it can also serve as an electron donor to increase methane production in dry AD system (Puyol et al., 2018). Daniels et al. (1987) reported that H_2 was released from the ZVI surface via anaerobic biocorrosion and captured by hydrogenotrophic methanogens for the conversion of CO_2 to CH_4. Zhu et al. (2020) indicated that the presence of ZVI that serves as a conductive material could establish DIET to enhance electron exchange among microbes and improve interspecies hydrogen transfer in dry AD systems. Besides, the presence of ZVI offers a more favorable environment for AD by decreasing oxidative–reductive potential (ORP) (Zhen et al., 2015).

The use of conductive materials is a promising strategy for the enhancement of dry AD performance, and it also has a great potential in industrial practice. To date, studies

investigating the effects of adding conductive materials on dry AD performance have largely been conducted on a laboratory scale or via batch operation models. More studies need to focus on long-term operation tests at full-scale. From the point of practical feasibility, the use of these additives has been restricted as disposal of these additives after AD could cause environmental issues, such as secondary pollution and threats to environmental safety. Additionally, the high cost for production of conductive materials weakens the economic feasibility in practice. Therefore, more attention should be given to the economic feasibility and environmental effects of the practical use of conductive materials in the dry AD process in the future studies.

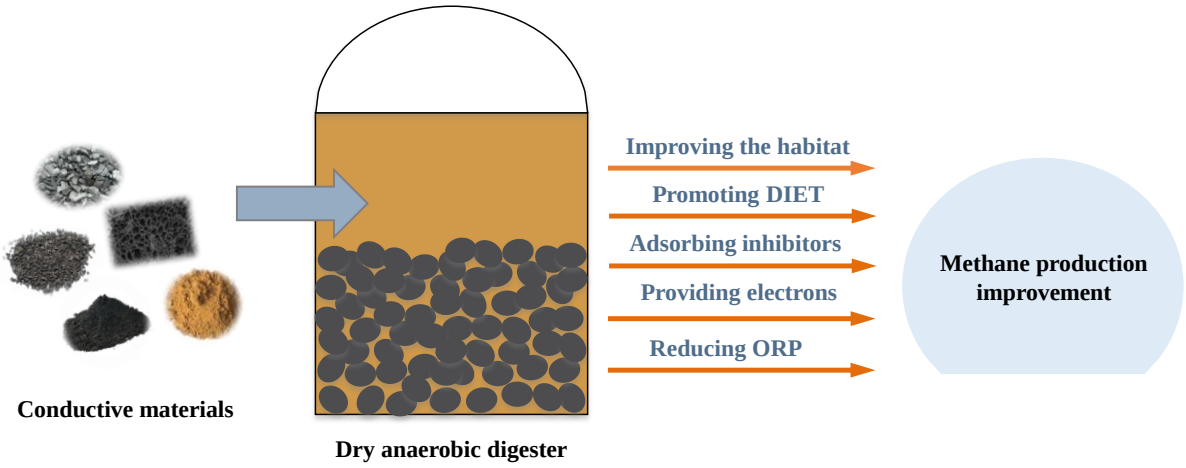


Figure 2-5. An overview of possible mechanisms for enhanced methane production in dry AD with conductive material supplementation.

Table 2-5. Different conductive materials used in recent studies for improving methane production in dry anaerobic digestion.

Type of additive		Particle size (mm)	Dosage (g/L)	Operational conditions for dry AD			Effectiveness	Possible explanations	References
				Type of substrates	Total solid	Reactor			
Carbon-based material	Cow manure-derived biochar	0.4-0.6	10	Beer lees	25%	Mesophilic, batch, lab-scale	+82.6% methane yield	promote DIET; the microbial colonization and biofilm development due to the high surface area and porous structure of biochar	(Sun et al., 2019)
						Thermophilic, batch; lab-scale	+46.8% methane yield		
	Pecan shell-derived biochar	0.46-1.10	15	Pig manure + food waste	15%	Mesophilic, batch, lab-scale	+12% methane yield	promote electron transfer	(Wang et al., 2021b)
	Wood-derived biochar	≤0.6	30	OFMSW [a]	16%	Mesophilic, batch, lab-scale	Allow execution of dry AD without a lag phase	-	(Salehiyoun et al., 2022)
					26%			-	
	PAC [b]	-	15	OFMSW	-	Mesophilic, batch, lab-scale	+17 % methane yield	-	(Dastyar et al., 2021)
		0.15	50% [c]	Sewage sludge	Around 16%	Mesophilic, batch, lab-scale	+49.9% methane yield	promote electron transfer; high adsorption due to porous structure of biochar	(Pan et al., 2020)
	GAC [d]	0.5–1.0	4.2%	Swine manure	28%	Mesophilic, semi-continuous, lab-scale	+10.6% biogas production	enhanced microbial adhesion, the provision of electronic bridges, and enrichment of functional microorganisms	(Xiao et al., 2019)

Metallic material	ZVI [e]	0.2	10	Food waste	Around 21%	Mesophilic, semi-continuous, lab-scale	Improved methane production and organic removal; resist to the stock of higher OLRs	Enhancing interspecies hydrogen transfer and direct interspecies electron transfer	(Zhu et al., 2020)
	Limonite	0.4	1%	Rice straw + cow manure	25%	Mesophilic, batch, lab-scale	+30.3% methane yield	promoted the growth rate and activity of methanogens, and direct interspecies electron transfer.	(Xu et al., 2019)

Notes: a. OFMSW: organic fraction of municipal solid waste. b. PAC: powdered activated carbon. c. the dosage of PAC is 50% of the volatile solids. d. GAC: granular activated carbon e. ZVI: zero-valent iron. DIET, direct interspecies electron transfer.

2.5.4 Percolate recirculation

As stated in Section 2.3.3, the mass transfer barrier inside dry AD systems is one of the major challenges to achieve efficient digestion. As a result of the high solids content in dry AD, mass transfer (such as substrates and metabolic intermediates) is restricted, thus limiting methane production and reaction kinetics. Percolate recirculation is one of the most commonly used strategies to improve mass transfer and thereby enhance dry AD performance (Meng et al., 2019). Benbelkacem et al. (2010) found that periodical leachate injections at a proper flow rate allowed to drastically speed up the biogas production in dry AD of municipal solid waste. Many studies have shown that percolate recirculation provides several benefits to dry AD systems, such as increased moisture content of the medium (Degueurce et al., 2016), elevated contact efficiency between microorganisms and nutrients (Lee et al., 2019); improved reactor uniformity (Rocamora et al., 2020); and provision of additional microorganisms that have a partial inoculation effect (Xu et al., 2014b). All these advantages could potentially reduce digestion time and increase the methane yield for dry AD.

There are two main patterns of recirculation: (1) percolation recirculation with leachate infiltrating through a leach-bed reactor; and (2) immersed digestate circulation with a liquid flow convection on the surface of a box-type reactor (Meng et al., 2019; Yu et al., 2019). Regardless of the use of either pattern, the intensity of percolate recirculation applied in dry AD is a significant factor in determining its effectiveness to a large extent. Usually, the intensity of percolate recirculation is determined by the recirculation rate that represents the ratio of the percolate recirculation volume to the digester volume per day (Luo et al., 2021). An excessive recirculation rate of percolate can lead to acidification, the buildup of VFAs, and a low pH level due to the different rates of hydrolysis, acidogenesis, acetogenesis, and methanogenesis, particularly in the start-up stage of a dry AD system (Degueurce et al., 2016). Rico et al. (2020) also suggest that low percolation recirculation rate must be applied at the start-up of the dry AD process.

More recent studies have paid more attention to the optimization of percolate recirculation strategies, in terms of the recirculation model and recirculation rate. Table 2-6 illustrates the different strategies of percolate recirculation used for enhanced methane production in dry

anaerobic digestion. Xing et al. (2020) evaluated the effects of recirculation models (continuous or intermittent) on methane production in Pennisetum hybrid dry AD, with the results showing that continuous recirculation led to a lower accumulative methane yield owning to washing effects and poorer distribution on lower layers. In contrast, Xu et al. (2014b) found that continuous leachate recirculation significantly improved the organic leaching with distinct extracellular enzyme activities. Therefore, it cannot be concluded that either continuous or intermittent recirculation is more effective in enhancing dry AD performance. Meng et al. (2019) explored the effects of different leachate circulation strategies on the AD performance co-digesting rice straw with pig urine, and the results showed immersing rice straw into leachate was more effective in improving methane production compared to leachate percolation. Pezzolla et al. (2017) demonstrated that recirculating percolate at an optimal frequency of 4 times per day had positive effects on biogas production and led to better process stability because it avoided the accumulation of VFAs in the liquid fraction.

The percolate recirculation is primarily designed to create a better environment for the growth of anaerobic microorganisms in dry AD systems, and it can improve the degradation of organic matters either via moisture movement within the reactor matrix or by directly affecting the moisture content of the feedstock. However, different feedstocks have different compactness, depending on the physicochemical properties of substrates used, which will directly influence the effectiveness of the percolate recirculation process. Thus, to enhance the performance of dry AD systems, it is vital to optimize percolate recirculation and select an appropriate recirculation strategy.

Table 2-6. Different strategies of percolate recirculation used for enhanced methane production in dry anaerobic digestion

Type of substrates	Reactor	Model	Conditions	Total solid	Recirculation strategy	PRR [a]	Effectiveness	Remarks	References
Pig slurry + straw	Leach-bed	Batch	Mesophilic	20.7%	1, 2 and 4 times per day; lasting 45 min for one time	0.133-0.533	+ (28%-105%) [b] biogas production	Avoid the accumulation of VFAs; induce the consumption of readily available compounds	(Pezzolla et al., 2017)
Cow manure	Leach-bed	Batch	Mesophilic	22.2%	Once per hour, lasting 20 s for one time	1.558	+ 95% methane production [c]	Inoculated leachates significantly affect the production of biogas	(Degueurce et al., 2016)
Rice straw	Box-type	Batch	Mesophilic	10%–12%	Immersion and recirculation;	0.5-2.0	No significant methane yield improvement	Enhanced VFAs release and methane production rate at the initial stage of AD	(Luo et al., 2021)
Rice straw + pig urine	Leach-bed	Batch	Thermophilic	-	Immersion and percolate recirculation, 1 h per 3 days	-	-	Combination of immersion and leachate recirculation showed a higher methane yield	(Meng et al., 2019)
Solid fraction of dairy manure	Leach-bed	Batch	Thermophilic	Around 26%	15 times per day; lasting 2 min for one time	0.333	Improved stability and speed of the process	-	(Rico et al., 2015)
Food waste	Box-type	Batch	Mesophilic	-	6, 12 times per day [d], lasting 1 min for one time	0.571-1.142	Increased methane production and shortened operation lime	Low PPR must be applied at the start-up of the process	(Rico et al., 2020)
Pennisetum hybrid	Leach-bed	Batch	Mesophilic	20%	Continuous reflux; and	3.6	Methane yield for intermittent reflux was	Shortened lag phase	(Xing et al., 2020)

					intermittent reflux (6 times per day, lasting less than 1 h)		40% more compared with continuous reflux		
Poultry litter	Leach-bed	Batch	Mesophilic	Around 33%	2, 3, 4 times per day; lasting 15 min for one time	-	Increased methane production	-	(Marchioro et al., 2018)
Corn stalks + cow dung	Leach-bed	Batch	Mesophilic	25%	based on equal interval times (2, 4, 6 and 8 h)	-	Increased methane yield	Having significant effects on methanogens and their key enzyme activities	(Yu et al., 2019)

Notes: a. PRR, percolate recirculation ratio, defined as the ratio of the percolate recirculation volume to fermentation volume per day, L/L/d.

b. These data was estimated based on the data present in the reference.

c. Compared with the group with the recirculation of autoclaved leachate.

d. 6 times per day during the start-up (days 1-20) and then increased recirculation frequency to 12 times per day.

2.6 Challenges and perspectives

Over the past few decades, the wet AD process, as a mature biotechnology, has been extensively applied in industrial projects and consolidated in the commercial market. Nevertheless, the by-product of wet AD, referred to as digestate, has posed a major issue (Wang et al., 2021b). Due to its high moisture content, large amounts of digestate are difficult to handle, and can be expensive to transport long distances. As a result, the high operating cost of post-treatment can be an economically negative aspect for wet AD. To address this issue and elevate AD effectiveness, many researchers have explored dry AD processes working with a high TS content in the last two decades. In comparison with wet AD, dry AD offers several additional benefits, including higher volumetric methane production, reduced digester volume and capital investment, improved feedstock flexibility, and reduced heating and mixing energy consumption.

Nevertheless, there are still some technical and economic challenges for the application of the dry AD process. From the technical perspective, one of the biggest challenges is to provide homogenization and good mass transfer in dry AD systems. The yield stress of anaerobically digested solid waste exhibits an exponential increase along with the TS content, which directly restricts the mass transfer in dry AD systems. In this case, the high yield stress not only restricts the availability of nutrients to microbes due to retarded mass transfer, but also makes it difficult to homogenize substrates through mechanical equipment and increases the mixing energy consumption. Hence, more research is required on the rheological behavior and mass transfer evaluation within the digester during the dry AD process as well as on optimizing the TS conditions. Secondly, many dry ADs often encounter the inhibition of ammonia and/or VFA accumulation. The TAN concentration is generally higher in dry AD due to the low water content. Because ammonia-induced inhibition has been extensively studied in wet AD processes, strategies for alleviating ammonia inhibition on dry AD systems can be developed from early developments in wet AD systems in future studies. On the other hand, the ammonia inhibition could be one of reasons for the build-up of VFAs in dry AD systems. The accumulation of VFAs is essentially due to imbalances between hydrolysis/acidogenesis/acetogenesis and methanogenesis, which can also be caused by the improper operational parameters such as temperature, OLRs, C/N ratios, and inoculation.

Therefore, the optimization of operational parameters is critical to prevent the accumulation of VFAs and, consequently, enabling better system performance and stability of the dry AD processes.

From the economic perspective, the dry AD performance in terms of methane production plays a vital role in cost effectiveness. The reduction of VS and methane production are relatively low in dry AD processes due to the technical hurdles (Rocamora et al., 2020; Wang et al., 2021b). The low methane production performance weakens the economic feasibility of the practical application of dry AD. Therefore, the development of improvement strategies for dry AD processes is one of important research topics in the future. In this chapter, a numerous of strategies for enhancing dry AD are summarized from previous studies. Some methods are mainly based on early developments on wet AD systems, in particular substrate pre-treatment processes. However, not all the strategies for methane enhancement are economically feasible. Therefore, it is necessary to perform a techno-economic analysis of methane production performance for those strategies to assess process costs, benefits, and feasibility for their practical implementation.

Currently, most dry AD studies and applications are based on the batch operation, and the studies on continuous/semi-continuous operation of the dry AD process are rarely reported. The engineering projects of dry AD are still lacking reliable practical data that could be used to support the design and build of the continuous processes. Therefore, the future research studies should pay more attention to scaling up lab-scale dry AD systems since environmental factors have potentially affect a commercial scale implementation under full scale conditions.

2.7 Summary

The dry AD process is a promising technology for the treatment and stabilization of organic wastes (e.g., agricultural residues, livestock waste) with high total solid contents (15-45%), while it simultaneously enables energy recovery through biogas production (methane, hydrogen). Dry AD possesses several advantages over wet AD, such as higher volumetric

methane productivity, reduced volume/size of the digester and capital investment, the flexibility of feedstock, decreased energy consumption for heating, less digested residual, and greater ease in dealing with the low moisture digestate. The focus of the present review is critically assessing the previous scientific literatures on dry AD, mainly including specific characteristics of dry AD, operational conditions, and enhanced strategies for dry AD. Additionally, it reveals the challenges of the dry digestion technology associated with mass transfer limitation, intermediate metabolites induced inhibition, limited methane production when compared with wet/liquid AD. This could benefit the development of a suitable and feasible dry AD process.

Chapter 3
Impact of total solids content on anaerobic co-digestion of pig manure and food waste: insights into shifting of the methanogenic pathway

3.1 Introduction

It was estimated that 141,622 breeding pigs were raised in Ireland and almost 3.2 million m^3 of liquid manure (with 4.8% solid content) was generated in 2019 (DAFM, 2019; Nolan et al., 2012). Pig manure (PM) without proper treatment and disposal could give rise to significant sanitation risks and environmental issues (Dennehy et al., 2017a; Dennehy et al., 2017c). Currently, land spreading is the preferred approach for PM management in Ireland (Jiang et al., 2018b; Xie et al., 2011), which leads to significant greenhouse gas (GHG) emissions, especially during PM storage and land application (Dennehy et al., 2017a; Dennehy et al., 2017c). Furthermore, considerable organic compounds in PM, which have great potential as bioenergy sources, are wasted during land spreading. Therefore, land spreading is far from a perfect option for PM management from the perspectives of sustainable development. Anaerobic digestion (AD) can not only degrade and stabilize biodegradable organic fractions of wastes, but also produce renewable energy in the form of biomethane. The application of AD for the treatment of PM has multiple benefits including generation of a gaseous biofuel that primarily contains methane, inactivation of pathogens, reduction of unpleasant odor, and its flexible application in different scales (Wu et al., 2017b). In addition, nutrient-rich digestate after digestion can be utilized as an agricultural fertilizer and thereby partly replace the chemical fertilizers (Dennehy et al., 2016). Hence, AD process could be one of the most eco-friendly, competitive and promising techniques for the management of PM.

However, due to the high nitrogen content of PM, mono-digestion of PM is prone to ammonia inhibition, which results in a long lag phase and low methane yield. Previous studies have demonstrated that total ammonia nitrogen (TAN) concentration of 5000 mg/L could reduce the methanogenic activity by 50% (Lay et al., 1998; Yadvika et al., 2007). Obviously, mono-digestion of ammonia-rich PM is likely subjected to process instability and low methane production, thus not a satisfactory option. Co-digestion with other substrates, turns out to be an economically feasible solution (Capson-Tojo et al., 2017). Food waste (FW), containing high bioavailable organic matters and volatile solids (VS, 80-98%), is an excellent co-substrate for AD of PM (Jiang et al., 2018a). Co-digestion of PM and FW favors the methanogenic processes by providing a more balanced carbon to nitrogen (C/N) ratio of

the feedstock, increasing the buffering capacity of the system, avoiding the accumulation of volatile fatty acids (VFAs) and subsequently maintaining an optimal pH for methanogens (Xie et al., 2011). Distinct synergistic effects on methane generation were observed in wet co-digestion of PM and FW, with the specific methane yield (SMY) increased by more than 20% relative to mono-digestion of PM (Dennehy et al., 2016).

Wet AD systems are usually fed with substrates with a total solid (TS) content below 10%, which means addition of a large amount of water is required in digesters when dealing with high-solid organic wastes. Furthermore, the generation of a large amount of digestate undermines the economic feasibility of AD systems, while being utilized as organic fertilizer. For example, the cost of transporting digestate could account for 30-70% of the total operation cost of AD systems (Dennehy et al., 2017a). Therefore, minimizing digestate generation is an effective approach to reduce the operational costs of AD. In this regard, dry AD, which is characterized by feeding substrates with high TS content (usually ≥15%) (Abbassi-Guendouz et al., 2012), could be a good alternative for the treatment of PM. Moreover, dry AD is advantageous over wet AD in some other respects, such as smaller reactor size, less energy requirement for heating, reduced water usage, and efficient inactivation of pathogens (Arelli et al., 2018; Jiang et al., 2018a; Jiang et al., 2018b).

The TS content of substrate has significant impacts on methane production. Abbassi-Guendouz et al. (2012) reported that the cumulative methane yield was reduced as the TS contents were increased from 10% to 25% in mesophilic digestion of cardboards. They identified mass transfer limitation at high TS contents being responsible for the low methane production. Similarly, Motte et al. (2013) observed a reduced methane production rate (VS-based) at different substrate/inoculum ratios (S/X, 28, 37.5 and 47 $g_{vs\text{-}S}/g_{vs\text{-}X}$) along with the increase of TS from 15% to 25% in AD of wheat straw. On the contrary, some studies have reported that biogas production was improved at higher TS content. Arelli et al. (2018) noticed an improvement of 70-85% in biogas production by increasing the TS content from 25% to 30% during dry anaerobic co-digestion of FW and cattle manure. Duan et al. (2012) demonstrated that a methane yield and VS reduction similar to those of wet AD systems could be obtained in the dry AD of dewatered sludge (TS=20%) under mesophilic conditions. As a result, it is difficult to draw a clear conclusion on the influence of TS content on

performance of the AD systems. Meanwhile, the AD performance in terms of methane production also depends on the substrates themselves which have different biochemical compositions (i.e., carbohydrate, protein, and lipid contents). So far, few studies have been carried out to investigate the effects of TS content on the performance in terms of methane production of anaerobic co-digestion of PM and FW. In addition, insightful analysis of the impacts of operation conditions (like TS content) on the methanogenic pathway is lacking, although lots of studies have investigated the microbial community structure in AD systems via high-throughput DNA sequencing (Arelli et al., 2018; Jiang et al., 2018a).

In this chapter, co-digestion of FW and PM at different TS contents (R_1, TS 5%; R_2, TS 10%; R_3, TS 15%; and R_4, TS 20%) was conducted to investigate (1) the impacts of the TS content on AD performance in terms of methane production; (2) the effects of TS content on the microbial (bacterial and archaeal) community structure and metabolic characteristics, in particular methanogenic pathways.

3.2 Materials and methods

3.2.1 Preparation of substrates and anaerobic inoculum

The PM was collected from manure storage tanks of a local pig farm in Galway, Ireland. It was stored in polyethylene (PE) drums in a cold room at 11 °C, which is the annual average temperature in Ireland, to mimic the practical storage condition in Ireland prior to utilization. The raw PM was centrifuged at 6000 rpm for 5 min (Hettich® ROTOFIX 32A centrifuge, London, UK) to gain the solid fraction (TS≈20%), which was used as the substrate for co-digestion. The fresh FW was obtained from two different cafeterias at the National University of Ireland, Galway. Then it was ground into particles < 2 mm by a food processor (Kenwood FPP210, Havant, UK) and mixed uniformly before use (Jiang et al., 2018b). Before grinding, bones and fruit peels, and non-biodegradable components (like gravel, plastic, etc.) were removed manually from the FW. The seed sludge was dewatered anaerobic sludge taken from a municipal wastewater treatment plant in Galway, Ireland. The sludge was anaerobically stored in a cold room at 11 °C for more than two months in order to deplete organics

available and completely release biogas before use (Dennehy et al., 2016; Jiang et al., 2018b). The main features of the prepared PM, FW and seed sludge are outlined in Table 3-1.

Table 3-1. Physiochemical features of pig manure, food waste and seed sludge *

No.	Parameters	Unit	Solid fraction of pig manure	Food waste	Seed sludge
1	pH	/	7.57±0.03	4.93±0.02	7.77±0.03
2	Moisture content	%	77.1±0.01	59.5±0.38	80.0±0.05
3	TS	%	22.90±0.01	40.52±0.38	20.02±0.05
4	VS	%	17.93±0.01	39.96±0.30	13.76±0.08
5	VS/TS	%	78.4	96.2	68.7
6	SCOD	g/L	40.9	126.8	7.1
7	TCOD	g/L	197.6	271.4	190.1
8	TVFAs	g HAc/L	24.04	8.79	0
9	FVFAs calculated	g HAc/L	0.80	0.02	0
10	TAN	mg N/L	4 156.3	240.2	1 793.3
11	FAN calculated	mg N/L	85.76	0.01	57.94

* TS: total solid; VS: volatile solid; SCOD/TCOD: soluble/total chemical oxygen demand; TVFAs/FVFAs: total/free volatile fatty acids; TAN/FAN: total/free ammonia nitrogen.

3.2.2 Experimental setup

The batch assays were conducted using 2-L glass digesters sealed with rubber stoppers. Two small holes were made in each rubber stopper: one was used for connecting a gas bag to collect biogas, while the other was used for installing a direct-reading thermometer. In addition, each reactor had a sampling port near the bottom.

According to the previous studies by Dennehy et al. (2016) on wet digestion and by Jiang et al. (2018a) on dry digestion, the blending ratio between PM and FW used in this study was 1:1 on VS basis and the S/X ratio was also 1:1 (VS basis). The mixture was diluted with tap water to obtain the target TS contents (R_1, TS 5%; R_2, TS 10%; R_3, TS 15%; and R_4, TS 20%) and then fed into the digesters after fully mixing. Each TS condition was conducted in triplicate. Before commencement, all reactors were purged with nitrogen gas for around 5 min to get rid of air from the headspace and then sealed tightly with rubber stoppers to

maintain anaerobic condition. These lab-scale digesters were subsequently placed into a lab incubator with a constant temperature of 37.0 ± 1.0 ℃. The digesters were shaken manually once every day.

3.2.3 Analytical methods

Biogas generated from each reactor was collected by using a biogas bag (Dalian Delin Gas Packing Co., Ltd, China). The biogas volume was measured with a volumetric flow meter (FMA-1620A-TOT, Omega, Deckenpfronn, Germany) and then converted to the volume under standard temperature and pressure (STP, 0 ℃ and 101kPa). The methane (CH_4) content in the biogas was determined by a gas chromatography (GC 7890 A, Agilent Technology, USA) equipped with a thermal conductivity detector and a stainless-steel column (13803-U, Sigma–Aldrich, USA). Argon gas was the carrier gas at a flow rate of 24 mL/min. The temperatures of the injector inlet and the detector were maintained at 90 ℃ and 200 ℃, respectively.

Digestate was regularly taken from each reactor to measure pH, soluble chemical oxygen demand (SCOD), TAN and VFA concentrations. According to Jiang et al. (2018b), to facilitate the measurements, 1 part of the digestate sample was diluted with 4 to 19 parts of deionized water (to obtain 5 to 20-fold dilutions, w/w) according to different parameter concentrations. The pH readings were taken from the diluted samples directly via a portable pH meter (pH3210, Germany). For all other parameters, the diluted samples were centrifuged at 13,500 rpm for 5 min (Sigma 2-16P, Germany) and the supernatants were further filtered using a syringe microfilter (<0.45 μm, Sarstedt Ltd., Germany) before analysis. VFAs were analyzed by a high-performance liquid chromatography (Agilent 1200, USA) (Xie et al., 2011). All the analyzed VFAs (propionic, iso-butyric, n-butyric, iso-valeric and n-valeric acids) were converted to acetic acid equivalents. TAN was analyzed using a Konelab nutrient analyzer (Thermo Clinical Labsystems, Vantaa, Finland). TS, VS, and COD were measured with the standard methods (APHA, 2012). To obtain a relatively precise SMY, the VS amount that had been previously taken out of the digesters due to periodic sampling was deducted from the total VS in calculation.

To investigate the shifting of the microbial community structures under different TS contents, 2 g mixture was withdrawn from each digester at different incubation time (0 d, 14 d, 32 d and 46 d for TS of 5%; 0 d, 14 d, 46 d, 74 d and 120 d for TS of 20%). According to the data of methane production, there was no significant difference among digesters under TS of 5%, 10% and 15%, while the 20% TS resulted in a significantly low SMY, so the TS contents of 5% and 20% were selected to represent wet and dry AD systems, respectively. The time for biomass sampling was determined in accordance with key anaerobic digestion phases: at the beginning, active methane production, slowed methane production, and at the end. Then DNA extraction and microbial community analysis were carried out. The DNA was extracted from the solid phase of each digestate sample using the PowerSoil DNA Extraction Kit (Laboratories Inc., CA, USA) according to the manufacturer's instructions. For each sample, two independent PCR reactions were conducted to amplify the extracted DNA in the V3-V4 region of bacterial 16S rRNA gene, using primers of 341 F (CCTACGGGNGGCWGCAG) and 805 R (GACTACHVGGGTATCTAATCC) for bacteria, and 340 F (CCCTAYGGGGYGCASCAG) and 1000 R (GGCCATGCACYWCYTCTC) for archaea (349 F: GYGCASCAGKCGMGAAW; 806 R: GGACTACVSGGGTATCTAAT). The sequences obtained were determined using the Illumina MiSeq platform. The Usearch (version 5.2.236) software was employed to cluster operational taxonomic unit (OTUs) according to the method by Yin et al. (2018). The clustered OTUs were annotated down to the different levels (phylum, class, order, family, and genus) on the basis of the RDP database.

3.2.4 Data analysis

The free ammonia nitrogen (FAN) concentrations were calculated according to Eq. (3-1) (Jiang et al., 2018b).

$$\frac{TAN}{FAN} = \left(1 + \frac{10^{-p\mathrm{H}}}{10^{-\left(0.09018 + \frac{2729.92}{T}\right)}}\right) \quad (3\text{-}1)$$

where, *FAN* and *TAN* are the free (NH_3) and total ($NH_3 + NH_4^+$) ammonia concentrations, respectively, mg/L; and *T* is the temperature in Kelvin, K.

Kinetic modelling is widely used in predicting methane yields, establishing key parameters for reactor design, and optimizing the performance of AD process. To describe the AD kinetics, two classics models, the first order and modified Gompertz models, and a new superimposed model, were employed in this study.

The first-order model (Eq. (3-2)) is established with the assumption that hydrolysis is the rate-limiting step in AD and is broadly applied to analyze the cumulative methane production of various substrates in AD systems (Dennehy et al., 2016).

$$P_{CH_4}(t) = P_{max}[1 - exp(-k \cdot t)] \quad (3\text{-}2)$$

where $P_{CH_4}(t)$ is the cumulative SMY at a certain incubation time (t), mL/gVS$_{added}$; P_{max} is the methane production potential (maximal SMY), mL/gVS$_{added}$; k is the hydrolysis rate constant, d^{-1}; and t is the incubation time, d.

The modified Gompertz model (Eq. (3-3)), initially developed by Zwietering et al. (1990) to predict bacterial growth, is another typical model to simulate the methane production. This model is based on the assumption that the methane production rate corresponds to the methanogenic bacterial growth rates (Syaichurrozi & Sumardiono, 2014).

$$P_{CH_4}(t) = P_{max} \cdot exp\left\{-exp\left[\frac{R_m \cdot e}{P_{max}} \cdot (\lambda - t) + 1\right]\right\} \quad (3\text{-}3)$$

where R_m is the maximum specific methane production rate, mL/(gVS$_{added}$·d); λ is the lag phase duration, days; and e is a mathematical constant, 2.71828.

In this study, the cumulative methane production curves at high TS contents (10-20%) exhibited a stepped shape with two daily methane production peaks. The first peak was possibly related to rapid utilization of the readily biodegradable organic compounds by microbes, and the second peak was likely linked to the subsequent degradation of poorly biodegradable organic compounds such as protein, fat and fiber (Wu et al., 2017b; Zhang et

al., 2014). Taking the degradation of these two groups of organic compounds into account, a new superimposed model (Eq. (3-4)) was developed in this study by coupling the first-order kinetic model with modified Gompertz model.

$$P_{CH_4}(t) = P_{1max}[1 - \exp(-k \cdot t)] + P_{2max} \cdot exp\left\{-exp\left[\frac{R_m \cdot e}{P_{2max}} \cdot (\lambda - t) + 1\right]\right\} \quad (3\text{-}4)$$

where P_{1max} is the methane production potential (maximal SMY) from the readily biodegradable organic matters, mL/gVS$_{added}$; P_{2max} is the methane production potential (maximal SMY) from the poorly biodegradable organic matters, mL/gVS$_{added}$.

The models were fitted to the experimental data using non-linear regression. All the data analyses were computed using Excel 2016 and Origin pro 2021.

3.3 Results and discussion

3.3.1 Ammonia and VFA profiles at high and low TS contents

In practice, AD often encounters process instability issues, such as acidification, declined methane production rate, etc. (Westerholm et al., 2015). Usually, pH, ammonia and VFAs are considered as significant and accessible criterions to assess AD process stability.

Acidification is a common issue in high-solid AD, especially in mono-digestion digestion (Wang et al., 2017b). In the present study, the pH profiles at different TS contents showed an identical tendency that decreased in the first several days, then increased gradually until reached constant values (Figure A-1). During Day11-25, pH in R_4 at TS of 20% experienced a lower value of around 7.5 while the pH of the other three digesters rose to around 8.0. The lower pH in R_4 during the lag phase was most likely attributed to the accumulation of VFAs, although it was partly counteracted by the increase of ammonia concentration (Wang et al.,

2016a; Yi et al., 2014). At the stable period, the pH values showed an increasing tendency with the increase of TS contents and reached 8.01, 8.32, 8.43 and 8.53 in R_1, R_2, R_3, and R_4, respectively, at the end of digestion. Herein, all the pH values in different digesters were within the acceptable range of 6.5-8.5 for AD (Yi et al., 2014). The high buffering capacity of PM effectively avoided the acidification problem at high TS contents, thus offering adaptable surroundings for all kinds of functional microorganisms.

It has been widely reported that high ammonia concentration has negative impacts on the performance of AD process, especially free ammonia (Karthikeyan & Visvanathan, 2013). In this study, higher TAN concentrations were detected in the digesters operated at higher TS contents (Fig. 3-1a), with the average TAN concentrations of 580.3-1093.9, 1201.5-2582.7, 1819.0-3862.4 and 2367.7-5293.4 mg N/L for R_1, R_2, R_3, and R_4 respectively. The maximum FAN concentrations were 115.0±8.2, 499.4±10.6, 1025.1±31.3 and 1585.2 ± 74.0 mg-N/L in R_1, R_2, R_3, and R_4 respectively. Former studies have demonstrated that FAN at concentrations above 150 - 1200 mg-N/L can exert inhibitory impacts on AD systems (Meng et al., 2018b; Wu et al., 2009). Hence, FAN inhibition may occur in the digesters with TS contents above 10% (R_2-R_4).

VFAs are the main metabolic intermediates in anaerobic digestion (Riggio et al., 2017). The accumulation of VFAs, derived from imbalanced rates between hydrolysis/acidogenesis/ acedogenesis and methanogenesis, is one of the crucial issues resulting in AD instability or even failure, especially under high organic/solid loading rates. As shown in Fig.1, two TVFA peaks were observed in R_2, R_3 and R_4, while only one peak occurred in R_1. The first TVFA peak occurred at the initial period (Day 0-5) in all the digesters, accompanied by the minimal pH value and a daily SMY peak. In R_1 at TS of 5%, the TVFAs gradually decreased after reaching the maximum value on Day 2, which was consistent with the trend of SCOD (Figure A-2). This indicates that all the AD processes functioned well under this condition. On the contrary, TVFAs gradually accumulated in R_2, R_3 and R_4 during the periods of day 10-24, day11-28, and day 7-28, respectively. For R_2, due to the relatively low concentration, such a TVFA accumulation did not negatively affect methanogenesis. However, the performances of R_3 and R_4 were more or less affected, especially for R_4 where there was almost no methane production during this period.

Regarding specific VAFs, the accumulation of propionic and acetic acids was more persistent than the others (Figure 3-1), especially in R_4 at the highest TS of 20% (Figure 3-1f). After the start-up, propionic acid gradually accumulated to a high level in all the digesters. Only after most of the other VFAs had been depleted, the propionic acid began to reduce. The standard free energy change for anaerobic degradation of propionic acid ($CH_3CH_2COO^- + 3H_2O \rightarrow HCO_3^- + CH_3COO^- + 3H_2 + H^+$, $\triangle G_0' = +76.1$ kJ/mol) is the highest compared with the other VFAs (Aymerich et al., 2013). Therefore, persistent accumulation of propionic acid is not uncommon in AD. Surprisingly, substantial accumulation of acetic acid was also observed for a long period in the digesters with high TS contents (e.g., R_2, day 10-28; R_3, day 11-25; R_4, day 7-28) (Figure 3-1[d-f]). The higher the TS content was, the more acetic acid accumulated. Especially in R_4, there was still considerable acetic acid remaining at the end of the experiment, along with propionic acid. Acetic acid is readily usable substrate for acetoclastic methanogens. The substantial accumulation of acetic acid suggests that its metabolism by acetoclastic methanogens was not satisfactory, especially in R_4 at TS of 20%, where the activity of acetoclastic methanogens was found to almost disappear during day 7 to 25, as indicated by the negligible methane production under high levels of acetic acid. This phenomenon may be explained with two factors: (1) at high solid contents, the diffusion of the intermediate products (VFAs) was slowed down due to high mass transfer resistant, thus they were not easily accessible to the anaerobic microbes; and (2) the acetoclastic methanogens were inhibited or outcompeted under high solids content, which will be further discussed in section 3.3.4.

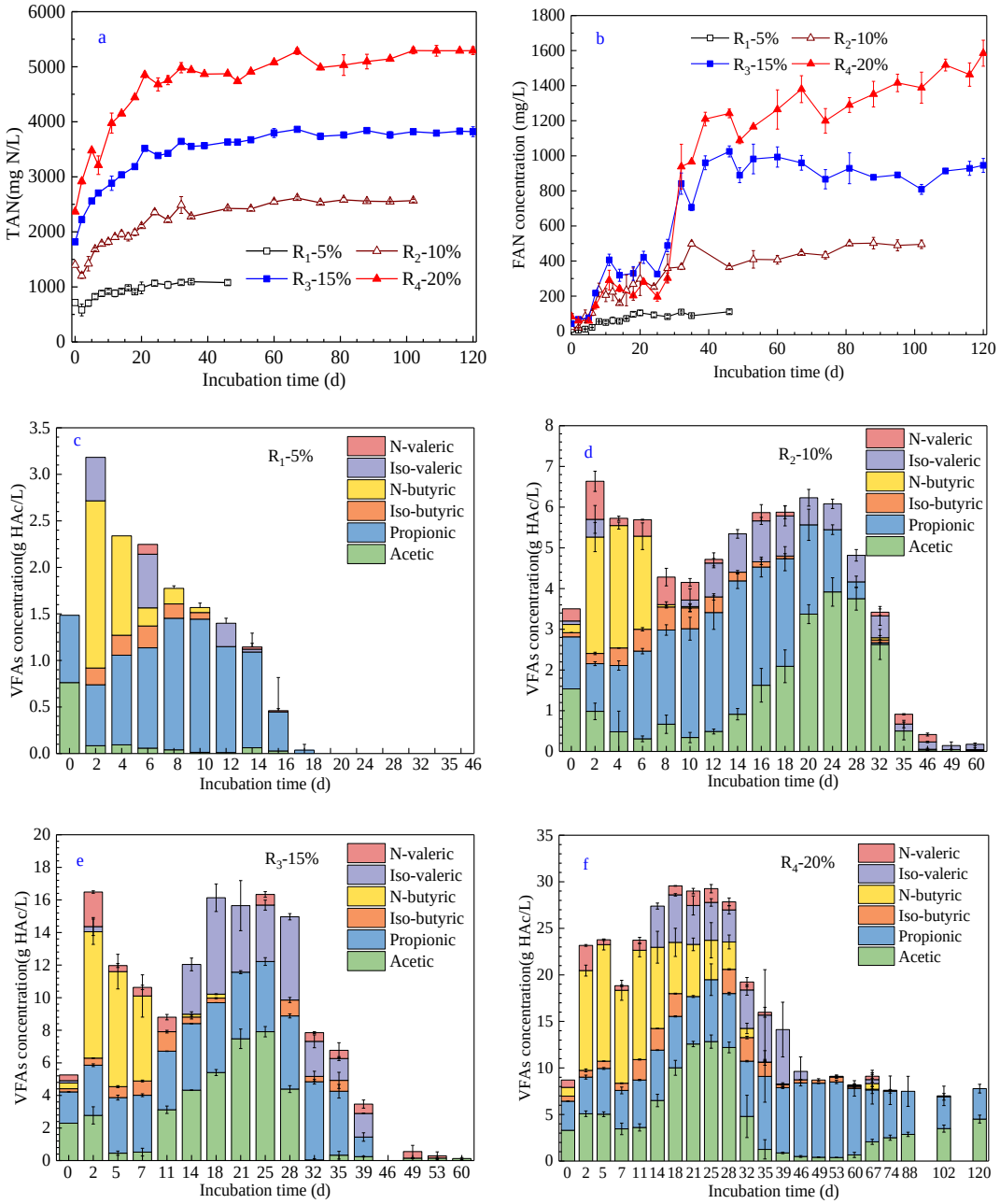


Figure 3-1. Evolution of ammonia and VFAs in digesters operated at different initial TS contents. (a) TAN; (b) FAN; (c)-(f) VFAs. T/FAN: total/free ammonia nitrogen; VFAs: volatile fatty acids.

3.3.2 Methane production

The anaerobic digesters were operated for 120 days, until methane production was negligible. The methane production under different initial TS contents is shown in Figure 3-2. Overall, all the digesters produced methane and no acidification or failure was observed.

As shown in the daily SMY results (Figure 3-2a), two methane production peaks were observed in R_2-R_4 at the TS of 10%-20%. There was a fluctuation of daily methane production at TS 5% indicating two production peaks (Dennehy et al., 2016), but the time gap between the two peaks was not as obvious as at other three TS contents. The first peak was possibly related to rapid utilization of the readily biodegradable organic compounds by microbes (Zhang et al., 2014), and the second peak was likely linked to the subsequent degradation of the poorly biodegradable organic compounds such as protein, fat and fiber (Wu et al., 2017b; Zhang et al., 2014). Therefore, the methane production started immediately after the start-up, then gradually declined, and maintained at a low production rate for a period of time, until it increased again towards the second peak. Finally, it decreased again along with the depletion of VFAs in the digesters. Specifically, with the highest TS content (20%), R_4 experienced a long period (day 7-25) of low methane production after the first peak, while this period was much shorter in the digesters (R_2 and R_3) with lower TS contents (10% and 15%).

The average cumulative SMYs under TS of 5%, 10%, 15%, and 20% at the end of experiments were 278.8±3.9, 291.7±2.3, 289.5±4.7 and 259.8±6.0 NmL/g VS_{added}, corresponding to the VS reductions of 78.6%, 79.5%, 77.1% and 71.7% respectively. It turned out that the cumulative SMY did not change significantly with the increase of TS contents from 5% to 15% ($p > 0.05$), while the 20% TS resulted in a significantly low SMY ($p < 0.05$). The results suggest that below 15% the increase of TS content had no negative impact on CH_4 production in anaerobic co-digestion of PM and FW. Conversely, initial TS content above 20% impacted the substrate utilization of microbes, thereby resulting in decreased methane production, which was in line with the VS reduction in the reactor (Table 3-2). The reasons for the reductions at 20% TS content were likely related to mass transfer, microbial activities and the structures of the microbial community present in digesters (section 3.3.4). Similar results were also observed in dry digestion of wheat straw (TS from 15% to 25%) in a previous study (Motte et al., 2013). Moreover, 95% of total methane yields

in R_1, R_2, R_3 and R_4 were obtained in 24, 43, 49 and 67 days, respectively. This indicates that a long retention time is required in high-solid AD.

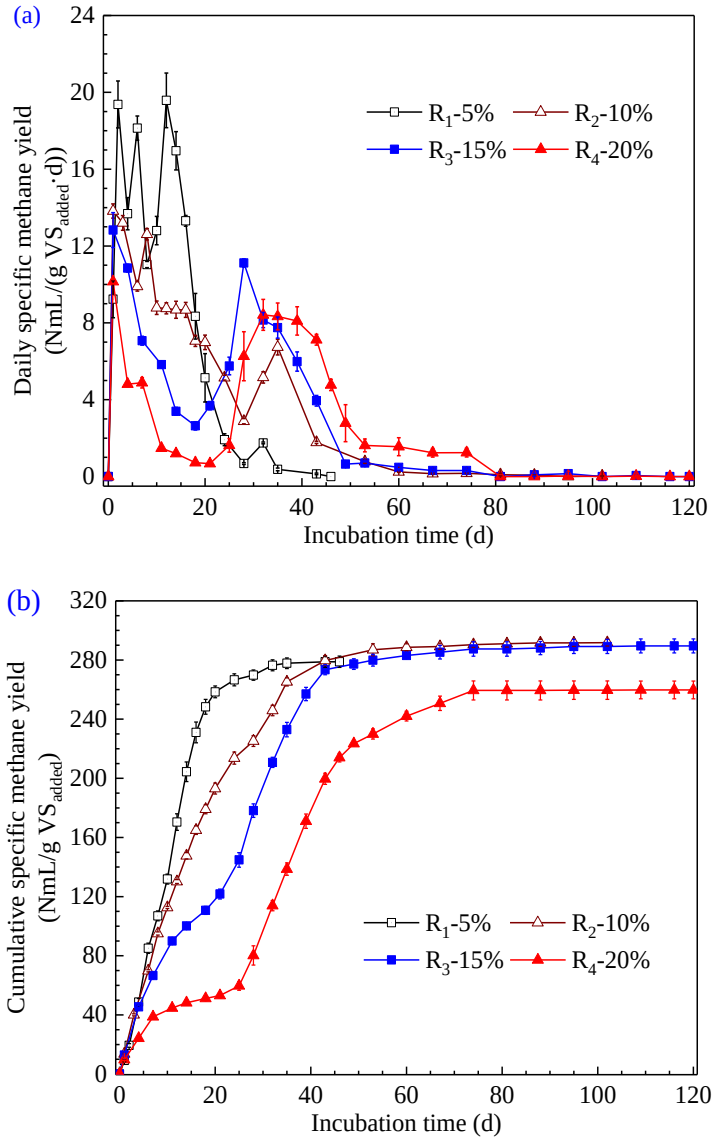


Figure 3-2. Methane productions under different TS contents: (a) Daily specific methane yield (on a daily average basis); (b) cumulative specific methane yield.

Table 3-2. Overall performance of the co-digestion systems at various total solid contents [a]

Parameters	R_1 (5% TS_{in})	R_2 (10% TS_{in})	R_3 (15% TS_{in})	R_4 (20% TS_{in})
Lowest pH observed	7.12 ± 0.10	7.14 ± 0.09	7.36 ± 0.01	7.23 ± 0.05
Final pH	8.01 ± 0.03	8.32 ± 0.03	8.43 ± 0.03	8.53 ± 0.02
TS reduction (%)	48.4 ± 1.8	33.3 ± 2.4	34.6 ± 3.2	35.4 ± 3.7

VS reduction (%)	78.6 ± 5.1	79.5 ± 2.6	77.1 ± 4.7	71.8 ± 5.1
Cumulative SMY (mL/g VS_{added})	278.8 ± 3.9	291.7 ± 2.3	289.5 ± 4.7	259.8 ± 6.0
Maximum CH_4 content (%)	72.7 ± 0.4	75.7 ± 0.3	74.9 ± 1.4	74.4 ± 0.6
$SCOD_{max}$ (g/L) [b]	7.78 ± 0.10	14.83 ± 0.54	25.66 ± 0.12	43.94 ± 0.89
$TVFA_{max}$ (g HAc/L)	3.18 ± 0.12	6.63 ± 0.49	16.48 ±0.89	29.55 ± 0.85
$TVFA_{max}$ (mg HAc/g VS_{added}) [c]	166.3 ± 0.0	173.3 ± 12.7	284.5 ±5.5	386.1 ± 11.1
TAN_{max} (mg N/L)	1093.9 ± 20.6	2582.7 ± 37.6	3862.4 ±26.1	5293.4 ±58.8
FAN_{max} (mg N/L)	115.0 ± 8.2	499.4 ± 10.6	1025.1 ±31.3	1585.2 ± 74.0

[a] Mean value ± standard deviation; TS: total solid; VS: volatile solid; SMY: specific methane yield; SCOD: soluble chemical oxygen demand; TVFA: total volatile fatty acid; TAN/FAN: total/free ammonia nitrogen.

[b] Detailed data was shown in Figure A-2.

[c] Detailed data was shown in Figure A-3.

3.3.3 Kinetic modelling of cumulative methane production

In this study, the first-order kinetic model and modified Gompertz model were first employed to simulate methane productions in different digesters (Table 3-3 and Table A-1). The results indicated that the modified Gompertz model fitted the AD systems operated at TS of 5% and 10% well with R^2 of 0.995 and 0.994, respectively, which are conventionally regarded as wet AD systems. However, neither the first order nor the modified Gompertz model described the two-peak digestion behavior at TS of 15% and 20%. Therefore, the superimposed model (Eq. (3-4)) was developed to simulate the methane production of digesters at high TS contents which commonly showed a two-peak digestion behavior.

Table 3-3. Kinetic parameters of the superimposed model *

Parameters	$P_{1\ max}$	k	$P_{2\ max}$	R_m	λ	Adj. R-Square
	mL CH_4/g VS	d^{-1}	mL CH_4/g VS	mL CH_4/g VS/d	d	
R_2(10% TS)	182.38	0.080	110.21	3.61	6.85	0.9989
R_3(15% TS)	128.67	0.107	159.11	8.93	22.10	0.9996
R_4(20% TS)	50.17	0.194	208.70	8.56	24.74	0.9993

* TS: total solid; VS: volatile solid.

The newly developed superimposed model (Eq. (3-4)) was used to fit the measured data of cumulative methane production of R_2, R_3 and R_4 and the relevant results are shown in Figure

3-3 and Table 3-3. The modelling results perfectly matched the measured results of these three AD systems (R^2 greater than 0.999). P_{1max} and k were linked to the degradation of the readily biodegradable organic compounds. It is worth nothing that the P_{1max} was decreased with the increase of TS content from 182.38 to 50.17 mL CH_4/g VS. This indicates that the utilization of readily biodegradable organic compounds by anaerobic microbes was adversely affected by the elevated TS content. The reason might be that mass transfer limitation occurring at high TS contents could reduce the availability of organic matters to microbes, which led to declined methane production (Abbassi-Guendouz et al., 2012). Interestingly, the hydrolysis rate constant k increased with the increase of TS content. It means that AD systems operated at higher TS contents had better hydrolysis rate of readily biodegradable organic compounds. However, no higher methane production rates were observed under higher hydrolysis rates, which could be attributed to inhibition of the methanogens' activities by some inhibitory factors such as high ammonia and VFAs concentrations (see section 3.3.1). P_{2max}, R_m and λ were related to the degradation of poorly biodegradable organic matters. In contrast with P_{1max}, P_{2max} increased with the increase of TS content from 110.2 to 208.7 mL CH_4/g VS. In addition, the lag phase showed an increasing trend with the increase of TS content, with the λ value of 6.85 d, 22.10 d and 24.74 d, respectively.

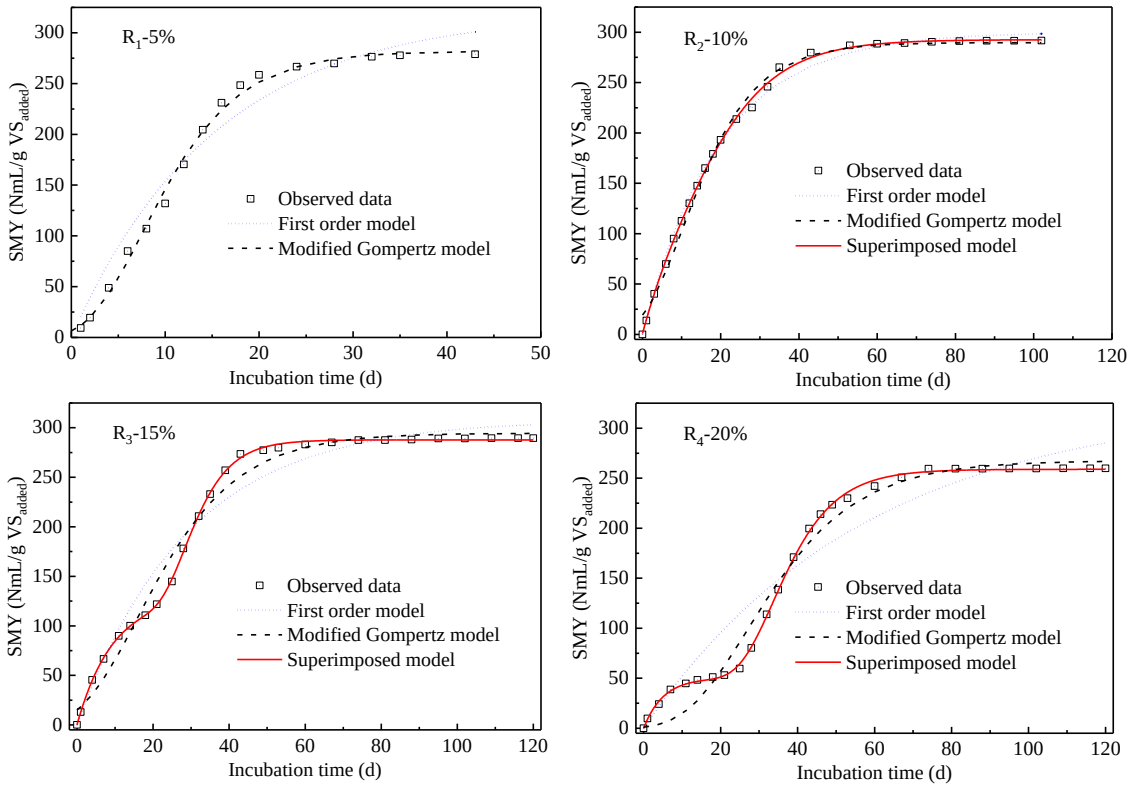


Figure 3-3. Cumulative methane productions in different digesters fitted with different models. SMY: specific methane yield; VS: volatile solid.

3.3.4 Microbial community analysis

3.3.4.1 Characteristics of bacterial community in AD systems

The bacteria detected in all the samples were mainly assigned into 12 phyla. Figure 3-4a illustrates the bacterial community structure at the phylum level. It should be noted that only the phylum with a relative abundance higher than 1% in at least one sample is shown in the figure. Overall, four phyla *Firmicutes*, *Proteobacteria* and *Chloroflexi* dominated. Within the mixture (undiluted mixture of substrates and inoculum), the relative abundances of *Firmicutes*, *Proteobacteria* and *Chloroflexi* were 33.9%, 19.5%, and 11.4%, respectively. The phylum-level relative abundance in the mixture, wet digesters (R_1) and dry digesters (R_4) varied considerably. Phyla *Firmicutes* (with relative abundance of 43.9-49.1%) and *Proteobacteria* (18.6-39.1%), dominated in the dry digesters, while *Chloroflexi* (29.8-38.8%),

Firmicutes (20.1-23.0%) and *Proteobacteria* (15.8-21.2%) dominated in the wet digesters. The relative abundances of phyla *Firmicutes* and *Proteobacteria* considerably rose in dry digesters relative to those in wet digesters, while phylum *Chloroflexi* declined partly. In wet digesters, the relative abundance of *Chloroflexi* rose significantly.

The taxonomic compositions of bacteria at the genus level are shown in Figure 3-4b. Overall, the taxonomic composition in the mixture, wet and dry digesters was distinctly different between each other. The results indicate that TS content had a significant impact on the bacterial community structure in AD.

The bacterial composition in the dry digesters varied significantly over the incubation time (Figure 3-4b). During the period of VFAs accumulation (D14, Figure 3-4b), unclassified genus in family *Ruminococcaceae* (31.7%) dominated, followed by *Clostridium sensu stricto* (7.2%). Members of family *Ruminococcaceae* are well known to be able to hydrolyze various polysaccharides and represented by cellulolytic bacteria (Poirier et al., 2016), which could persist in fibrinolytic communities and decompose a variety of recalcitrant substrates (Biddle et al., 2013). Its dominance in the dry digesters and very low relative abundance (<1%) in the wet digesters indicated that *Ruminococcaceae* was more adaptable to dry AD and played a significant role in it. Members in *Clostridium sensu stricto* can syntrophically produce methanogenesis precursors such as acetate, butyrate, H_2 and CO_2 by utilizing various sugars as carbon and energy sources (Kaur et al., 2014). During the active methane production period (D46, Figure 3-4b), members of family *Ruminococcaceae* (14.4%) and *Clostridium sensu stricto* (13.6%) were dominant in digesters. The relative abundance of *Clostridium sensu stricto*, *Syntrophomonas* and *Terrisporobacter* rose notably in comparison to those on day 14. *Syntrophomonas* is a metabolic specialist that can syntrophically metabolize short chain fatty acids of four to eight carbon atoms to acetate using the beta-oxidation pathway (Crable et al., 2016; Sieber et al., 2010; Sousa et al., 2007), which might play a key role in the complete degradation of butyric acids in dry digesters. Consequently, it is inferred that *Clostridium sensu stricto, Syntrophomonas* and *Terrisporobacter* may promote methane production in dry AD by enhancing syntrophic degradation of the organic acids.

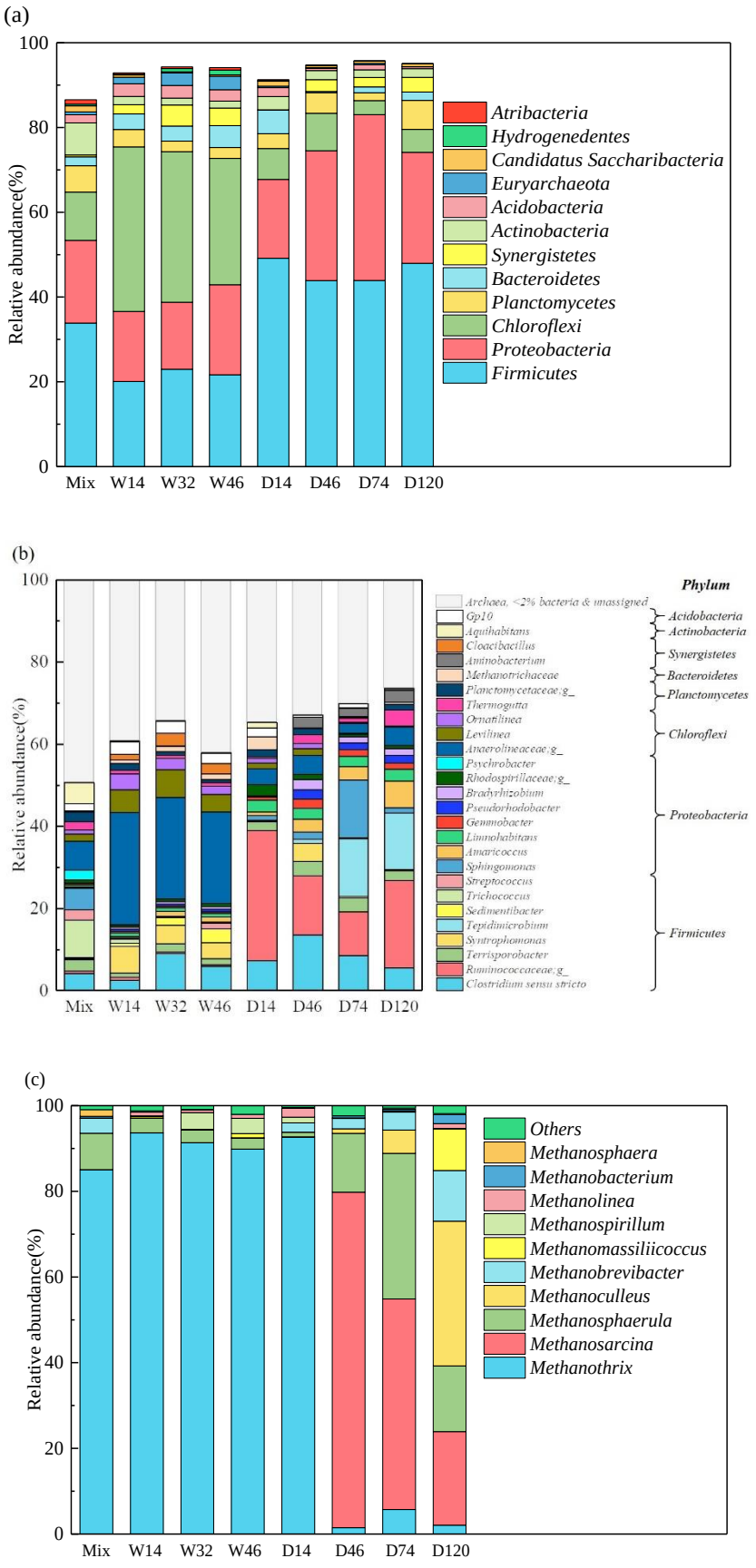

(a)
Relative abundance(%)
100
80
60
40
20
0
Mix W14 W32 W46 D14 D46 D74 D120
Atribacteria
Hydrogenedentes
Candidatus Saccharibacteria
Euryarchaeota
Acidobacteria
Actinobacteria
Synergistetes
Bacteroidetes
Planctomycetes
Chloroflexi
Proteobacteria
Firmicutes
(b)
Relative abundance(%)
Phylum
Archaea, <2% bacteria & unassigned
Gp10
Aquihabitans
Cloacibacillus
Aminobacterium
Methanotrichaceae
Planctomycetaceae;g_
Thermogutta
Ornatilinea
Levilinea
Anaerolineaceae;g_
Psychrobacter
Rhodospirillaceae;g_
Bradyrhizobium
Pseudorhodobacter
Gemmobacter
Limnohabitans
Amaricoccus
Sphingomonas
Streptococcus
Trichococcus
Sedimentibacter
Tepidimicrobium
Syntrophomonas
Terrisporobacter
Ruminococcaceae;g_
Clostridium sensu stricto
Acidobacteria
Actinobacteria
Synergistetes
Bacteroidetes
Planctomycetes
Chloroflexi
Proteobacteria
Firmicutes
(c)
Relative abundance(%)
Others
Methanosphaera
Methanobacterium
Methanolinea
Methanospirillum
Methanomassiliicoccus
Methanobrevibacter
Methanoculleus
Methanosphaerula
Methanosarcina
Methanothrix

Figure 3-4. Microbial community structure in wet and dry digesters: (a) bacteria at the phylum level, (b) bacteria at the genus level, and (c) archaea at genus level. Mix refers to mixture of substrates and inoculum before anaerobic digestion; W/D refers to samples from wet digesters (TS=5%) and dry digesters (TS=20%); numbers of 14, 32, 46, 74, and 120 refer to incubation time, day.

3.3.4.2 Characteristics of archaeal community in AD systems

As shown in Figure 3-1, high VFA and ammonia concentrations occurred in the dry AD systems, which inevitably hindered the methanogenic process. The revival of methanogenesis implied an enhanced resistance capacity of the functional groups to the unfavourable environment in dry AD. Figure 3-4c shows the shifting of the archaeal compositions over time in the wet and dry digesters. For the wet digesters, *Methanothrix* (89.8-93.6%) overwhelmingly dominated in methanogens. Compositions of methanogens in wet digesters and the mixture were almost identical, indicating that wet digestion did not give rise to unadaptable or suppressed impacts on methanogens.

However, the methanogenic community of the dry digesters varied greatly over time. At the beginning (Day 14), the composition of methanogens was extremely similar to that in the wet digesters as well as the mixture, with *Methanothrix* (92.7%) as the predominant methanogens. After the period of VFAs accumulation, *Methanothrix* diminished to 1.5-5.7% and the archaeal community changed to a new structure with the dominance of *Methanosarcina* (21.8-78.3%), *Methanosphaerula* (13.7-34.0%), *Methanoculleus* (1.1-33.8%) and *Methanobrevibacter* (2.4-11.8%). The high concentration of metabolic intermediates such as VFAs and ammonia, might be severely adverse to the survival of *Methanothrix*, causing its diminishment in dry AD. In contrast, *Methanosarcina*, which is versatile and can utilizes acetate, H_2/CO_2, methanol as well as methylamine for growth and methane production (Demirel & Scherer, 2008; Guo et al., 2015; Karakashev et al., 2005), was adapted to the severe conditions in dry AD. Moreover, *Methanosarcina* have high growth rates and thick cell clumps, which protect them from inhibitors, thereby enhancing their resistance to stressed conditions (Demirel & Scherer, 2008; Guo et al., 2015; Karakashev et al., 2005). Both *Methanosphaerula* and *Methanoculleus* are capable of utilizing H_2 and CO_2

as the substrates for methane production. High tolerance of *Methanoculleus* to inhibitors like ammonium and VFAs has also been observed (Schnürer et al., 1999).

According to the substrates they can use, methanogens are generally divided into acetoclastic, hydrogenotrophic and methylotrophic methanogens, corresponding to three types of methanogenic pathways. The shifting of methanogenic pathways in the wet and dry AD digesters is shown in Figure 3-5. In this study, *Methanosarcina* is treated as a separate category because it is quite versatile to utilize different substrates including acetate, H_2/CO_2, methanol as well as methylamine. As shown in Figure 3-5, acetoclastic methanogenesis was always overwhelmingly predominate in the wet digesters, up to 89.9-93.7%. However, an obvious shifting of methanogenic pathways was observed in the dry digesters. In detail, the proportion of hydrogenotrophic communities increased sharply (from 1.1% to 64.3%), while the acetoclastic methanogens (*Methanothrix*) quickly declined from 92.7% on Day 14 to 1.5% on Day 46 during the active methane production period. Clearly, hydrogenotrophic pathway dominated at the end of the dry digestion. These results demonstrate that hydrogenotrophic methanogens were more competitive than acetoclastic methanogens under high solids conditions. Acetoclastic methanogens are reported to be easily restrained by high VFA or ammonia concentrations (Zheng & Raskin, 2000), while hydrogenotrophic methanogens are more resistant. The diminishment of acetoclastic methanogens can be well explained by the persistent acetic acid accumulation and high TAN concentrations in dry AD (Figure 3-1). Without acetoclastic methanogens, acetic acid can only be degraded by syntrophic acetate oxidation bacteria (SAOB) to generate H_2 and CO_2, which can be further utilized in hydrogenotrophic methanogenesis (Wu et al., 2017a). However, the degradation of acetic acids by SAOB is thermodynamically unfavorable ($CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 4H_2 + H^+$, $\triangle G^{0'} = +104.6$ kJ/mol) (Zinder & Koch, 1984), thus resulting in high levels of acetic acid accumulation. Additionally, the mixotrophic methanogens, *Methanosarcina,* played an important role during the transition period, whose proportion increased from 0.02% on Day 14 to 78.3% on Day 46 and then decreased to 21.8% at the end of digestion. As shown in Fig. 1, most methane in R_4 was generated between Day 21-53, accounting for 68% of the total methane production (Figure A-4). The dominance of *Methanosarcina* during this period indicates its significant role in dry AD systems.

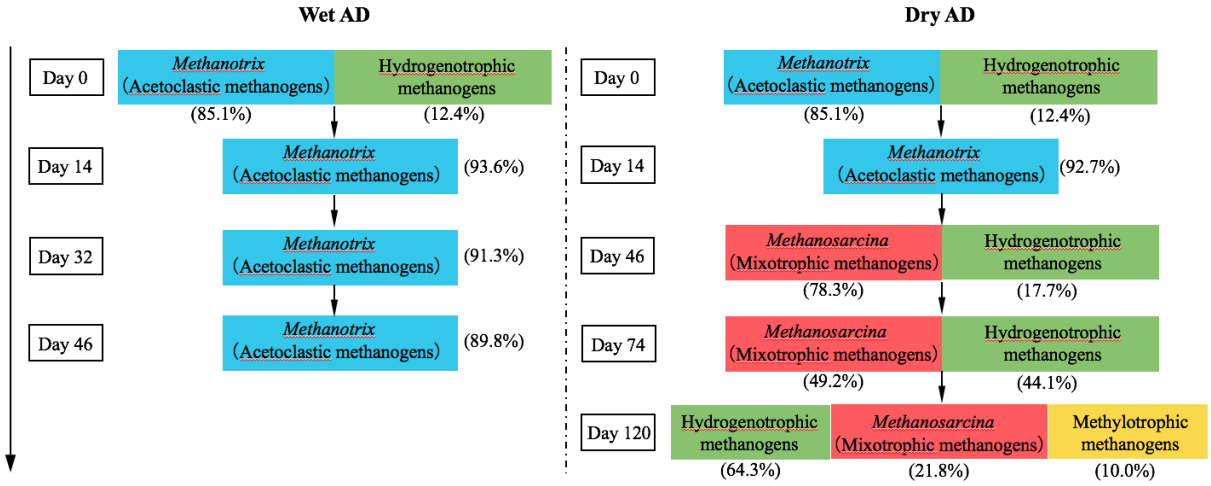


Figure 3-5. Shifting of methanogenic pathways in wet and dry AD digesters (TS-5% and TS-20%). Wet/Dry AD refers to samples from wet digesters (TS=5%) and dry digesters (TS=20%).

In summary, the composition of methanogens in dry digesters highly differed from that in wet digesters, and methanogenic pathways in dry digesters varied greatly over time. Hydrogenotrophic methanogenesis increased along with the incubation time in dry digesters and dominated at the end of digestion. Meanwhile, mixotrophic methanogens of *Methanosarcina* accounted for a large proportion and played a significant role during the transition period in dry AD systems. Hence, it was concluded that the dominance of mixotrophic and hydrogenotrophic methanogens might be responsible for the enhanced resistance of dry co-digestion of PM and FW under mesophilic conditions.

3.4 Summary

The effects of TS contents (ranging from 5% to 20%) on anaerobic co-digestion of FW and PM and the methanogenic pathway were assessed. The cumulative SMY of digesters had no significant change with the increase TS from 5% to 15%, while a 20% TS content reduced SMY. A new superimposed model by coupling the first-order kinetic model with the modified Gompertz model was developed to describe the two-peak methane production behavior in digesters operated at high TS contents. The shifting of methanogenic pathways was observed over the incubation time in dry digesters, where hydrogenotrophic

methanogenesis increased with time and dominated at the end of digestion, and mixotrophic methanogen played important roles during the transition period. The insightful view of the impacts of TS content on the methanogenic pathway, revealed in this study, can contribute to an in-depth understanding of wet and dry AD, and provide an important reference in the field of AD.

Chapter 4
Distinguishing responses of acetoclastic and hydrogenotrophic methanogens to ammonia stress in mesophilic mixed cultures

4.1 Introduction

Anaerobic digestion (AD) is a well-established biotechnology for the treatment of organic wastes (such as livestock manure, sewage sludge, food waste, etc.) while enabling energy recovery through the production of biogas (primarily methane). The conversion of organic wastes into biogas in AD is achieved via four successive steps (hydrolysis, acidogenesis, acetogenesis, and methanogenesis) which are catalyzed by distinct groups of microorganisms including hydrolytic bacteria, fermentative bacteria, acetogenic bacteria, and methanogenic archaea (including acetoclastic methanogens and hydrogenotrophic methanogens). This complex bioprocess is prone to inhibition by certain chemical compounds, among which ammoniacal nitrogen is one of the most significant inhibitors (Yenigün & Demirel, 2013), especially when treating nitrogen-rich feedstock, operating under high loading rates, and applying dry AD processes (Capson-Tojo et al., 2020; Rajagopal et al., 2013; Wang et al., 2021b). In aqueous solutions, ammoniacal nitrogen exists in two forms, i.e., free ammonia nitrogen (FAN or NH_3) and ammonium ions (NH_4^+), which are in a pH and temperature-dependent equilibrium as shown in Eq. (4-1) (Wu et al., 2009). FAN is generally considered to be more responsible for the ammonia inhibition than NH_4^+ and often treated as a key factor in many studies (Müller et al., 2006; Rajagopal et al., 2013).

$$NH_3(aq.) + H_2O(l.) \leftrightarrow NH_4^+(aq) + OH^+(aq.) \quad (4\text{-}1)$$

Ammonia inhibition may affect all the stages of AD, but methanogens are particularly sensitive to ammonia stress (Capson-Tojo et al., 2020; Kalamaras et al., 2020). Consequently, a decline in the methanogenic activity is often observed at high ammonia levels, which causes a feedback inhibition loop and may eventually lead to AD failure. Another common observation under ammonia stress is the shift of the methanogenesis pathway from the acetoclastic pathway to the hydrogenotrophic pathway (Capson-Tojo et al., 2020). For example, Hao et al. (2017) found that the biodegradation of acetate gradually shifted from the acetoclastic methanogenesis to the hydrogenotrophic methanogenesis in mesophilic AD reactors when the total ammonia nitrogen

(TAN) was elevated from 0.14 to 7 g/L. Tian et al. (2018b) indicated that syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis (SAO-HM) pathway was enhanced after the ammonia acclimation process (up to 10 g NH_4^+-N/L) in mesophilic reactors co-digesting cattle slurry and microalgae, as evidenced by the increase of the relative abundance of *Clostridium ultunense* and *Methanoculleus*. Similarly, Wang et al. (2020c) found that a manifest shifting from the acetoclastic pathway to the hydrogenotrophic pathway occurred in dry digestion (TS=20%) of pig manure and food waste, in which there was a high TAN concentration of 5293 mg N/L.

The shift of the methanogenesis pathway under ammonia stress has attracted considerable research interest due to its significance in understanding the tolerance response of methanogens to *in situ* ammonia stress (Gao et al., 2015), developing mitigation strategies (Tian et al., 2019), and exploring novel processes for bioconversion of carbon dioxide to methane (Wang et al., 2016a; Zabranska & Pokorna, 2018). The consensus from different studies is that hydrogenotrophic methanogens (HMs) are much more resistant to ammonia inhibition than acetoclastic methanogens (AMs) (Capson-Tojo et al., 2020). However, there are still considerable knowledge gaps to be addressed. For example, in most studies, the different tolerance of HM and AM to ammonia inhibition was inferred from microbial community changes or metabolic shifts under ammonia stress (Hao et al., 2017; Tian et al., 2018b; Wang et al., 2020c), while direct comparisons of the ammonia tolerance levels of these two types of methanogens are surprisingly rare (Angelidaki & Ahring, 1993; Tian et al., 2018a). In addition, the impacts of NH_4^+ and FAN have rarely been distinguished, especially for HMs. More specifically, in almost all studies, the inhibitory effects were investigated based on FAN and TAN, while NH_4^+ was overlooked (Capson-Tojo et al., 2020). Furthermore, studies on the viability and recoverability of AMs and HMs after severe ammonia inhibition are also rare.

To address the above knowledge gaps, this study systematically investigated the responses of acetoclastic and hydrogenotrophic cultures to ammonia inhibition at different TAN and pH values under mesophilic conditions. The specific objectives were: (1) to quantitatively compare the ammonia tolerance levels of AMs and HMs; (2) to distinguish the impacts of FAN and NH_4^+

on AMs and HMs; (3) to investigate the viability and recoverability of HMs and AMs after acute ammonia inhibition; and (4) to explore the possible mechanisms causing the different responses of AMs and HMs to ammonia inhibition. It is expected that the findings in this study could contribute to a better understanding of the different responses of AMs and HMs to ammonia stress and provide useful information for developing tailored operating strategies to mitigate ammonia inhibition in practical applications.

4.2 Materials and methods

4.2.1 Selective enrichment of AMs and HMs cultures

Two 2-L anaerobic sequencing batch reactors (referred to as $ASBR_{AM}$ and $ASBR_{HM}$) were used as selective enrichment systems. $ASBR_{AM}$ was used to selectively enrich the culture of AMs with 2000 mg COD/L acetate as the sole carbon source, while $ASBR_{HM}$ was fed with 1000 mg COD/L formate to enrich the culture of HMs (Sun et al., 2020). Dewatered anaerobic sludge collected from a mesophilic digester at a local municipal wastewater treatment plant in Galway, Ireland, was used as the inoculum in both bioreactors. The total solids (TS) and volatile solids (VS) of the raw anaerobic sludge were 20.0% and 12.8%, respectively. Prior to incubation, the raw sludge was diluted with the mineral medium to reach 4.5% TS content and filtered through a 1.10-mm sieve mesh to remove large particles. The two bioreactors were incubated on an orbital platform shaker at a rotational speed of 150 rpm, which was placed inside a chemostat incubator at 37.0±1.0 ℃. The hydraulic retention time of the two bioreactors was 48 h, and the reaction cycle was 24 h. The typical operation cycle of the two ASBRs consisted of four consecutive stages: 5 min of feeding, 23 h of anaerobic reaction, 50 min of settlement, and 5 min of discharge. All these steps were operated anaerobically. The pH of the influent for the two ASBRs was adjusted to 7.0 by using either 2M HCl or 2M NaOH solutions.

The mineral medium was modified from the study of Wang et al. (2018c), and consisted of 500 mg/L NH_4Cl, 150 mg/L $CaCl_2$, 200 mg/L $MgSO_4 \cdot 7H_2O$, 1062.5 mg/L K_2HPO_4, 467.84 mg/L

NaH_2PO_4, 3 g/L $NaHCO_3$, and 0.5 ml/L trace element solution. Trace elements were composed of 2 g/L $FeCl_3 \cdot 4H_2O$, 2 g/L $CoCl_2 \cdot 6H_2O$, 0.5 g/L $MnCl_2 \cdot 4H_2O$, 30 mg/L $CuCl_2 \cdot 2H_2O$, 50 mg/L $ZnCl_2$, 50 mg/L HBO_3, 90 mg/L $(NH_4)_6Mo_7O_2 \cdot 4H_2O$, 100 mg/L $Na_2SeO_3 \cdot 5H_2O$, 50 mg/L $NiCl_2 \cdot 6H_2O$, 1 g/L EDTA, and 1mL/L HCl 36% (van Loosdrecht et al., 2016).

4.2.2 Batch experiments

4.2.2.1 Preliminary tests under non-inhibited conditions

After over four months of successive incubation with the $ASBR_{AM}$ and $ASBR_{HM}$, preliminary batch experiments were conducted to test whether the targeted methanogenic cultures (AMs or HMs) were sufficiently enriched in each reactor. In the experiments, two carbon sources, 2.0 g COD/L acetate (prepared with sodium acetate) and H_2/CO_2 (80:20, v/v) gas mixture, were used individually to determine the specific methanogenic activity (SMA) of the anaerobic sludge enriched in each reactor and of the raw sludge (without enrichment). Meanwhile, 2.0 g anaerobic sludge was drawn from each reactor and the inoculum for the investigation of the microbial community structure.

4.2.2.2 Inhibition tests

Following successful enrichment of the two types of methanogenic cultures, batch inhibition experiments were performed using 160 mL serum bottles. Considering the fact that chloride was less inhibitory than ammonium (Lay et al., 1998), ammonium chloride, rather than any other ammonium salts, was used to generate the required levels of ammonium in the inhibition experiments. The pH was adjusted to the desired level (Table 4-1) by using either 2M HCl or 2M NaOH solutions. The experimental conditions for the ammonia inhibition tests are summarized in Table 4-1. All the experiments were conducted in triplicate for each condition.

Before the commencement of the inhibition experiments, the enriched sludge taken from each bioreactor was centrifuged at 6000 rpm for 10 minutes (Hettich® ROTOFIX 32A, Andreas Hettich GmbH & Co. KG, Germany) and then washed with phosphate buffer three times. In the batch inhibition experiments with acetoclastic methanogens (Set-I), a certain amount of sludge pellets was resuspended with 50 mL of the prepared medium (consisting of 2.0 g COD/L acetate prepared with sodium acetate and different concentrations of ammonia, Table 4-1) in each serum bottle to obtain an initial biomass concentration of 3.0 g VSS/L. The total Na^+ concentration introduced by the medium solution (i.e., sodium acetate) and NaOH (for pH adjustment) was ≤ 1.88 g/L, which was much lower than the inhibition threshold of 3-5 g/L (Rinzema et al., 1988; Chen et al., 2003). Therefore, sodium toxicity to AMs was considered negligible and no additional control was used assess the impacts of sodium ions. In the batch inhibition experiments with hydrogenotrophic methanogens (Set-II), aliquots of sludge pellets were dispensed into the serum bottles with the prepared medium (containing different concentrations of ammonia, Table 4-1) to form 30-mL mixed liquors at a biomass concentration of 3.0 g VSS/L. Subsequently, all the serum bottles were made anaerobic by flushing with pure nitrogen gas and then immediately closed with butyl rubber stoppers and aluminum crimp seals. Additionally, in Set-II experiments, all the serum bottles were purged with H_2/CO_2 (80:20) gas mixture and pressurized to the required pressure (partial hydrogen pressure P_{H_2} is 101 kPa). In order to avoid the decrease of pH caused by the introduction of H_2/CO_2 (80:20) gas mixture, 3 g $NaHCO_3$/L, 1062.5 mg/L K_2HPO_4, and 467.84 mg/L NaH_2PO_4 were used in the medium solution in each serum bottle. Afterwards, all the serum bottles were placed on an orbital platform shaker at 150 rpm in an air bath incubator (37.0±1.0 ℃). The headspace pressure in each bottle was periodically recorded, and then 100 μL of biogas was taken with a gas syringe to determine biogas composition.

Table 4-1. Experimental conditions for ammonia inhibition and recovery of cultures of acetoclastic and hydrogenotrophic methanogens

Batch experiments		**Inoculum**	**pH levels**	**TAN (g N/L)**
Inhibition experiments	Set-I	Enriched culture taken from $ASBR_{AM}$	7.0, 7.5, 8.0 and 8.5	0.2, 0.5, 1.0, 2.0, 3.0, 4.0 and 6.0.
	Set-II	Enriched culture taken from $ASBR_{HM}$	7.0, 7.5, 8.0 and 8.5	0.2, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0 and 10.0.
Recovery experiments	Set-III	Refreshed sludge from Set-I [a]	7.0	0.18 [b]
	Set-IV	Refreshed sludge from Set-II	7.0	0.18

Notes: a. Sludge from each serum bottle in Set-I (or Set-II) experiments was centrifuged and washed three times with phosphate buffer solution, referred to as refreshed sludge.

b. The TAN concentration used was derived from van Loosdrecht et al. (2016).

4.2.2.3 Batch recovery experiments

In the recovery experiments (Set-III and Set-IV), after the short-term inhibition experiments (lasting two days) in Set-I and Set-II, the mixed liquor in these serum bottles was centrifuged at 6000 rpm for 10 minutes. Then the sludge pellets were washed three times with phosphate buffer and subsequently dispensed with the mineral medium into the serum bottles. Afterwards, 0.18 g N/L was added to each bottle as nitrogen source, and the original pH in each bottle was maintained at 7.0 (as shown in Table 4-1). All the other conditions were the same as those used in the Set-I and Set-II inhibition experiments.

4.2.3 Determination of specific methanogenic activity

The specific acetoclastic methanogenic activity (SAMA) (Regueiro et al., 2012; van Loosdrecht et al., 2016) and hydrogenotrophic methanogenic activity (SHMA) (Coates et al., 1996) of the

AMs and HMs cultures at different ammonia levels were determined based on the methane production rate with acetate (2.0 g COD/L) and H_2/CO_2 (80/20) as the sole carbon source, respectively. For the measurement of SAMA, the initial food to microorganisms (F/M) ratio was 2/3 g COD/g VSS. The components of the medium were described in detail in van Loosdrecht et al. (2016). The methane production was measured based on the measurement of headspace pressure and methane content. For the measurement of SHMA, the methodology was based on the measurement of the headspace pressure, according to the protocol proposed by Coates et al. (1996). In an enclosed AD system, the inside pressure depletion is stoichiometrically correlated with methane production for the conversion of H_2/CO_2 to methane according to Eq. (4-2). Hence, the methane yield (Δn_{CH_4}, mol) generated from H_2/CO_2 during a specific interval of time can be estimated based on the pressure change as shown in Eq. (4-3) (Ripoll et al., 2020), where, ΔP is the pressure change within the headspace, kPa; V is the volume of the headspace, L; R is the ideal gas constant, 8.314 L·kPa/K/mol; and T is the thermodynamic temperature, K.

$$4H_{2(g)} + CO_{2(g)} \rightarrow CH_{4(g)} + 2H_2O_{(l)} \quad (4\text{-}2)$$

$$\Delta n_{CH_4} = -\frac{1}{4}\Delta n_{Biogas} = -\frac{\Delta P \cdot V}{4 \cdot R \cdot T} \quad (4\text{-}3)$$

All the tests lasted no more than two days. The methane production during the SMA tests (Table 4-1) was monitored every 1 or 2 h. On the completion of the test, the SMA (SAMA or SHMA) was estimated from the maximum rate of methane production, which was determined from the slope (S, N-mL/h) of the cumulative methane (N-mL) produced against the reaction time (h). All the SMA values were normalized to biomass (VSS, g) used and expressed as g COD-CH_4/g VSS/d according to Eq. (4-4), where f (N-mL/g COD-CH_4) is the stoichiometric volume of methane in mL equivalent to 1 g COD at standard temperature and pressure (STP).

$$SMA = \frac{S \cdot 24}{f \cdot VSS} \quad (4\text{-}4)$$

4.2.4 Inhibition models

Assuming that the methanogenic activity is inhibited by both NH_4^+ and NH_3, an inhibition model that considers both the inhibitors can be expressed in Eq. (4-5) (Astals et al., 2018). The SMA (SAMA or SHMA) measured from the inhibition experiments was mathematically analyzed based on the change in SMA from its maximum value (SMA_{max}) along with increasing concentrations of NH_4^+ and NH_3. $I_{NH_4^+}$ (or I_{NH_3}) represents the inhibition degree caused by NH_4^+ (or NH_3). SMA_{max} is the maximum SMA obtained under uninhibited conditions (g COD-CH_4/g VSS/d).

$$SMA = SMA_{max} \cdot I_{NH_4^+} \cdot I_{NH_3} \quad (4\text{-}5)$$

In this study, the inhibitory effect of NH_4^+ or NH_3 concentration on SMA was evaluated by using the following three inhibition models: (i) a simple Monod inhibition model (Eq. (4-6)) (Astals et al., 2018), (ii) a modified Monod inhibition model (Eq. (4-7)) (Siegrist et al., 2002), and (iii) a sigmoidal inhibition model (Eq. (4-8)) (Wett et al., 2009)).

$$I_X = \frac{K_X}{K_X + C_X} \quad (4\text{-}6)$$

where C_X is the inhibitor concentration (NH_4^+ or NH_3, mg N/L), and K_X ($K_{NH_4^+}$ or K_{NH_3}) is the inhibitor concentration that halves the maximum SMA (i.e., I_X=50%).

$$I_X = \frac{{K_X}^n}{{K_X}^n + {C_X}^n} \quad (4\text{-}7)$$

where n is used to describe the strong increase of the inhibition with increasing ammonia or ammonium concentration.

$$I_X = \frac{1}{1 + e^{-m \times (K_X - C_X)}} \quad (4\text{-}8)$$

where m is related to the slope of the logistic curve.

4.2.5 Analytical methods

Suspended solids (SS) and volatile suspended solids (VSS) were analyzed according to the standard method (APHA, 2012). The pH readings were recorded with a portable pH meter (pH3210, WTW, Weilheim, Germany). TAN was determined through a nutrient analyzer (Konelab 20, Thermo Clinical Labsystems, Finland). The FAN concentration was calculated according to Eq. (4-9) and Eq. (4-10) (Jiang et al., 2018b), where C_{FAN} and C_{TAN} are the free and total ammonia concentrations, respectively, mg/L; pK_a is the dissociation constant for ammonium ion, 8.892 at 37 °C and T is the temperature, ℃. The headspace pressure in the serum bottles was measured with a portable pressure gauge (Testo 512, Testo, USA). The composition of the biogas (CH_4, CO_2, and H_2) was determined using gas chromatography (GC 7890 A, Agilent Technologies, USA) as described by Wang et al. (2020c).

$$C_{FAN} = \frac{C_{TAN}}{1+10^{(pK_a-pH)}} \quad (9)$$

$$pK_a = 0.09018 + \frac{2729.92}{T+273.15} \quad (10)$$

The microbial community was analyzed using high-throughput 16S rRNA gene sequencing according to Yin et al. (2018)'s protocol. DNA was extracted from the solid phase of each sample using the PowerSoil DNA Extraction Kit (Laboratories Inc., CA, USA), following the manufacturer's instructions. For each sample, two independent PCR reactions were performed to amplify the extracted DNA. Then, the obtained sequences were determined using the Illumina MiSeq platform. The Usearch (version v7.0.1090) software was employed to cluster the operational taxonomic units (OTUs). The clustered OTUs were annotated down to the different levels (phylum, class, order, family, and genus) based on the RDP database.

The LIVE/DEAD® BacLight™ Bacterial viability kit (Molecular Probes, L-7012, Thermo Fisher Scientific), composed of two nucleic acid-binding stains (green-fluorescent SYTO™ 9 and red-fluorescent Propidium Iodide (PI)), was employed to assess the viability of the AMs and HMs cultures after different degrees of ammonia stress (Table 4-1). This method is based on the principle that the SYTO™ 9 stain selectively reacts to living cells to give green fluorescence, whereas the PI stain selectively reacts to dead cells to give red fluorescence (Du et al., 2020; Hao et al., 2009). Images with red and green fluorescence were respectively photographed under a fluorescence microscope (Nikon 90i), and then the red and green areas were quantified with Image J software. The ratio of green fluorescence to total fluorescence (red + green fluorescence) gives the proportion of living cells to total cells (Hao et al., 2009). The procedures for the determination are elaborated in the supplementary material.

4.2.6 Data analysis

All the statistical analyses and plotting of data were conducted using Microsoft Office 365 and OriginPro 2021. The statistically significant differences ($p < 0.05$) of the SMA data were evaluated by one-way analysis of variance (ANOVA) with p-values calculated at a 95% confidence level. Spearman correlation analysis was performed to determine the relationship between the activity of methanogens and the different factors by using SPSS 28. The inhibition modeling was conducted by non-linear curve fit with OriginPro 2021.

4.3 Results and discussion

4.3.1 Activity and community structure of the enriched methanogenic cultures

To ensure that the targeted AMs and HMs cultures were selectively, and adequately enriched, preliminary batch experiments were carried out to estimate the SAMA and SHMA of the methanogenic cultures in the $ASBR_{AM}$ and $ASBR_{HM}$. Furthermore, the enriched cultures and the

raw sludge (inoculum) were analyzed by high throughput 16S rRNA gene sequencing. The results are present in Figure 4-1.

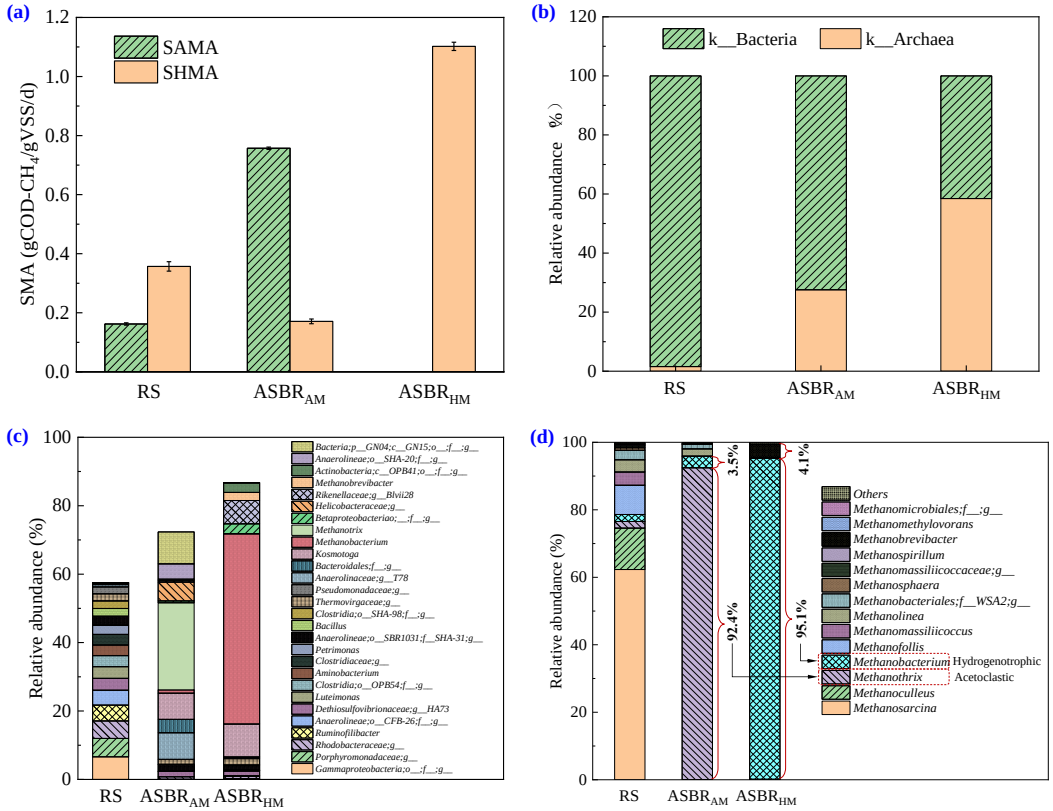


Figure 4-1. Specific methanogenic activity of anaerobic sludge with two kinds of carbon sources (a) and microbial community structure: (b) bacteria at the kingdom level, (c) bacteria at the genus level, and (d) archaea at the genus level. RS refers to the raw sludge (inoculum); $ASBR_{AM}$ refers to the AMs culture; and $ASBR_{HM}$ refers to the HMs culture.

The SAMA of the $ASBR_{AM}$ culture increased from 0.162±0.004 to 0.757±0.004 g COD-CH_4/g VSS/d after incubation with acetate as the sole carbon source for 4 months (Figure 4-1a). The SHMA of the HMs culture reached 1.102±0.014 g COD-CH_4/g VSS/d which was roughly 3.1 times the raw sludge's SHMA (0.357±0.004 g COD-CH_4/g VSS/d). Both the SAMA for the $ASBR_{AM}$ culture and the SHMA for the $ASBR_{HM}$ culture were close to those values reported in former studies (Ripoll et al., 2020). Noteworthy, the HMs culture had no acetoclastic methanogenic activity (SAMA=0, Figure 4-1a), indicating the HMs culture did not consist of

metabolically active AMs. However, for AMs culture, hydrogenotrophic methanogenic activity of 0.171±0.008 g COD-CH_4/g VSS/d was still measured, which was about 22.6% of the culture's acetoclastic methanogenic activity (SAMA). There were three possible reasons for this phenomenon: (1) some species in AMs culture were capable of utilizing H_2/CO_2 to produce CH_4, or (2) the AMs culture in $ASBR_{AM}$ still contained some HMs, which were capable of utilizing H_2/CO_2 to produce CH_4, or (3) homoacetogenic organisms in the AMs culture converted H_2/CO_2 to acetate, which was then utilized by the AMs to produce CH_4. This issue will be clarified in the following paragraphs.

After the selective enrichment, the relative abundance of the archaeal population was significantly increased in both the AMs and HMs cultures, reaching 27.6% and 58.5% respectively at the kingdom level from 1.6% in the raw sludge (Figure 4-1b). The archaeal community structure (Figure 4-1d) shows that acetoclastic genera *Methanothrix* (former *Methanosaeta*), in which species utilize acetate as their sole source of energy, were the most dominant methanogens (92.4%) in the AMs culture, followed by hydrogenotrophic genera *Methanobacterium* (3.5%). Meanwhile, no defined homoacetogenic bacteria were detected in both the AMs and HMs cultures (Figure 4-1c). The above results indicate that the hydrogenotrophic methanogenic activity measured in the enriched AMs culture (Figure 4-1a) solely resulted from the HMs. Nonetheless, the presence of HMs in the AMs culture would not affect its representativeness in assessing the ammonia tolerance of AMs because of the following reasons: (1) the proportion (3.5%) of the HMs in the enriched AMs culture was very small as compared with the AMs (92.4%); and (2) the HMs could not contribute to the acetoclastic methanogenic activity due to the absence of homoacetogenic bacteria which may otherwise facilitate hydrogenotrophic methanogenesis via the SAO-HM pathway. In the HMs culture, hydrogenotrophic genera *Methanobacterium* (accounted for 95.1%) overwhelmingly dominated, followed by hydrogenotrophic genera *Methanobrevibacter* (4.1%). The results show that most archaea (99.2%) in the HMs culture were hydrogenotrophic methanogens. So, the SHMA of the HMs culture measured (Figure 4-1a) practically represented the hydrogenotrophic methanogenic activity.

In summary, the above results prove that the target AMs and HMs cultures were selectively and adequately enriched. Hence, the enriched cultures could be suitably used to assess the tolerance responses of AMs and HMs to ammonia stress.

4.3.2 Tolerance of AMs and HMs to ammonia stress

The inhibitory effects of different TAN concentrations (0.2-10 g-N/L) at varying pH (7.0-8.5) on the activity of AMs (SAMA) and HMs (SHMA) are shown in Figure 4-2a and Figure 4-2b, respectively. The activities of AMs and HMs both decreased with the increase of TAN concentrations at given pH conditions. For instance, at the pH of 7.0, the SAMA significantly decreased ($p<0.05$) from 0.753±0.004 to 0.221±0.011 g COD-CH_4/g VSS/d when the TAN concentration increased from 0.2 to 4 g-N/L. Similarly, the SHMA declined from 1.100±0.019 to 0.217±0.010 g COD-CH_4/g VSS/d when the TAN concentration rose from 0.2 to 10.0 g-N/L. Consistent with previous studies (Werner et al., 2014), AMs were much more sensitive to TAN than HMs under given pH conditions. It should be noted that, the SAMA slightly decreased when TAN was still not greater than 1.0 g N/L and pH was moderate, e.g., 7.0 and 7.5. The results showed that AMs were extremely sensitive to ammonia exposure even at low TAN levels (≤ 1.0 g N/L). As depicted in Figure 4-2a, the SAMA decreased by more than 50% when the TAN concentration exceeded 2.0 g-N/L regardless of the pH conditions. Furthermore, almost complete inhibition of the SAMA occurred when the TAN concentration exceeded 3.0 g N/L under high pH conditions (8.0-8.5). Conversely, TAN exerted no severe inhibition on the hydrogenotrophic methanogenic activity (SHMA) even at a high TAN of 4.0 g N/L under high pH stress (8.5) (Figure 4-2b), which only resulted in around 32.9% inhibition. Moreover, complete inhibition of SHMA only occurred when the TAN rose to 10.0 g N/L at pH=8.5. Figure 4-2c and Figure 4-2d illustrate the inhibition degree of SAMA and SHMA at different TAN concentrations and pH values. The IC_{50} values of TAN for SAMA (50% inhibition) ranged from 0.4 to 1.7 g N/L, whereas it was an order of magnitude higher (4.7-6.8 g N/L) for SHMA. These observations further indicated that the HMs were more resistant to TAN in comparison with AMs (Chen et al., 2008). Another important observation is that the inhibition of AMs (SAMA)

by TAN was worsened significantly at higher pH, while the inhibition of HMs (SHMA) by TAN was much less dependent on pH, especially at TAN levels ≤6.0 g N/L (Figure 4-2c and Figure 4-2d). This implies that the HMs were much less sensitive to FAN inhibition, as FAN rises rapidly with increasing pH (Eq.s (4-9) and (4-10)). Therefore, the conventional strategy of alleviating ammonia inhibition by lowering pH (Rajagopal et al., 2013) is less effective for HMs.

To further clarify the responses of AMs and HMs to FAN stress, their activities under different FAN levels are depicted in Figure 4-2e and Figure 4-2f. The results show that the activity of AMs decreased at higher FAN levels and was completely inhibited at a FAN concentration of around 500 mg N/L (Figure 4-2e), which agrees with previous findings that FAN is the key inhibitor (Jiang et al., 2019). In contrast, the HMs have a much higher tolerance to FAN and their response to FAN stress was much more dispersed, although a decreasing trend of the SHMA with rising FAN was still observed (Figure 4-2f). More interestingly, the inhibition degree of FAN to the HMs seemed to decrease with the rising pH levels. These results suggest that compared with AMs, the HMs were less sensitive to FAN inhibition and could be affected by other factors besides FAN.

Consistent with previous findings (Yuan & Zhu, 2016), the above results indicate that the ammonia inhibition of methanogenesis was affected by several factors including TAN, FAN, NH_4^+, and pH. Hence, Spearman's correlation between the methanogens' activity and the different factors was analyzed. The results (Table 4-2) show that the activity of acetoclastic methanogens (SAMA) was negatively correlated with TAN, FAN, and NH_4^+ concentrations ($P < 0.01$), as well as pH values ($P < 0.05$). Besides, FAN had the greatest impact on SAMA as indicated by the correlation coefficient r_s, which was in line with the above results and previous findings (Müller et al., 2006; Rajagopal et al., 2013). Similarly, a negative correlation between SHMA and ammonia concentrations (TAN, FAN, NH_4^+) ($P < 0.01$) was also observed. However, there was no significant correlation between SHMA and pH. Contrary to the AMs, the HMs were more impacted by NH_4^+ than FAN. These results clearly show that HMs had a different response to ammonia inhibition from AMs, in terms of both tolerance and ammonia species.

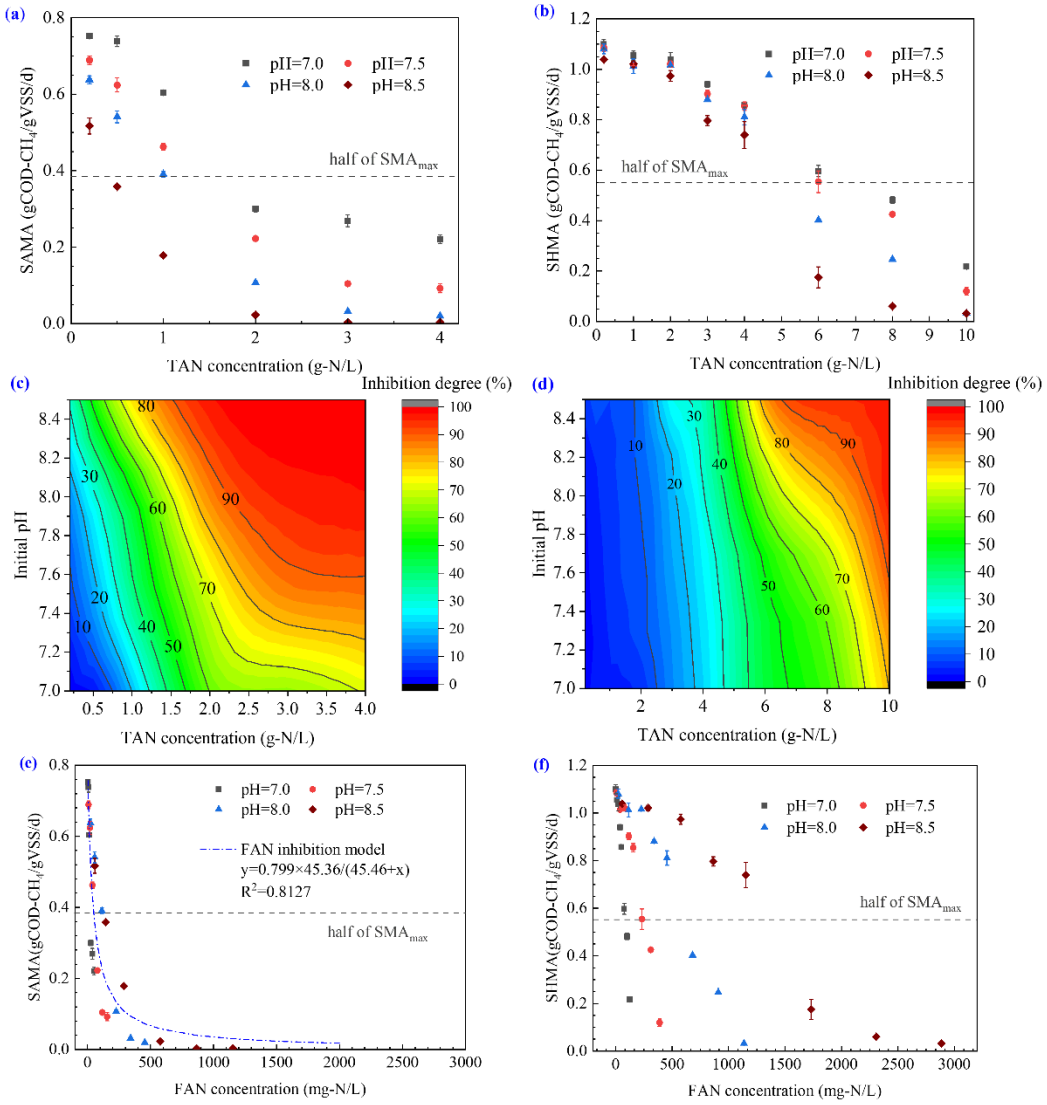


Figure 4-2. Inhibitory effects of TAN and FAN concentrations on the activity of AMs (a, c, and e) and HMs (b, d, and f). AMs refers to acetoclastic methanogens; HMs refers to hydrogenotrophic methanogens.

Table 4-2. Spearman's correlation between the activity of methanogens and different factors (TAN, NH_4^+, FAN, and pH)

Type	SMA	Spearman correlation coefficient r_s			
		TAN	NH_4^+	FAN	pH
AMs	SAMA	-0.857**	-0.755**	-0.923**	-0.474*
HMs	SHMA	-0.947**	-0.905**	-0.811**	-0.232

Notes: statistically significant values are indicated by symbols: *P < 0.05; **P < 0.01.

4.3.3 Inhibition modelling

In this study, the simple Monod inhibition model (Eq. (4-6)), the modified Monod inhibition model (Eq. (4-7)), and the sigmoidal inhibition model (Eq. (4-8)) were all employed to simulate the inhibitory effects of both FAN and NH_4^+ on AMs and HMs. The modelling results (Table 4-3, Figure B-2 and Figure B-3) show that the modified Monod inhibition model matched the experimental data (from the experiments Set-I and Set-II) best. The simple Monod inhibition model also satisfactorily described the experimental data. However, the fitting of the sigmoidal inhibition model was non-convergent. Therefore, the simple and modified Monod inhibition models were selected for the modelling study, and the modeled parameters are summarized in Table 4-3.

Table 4-3. Modeled parameters of batch inhibition experiments (Set-I and Set-II) with the simple Monod model and Modified Monod model

Parameters	Simple Monod model [a]		Modified Monod model [b]	
	Set-I	Set-II	Set-I	Set-II
SMA_{max} [c] (g COD-CH_4/g VSS/d)	0.89±0.04	1.21±0.06	0.76±0.03	1.05±0.02
$K_{NH_4^+}$ (mg N/L)	2018.9±395.5	6576.2±1541.3	2132.6±196.0	6049.9±223.5

K_{NH_3} (mg N/L)	105.5±18.1	1279.2±659.2	122.9±14.3	1344.7±93.5
n_1	-	-	1.73±0.29	2.93±0.30
n_2	-	-	1.28±0.18	2.96±0.54
Adj. R-Square	0.959	0.826	0.974	0.966
Reduced Chi-Square	0.00271	0.02266	0.00175	0.00443

Notes: a. $SAMA = SAMA_{max} \cdot \frac{K_{NH_4^+}}{K_{NH_4^+}+C_{NH_4^+}} \cdot \frac{K_{NH_3}}{K_{NH_3}+C_{NH_3}}$;

b. $SHMA = SHMA_{max} \cdot \frac{(K_{NH_4^+})^{n_1}}{(K_{NH_4^+})^{n_1}+(C_{NH_4^+})^{n_1}} \cdot \frac{(K_{NH_3})^{n_2}}{(K_{NH_3})^{n_2}+(C_{NH_3})^{n_2}}$;

c. SMA_{max} represents $SAMA_{max}$ or $SHMA_{max}$.

The results from the modified Monod inhibition model demonstrate that a FAN concentration of 122.9±14.3 mg N/L or an NH_4^+ concentration of 2132.6±196.0 mg N/L led to 50% inhibition of the activity of the AMs, whereas these values increased to 1344.7±93.5 mg N/L (FAN) and 6049.9±223.5 mg N/L (NH_4^+) for the HMs. The simple Monod inhibition model yielded similar results. The results show that tolerance of the HMs to ammonia inhibition was much higher than the AMs. Specifically, the tolerance of the HMs to FAN inhibition was one order of magnitude higher than the AMs, and tolerance of the HMs to NH_4^+ inhibition was 3 times higher than the AMs. Moreover, the results show that the AMs were much more sensitive to FAN inhibition than NH_4^+ inhibition, as compared with the HMs. Specifically, the tolerance of the AMs to FAN inhibition was one order of magnitude lower than NH_4^+ inhibition, whereas the tolerance of the HMs to FAN and NH_4^+ inhibition was in the same order although the HMs were still more sensitive to FAN inhibition in terms of IC_{50} (K_{NH_3} and $K_{NH_4^+}$).

A new indicator, the $I_{NH_4^+}/I_{NH_3}$ ratio, was introduced to evaluate individual contributions of FAN and NH_4^+ to the ammonia inhibition. If =1, NH_4^+ and NH_3 would make the equal contributions to the inhibition; >1 means that NH_3 would make more contributions to the inhibition; <1 means that NH_4^+ would make more contributions to the inhibition. The $I_{NH_4^+}/I_{NH_3}$ ratios for AMs and HMs with the increase of TAN concentration under different pH conditions

are depicted in Figure 4-3. The results show that the AMs were more impacted by FAN. Specifically, when the pH was above 8.0, the inhibition of the AMs predominately resulted from FAN. When the pH was below 8.0, FAN and NH_4^+ had a similar contribution to the inhibition at TAN ≤ 1 g N/L, while NH_4^+ had more contribution than FAN at TAN> 1 g N/L. On the contrary, the HMs were more impacted by NH_4^+ despite the lower IC_{50} of FAN than NH_4^+ for the HMs. Specifically, when the pH was below 8.5, the inhibition of the HMs predominately resulted from NH_4^+. The contribution of FAN exceeded NH_4^+ only at the highest pH (8.5). All these trends were exacerbated by increasing TAN. These results can explain why lowering pH was less effective in relieving ammonia inhibition for the HMs (Figure 4-2d), i.e., when relieving the FAN impact by lowering pH, the inhibition from NH_4^+ increased. These insights provide useful information for developing tailored mitigation strategies.

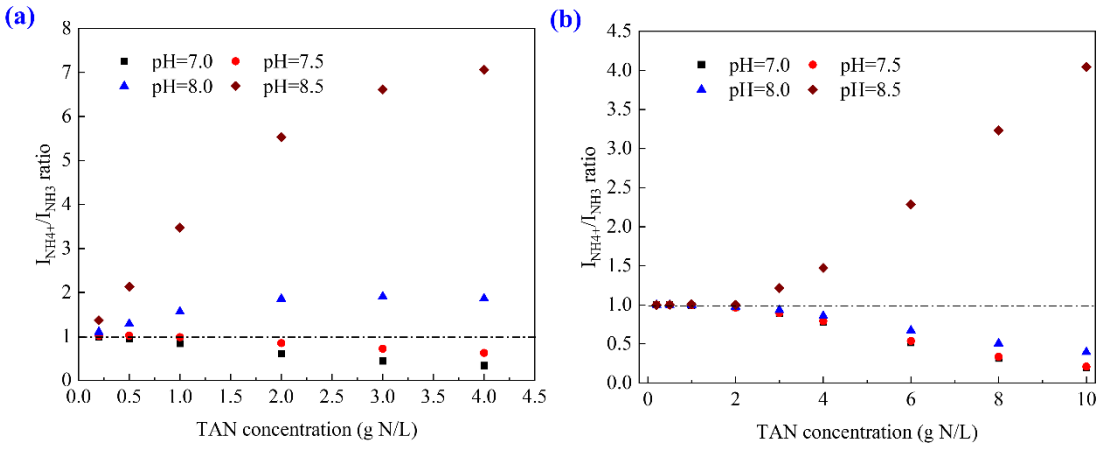


Figure 4-3. $I_{NH_4^+}/I_{NH_3}$ ratios for AMs (a) and HMs (b) with the increase of TAN concentration under different pH conditions.

4.3.4 Recoverability of methanogenic activity after ammonia inhibition

After the inhibition experiments, ammonia in bioreactors was completely removed and then the recovery of SAMA and SHMA was evaluated under non-inhibition conditions with fresh culture media (Table 4-1). The results are shown in Figure 4-4. For AMs, the recovery of SAMA was very limited, although a partial recovery of SAMA was observed with the biomass exposed to

low degrees of ammonia stress (Figure 4-4a). For example, the samples exposed to 1.0 g/L TAN at pH 7.0 recovered their SAMA from 0.604±0.006 COD-CH_4/g VSS/d to 0.655±0.001 g COD-CH_4/g VSS/d after the inhibition was removed (Figure 4-4a), which was still much lower than the maximum SAMA (0.757±0.004 g COD-CH_4/g VSS/d). Additionally, the recovery value of SAMA showed a downtrend along with the increase of FAN concentrations used in the inhibitory tests. These results indicated that even a low TAN concentration (1.0-4.0 g N/L) might have caused irreversible inhibition of the acetoclastic methanogenic activity during the short-term inhibition process.

For HMs, their activity could completely recover after exposured to severe FAN or TAN stress (FAN≤ 0.9 g N/L or TAN≤10 g N/L) when the pH was below 8.0 (Figure 4-4b). These results mean that the inhibitory effects of ammonia on HMs under a certain level were reversible. Interestingly, under certain conditions (TAN ≤6.0 g N/L and pH < 8), the recovery value of SHMA even exceeded the maximum SHMA measured before the ammonia stress. For example, the recovery value reached as high as 1.57 g COD-CH_4/g VSS/d for the HM culture exposed to 6.0 g N/L TAN at pH 7.5, which was approximately 40% above the maximum SHMA. It could be speculated that the activity of HMs was stimulated by the ammonia stress. A similar hormetic response of microorganisms was found in a study of Fan et al. (2021), where soil microorganisms were exposed to different levels of heavy metals. This phenomenon could be used to develop novel acclimation strategies. The inhibitory effect became irreversible when the FAN concentration exceeded 1.14 g N/L, which occurred at high TAN and pH levels (e.g., TAN 10.0 g N/L at pH ≥8.0; or TAN 8.0 g N/L at pH 8.5).

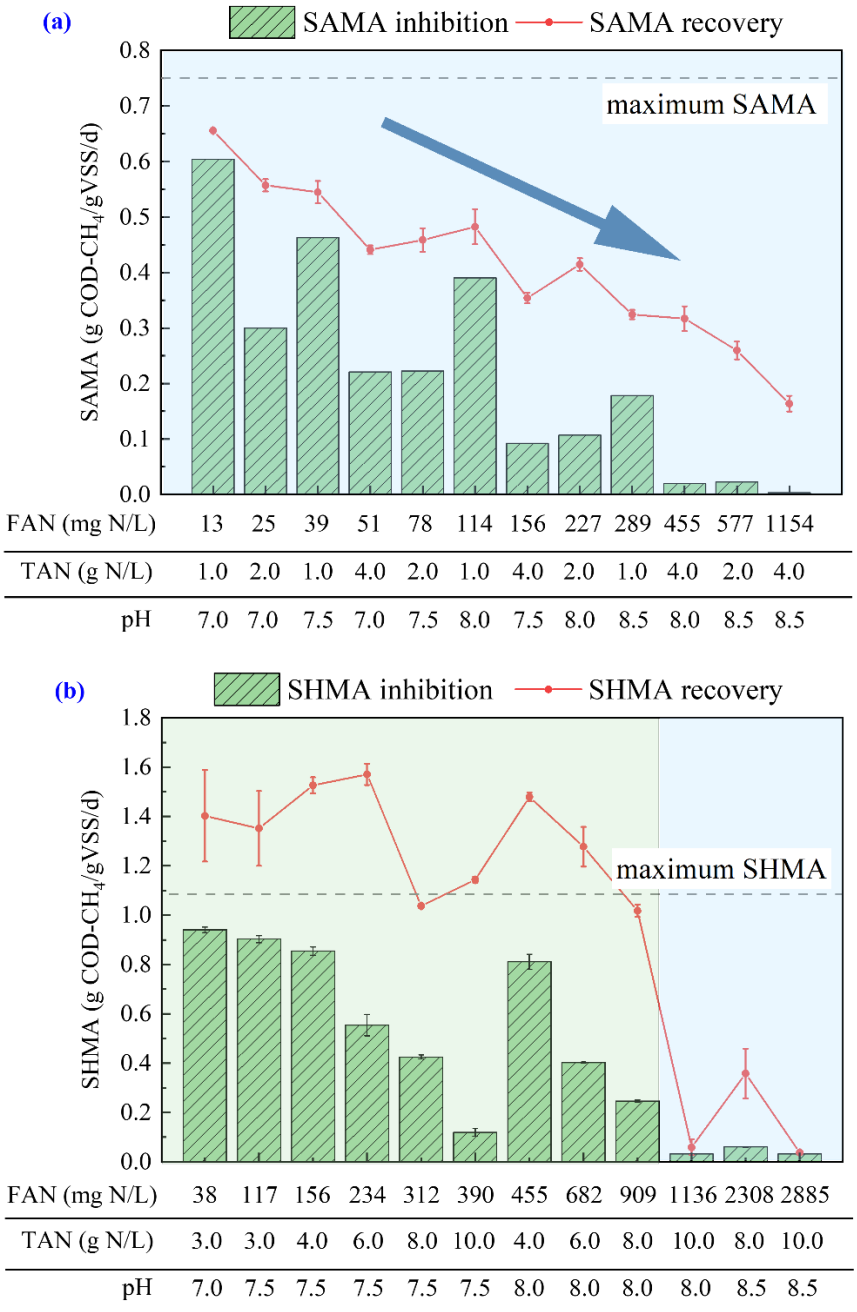


Figure 4-4. Recovery of the activity of AMs (a) and HMs (b) after ammonia inhibition

In summary, the above results show that a low degree of ammonia stress could cause irreversible inhibition to AMs, whereas HMs could fully or even over recover their activity from severe ammonia stress. This capability of HMs, along with their high tolerance to ammonia inhibition,

explains the common observation that the methanogenesis pathway shifts from the acetoclastic route to the hydrogenotrophic pathway under ammonia stress.

4.3.5 Possible mechanisms of AMs and HMs' different tolerance responses to ammonia stress

As evident from the above, HMs are much more robust to ammonia stress than AMs. Another direct evidence that supports this conclusion is the much higher cell viability of HMs than AMs under ammonia stress (Figure 4-5, Figure B-4 and B-5). The living cell proportion in AMs culture was significantly decreased with rising ammonia concentrations, with a 27.7% reduction at the TAN of 4 g N/L compared with the $Control_{AM}$ (Figure 4-5). In contrast, there was no significant change in the living cell proportion in HMs culture with increasing ammonia concentrations (below 8 g N/L). Only a 15.2% reduction was observed for HMs at much higher TAN concentrations of 10 g N/L relative to $Control_{HM}$. These results show that ammonia stress caused more severe death and destruction of the AMs populations than of the HMs populations, which explains the poor recoverability of the AMs after the ammonia inhibition.

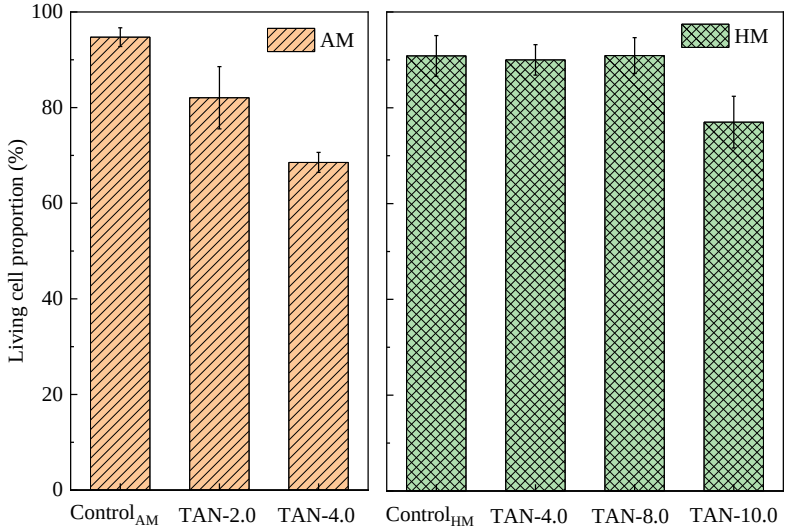


Figure 4-5. Evolution of fluorescently stained living cells of AMs under different TAN concentrations. AM, acetoclastic methanogen. HM, hydrogenotrophic methanogen.

Although no solid conclusions have been reached regarding the exact mechanism of ammonia toxicity in methanogens, several pathways for ammonia inhibition have been proposed based on the transmembrane electrical principle and pH gradients theory, such as changes in intracellular pH, ammonium ion accumulation, and interference with biomethane synthesizing enzymes (Figure 4-6) (Jiang et al., 2019). Previous studies have proposed that the energy requirements for the potassium (K^+) efflux process for proton exchange by the K^+ antiporter of the cell should increase to maintain intracellular pH levels (Sprott et al., 1984; Wittmann et al., 1995), which could potentially cause inhibition of specific enzyme reactions (Yin et al., 2020). When the K^+ pump cannot work efficiently to compensate for the excessive consumption of intracellular protons by FAN, an intracellular pH imbalance occurs, which then leads to cytotoxicity (Ling et al., 2021; Sprott & Patel, 1986). Furthermore, high NH_4^+ concentrations inside cells were reported to affect the cytosolic biomethane synthesizing process and the uptake of essential trace elements (such as Ca^{2+}, Mg^{2+}) required for cell function (Kadam & Boone, 1996; Yin et al., 2020).

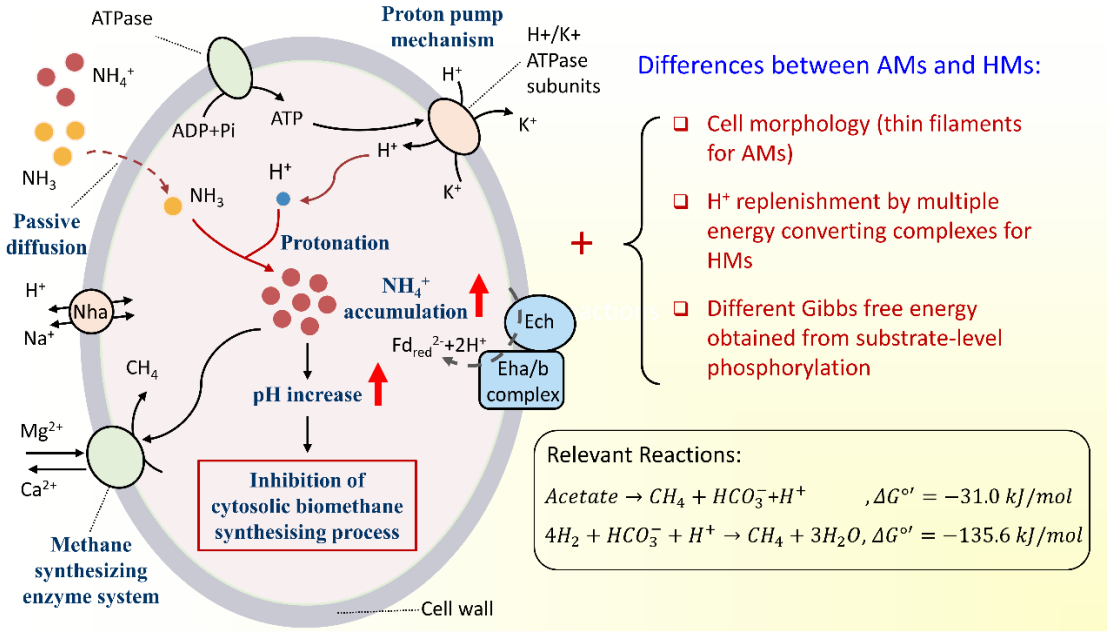


Figure 4-6. Mechanism of ammonia toxicity on AMs and HMs. AMs, acetoclastic methanogens; HMs, hydrogenotrophic methanogens; ATPase, ATP- synthase; Ech, Energy-converting hydrogenase; Eha/b, energy-converting hydrogenase A/B; Nha, Sodium/proton-antiporters.

Based on the above toxicity pathways, several mechanisms may explain the different tolerance responses of AMs and HMs to ammonia stress. First, FAN may more quickly diffuse into the AMs cells compared with HMs cells. As shown in Figure 4-6, the un-dissociated ammonia molecules (FAN) passively diffuse across cell membranes into the cytoplasm to maintain an equilibrium between the intracellular and extracellular FAN concentrations. After entering the archaeal cells, FAN can capture intracellular protons to form ammonium ions (NH_4^+), which causes the depletion of intracellular protons and the accumulation of NH_4^+ (Ling et al., 2021). A previous study suggested that the high sensitivity of AMs (e.g., *Methanothrix*) to ammonia stress was attributed to their cell morphology (thin filaments) (Liu et al., 2016). The thin filaments of AMs enlarge their surface as compared with HMs (growing as rods) so that un-dissociated NH_3 can diffuse more quickly into the AMs cells, thereby making them more responsive to FAN than the HMs (Demirel & Scherer, 2008; Ling et al., 2021). The existence of multiple energy-converting hydrogenases in HMs, such as the Eha/Ehb and Ech complexes, could be another reason for the high resistance of HMs to ammonia stress. Yan et al. (2020) found that hydrogenotrophic methanogen *Methanothermobacter* with the Eha/b and Ech energy-converting system had an extraordinary adaption ability to ammonia stress conditions compared to the acetoclastic methanogen *Methanothrix* which did not have the Eha/b and Ech complexes. Hence, methanogens with multiple energy-converting systems are more energy-efficient to meet energy requirements for regulating proton balance and replenishing H^+ when exposed to high ammonia levels. Besides, the higher tolerance of HMs to ammonia stress might be attributed to the higher energy gain (Gibbs free energy) from substrate-level phosphorylation (Figure 4-6). According to previous studies, K^+ uptake and the synthesis or transport of osmoprotectants are important for the microbial cells to overcome ammonia stress and maintain osmotic balance (Sprott et al., 1984; Martin et al., 1999). Thus, more energy is required for cell maintenance under ammonia stress, which can be obtained from substrate-level phosphorylation during methanogenesis (Yan et al., 2020). Methanogenesis from H_2/CO_2 ($\Delta G^{\circ\prime}$=-135.6 kJ/mol-CH_4) is far more exergonic

than from acetate ($\Delta G^{\circ\prime}$=-31.0 kJ/mol-CH_4) (Yan et al., 2020; Zhang et al., 2018b). Moreover, the methane production capacity of the HMs culture (1.102±0.014 g COD-CH_4/g VSS/d) was much higher than that of the AMs (0.757±0.004 g COD-CH_4/g VSS/d) under non-inhibited conditions (Figure 4-1a). As a result, HMs may obtain more energy from methanogenesis for cell maintenance and overcoming bioenergetic barriers induced by high ammonia levels.

In summary, the mechanism of ammonia toxicity in methanogens is attributed to changes in intracellular pH, ammonium ion accumulation, and interference with biomethane synthesizing enzymes. The different tolerance responses of AMs and HMs to ammonia might be related to their different cell morphologies, multiple energy-converting systems, and Gibbs free energy from substrate-level phosphorylation.

4.4 Summary

In this study, the ammonia tolerance and recoverability of AMs and HMs were assessed under mesophilic conditions. The results showed that HMs were much more robust to ammonia stress than AMs, with a tolerance level to free ammonia (IC_{50}=1345 mg N/L) and NH_4^+ (IC_{50}=6050 mg N/L) nearly 11 times and 3 times that of AMs (NH_3, IC_{50}=123 mg N/L; NH_4^+, IC_{50}=2133 mg N/L), respectively. Consistent with general belief, the AMs were more impacted by free ammonia. However, the HMs were more impacted by NH_4^+ when the pH was ≤ 8.0. A low TAN (1.0-4.0 g N/L) could cause irreversible inhibition of the AMs due to significant cell death, while the activity of HMs could be fully or even over recovered from severe ammonia stress (FAN ≤ 0.9 g N/L or TAN ≤ 10 g N/L) when the pH was ≤ 8.0. Different tolerance of AMs and HMs to ammonia stress might be associated with the cell morphology, multiple energy-converting systems, and Gibbs free energy from substrate-level phosphorylation. These new insights can contribute to an in-depth understanding of ammonia inhibition in methanogenesis and provide useful information for developing tailored operating strategies to mitigate ammonia inhibition in practical applications.

Chapter 5
Stimulatory effects of biochar addition on dry anaerobic co-digestion of pig manure and food waste under mesophilic conditions

5.1 Introduction

Dry AD systems can offer several benefits, including smaller digester size and less capital investment, reduced water usage, lower energy consumption for heating, and more flexible management of digestate (Dennehy et al., 2017b; Jiang et al., 2018a; Jiang et al., 2018b). Additionally, the need for material handling during pre- and post-processing is minimized in the dry AD process (Yi et al., 2014). Dry AD can also enhance the inactivation of pathogens (Jiang et al., 2020; Wang et al., 2021a; Wang et al., 2020a). Therefore, AD operated under high TS content seems promising, by which it could distinctly reduce the total operating expense of AD for minimal digestate generation (Wang et al., 2020c).

However, dry AD is also subjected to several drawbacks in biological and technological aspects. Due to the high solids content, it is very challenging to provide homogenization in dry AD systems, which is vital to the routine operation of AD and CH_4 production. Besides, dry AD is prone to ammonia or volatile fatty acid inhibitions as a result of the low water content. A previous study demonstrated that the specific methane yield (SMY) was decreased by 11% at a high TS of 20% relative to that at 10% TS (Wang et al., 2020c).

The utilization of various additives (such as activated carbon, graphite) to improve AD performance has drawn considerable interest from many researchers (Xie et al., 2020; Yin et al., 2018). Generally, the additives can offer a better habitat for the microorganisms participated in AD processes due to their porous structure and biostability, thus increasing biomass density and promoting microbial metabolic activity (Sun et al., 2019). Besides, the adsorption of inhibitory compounds such as ammonia by additives can also improve AD performance (Jang et al., 2018). However, the practical application of these additives has been restricted as disposal of these additives after AD could cause environmental issues, such as secondary pollution and threats to environmental safety. Additionally, the high cost for production weakens the economic feasibility of their practical application.

As a cost-effective material, carbon-rich biochar is a promising additive for enhancement of CH_4 production in AD process. Ma et al. (2020) indicated that the shortened lag phase by 44 % and the 25% increment in maximum CH_4 production rate were achieved when supplementing 15 g/L rice husk biochar in sorghum AD. The authors reported that biochar promoted the efficiency of electron exchange between acidogens and methanogens and had a positive effect on process stability during AD (Chen et al., 2014a; Mumme et al., 2014). Besides, the application of biochar to AD is advantageous over other additives. Biochar is generally produced by pyrolysis process at relatively low temperature in comparison to activated carbon (Lehmann & Joseph, 2009), which substantially reduces its production cost. Meanwhile, a wide range of materials or wastes have proven to be suitable feedstock for biochar production, such as wood, agricultural and forest residues, even digestate from AD systems (Chiappero et al., 2020; Jang et al., 2018; Wang & Wang, 2019). Additionally, biochar blended in digestate after digestion could directly be used as soil amendments without any environmental threats. Thus, eco-compatible and accessible biochar may serve as a good additive candidate for the promotion of CH_4 production in AD process. Currently, many studies have assessed the viability of improving anaerobic bioprocess performance by the supplementation of biochar. They have indicated that biochar could significantly promote the CH_4 production of digesters fed with various feedstocks, such as FW, municipal solid waste and algae (Qin et al., 2017; Shanmugam et al., 2018; Viggi et al., 2017). Nonetheless, the effect of biochar addition on dry AD has been rarely reported. Sun et al. (2019) studied the effect of cow manure-derived biochar addition on dry AD of beer lees and found that the biochar addition exerted a positive influence in promoting CH_4 production in dry AD systems. However, it is still unknown whether the use of biochar can enhance dry co-AD of PM and FW. As typical feedstocks for biochar production, bamboo, rice husk, and pecan shell derived biochars (BB, RHB, and PSB) were reported to be widely used as good adsorbents for wastewater treatment and soil amendments to improve soil fertility (Chiappero et al., 2020), but the application of them to improve AD performance is seldom explored.

The main objective of this study was to investigate the effects of commonly used biochars (bamboo, rice husk, and pecan shell) on CH_4 production performance of dry AD under mesophilic conditions. Additionally, possible mechanisms of enhanced CH_4 production with the

addition of biochar were discussed. It is expected that the findings of this present study could offer useful information on the practical application of biochar in dry AD process.

5.2 Materials and Methods

5.2.1 Anaerobic inoculum and substrates

Anaerobic sludge obtained from a mesophilic digester at a local municipal wastewater treatment plant in Galway, Ireland, was used as inoculum (seed sludge) in this study. Before use, it was stored in a cold room for almost 60 days to exhaust all the biodegradable organics (Dennehy et al., 2016; Jiang et al., 2018b). The PM used for this work was collected from a local pig farm in Galway, Ireland. After collection, the solid fraction of PM was obtained through centrifugation at 6000 rpm for 5 min, which directly served as one of the substrates for dry AD. The other substrate was FW collected from two canteens situated in the university campus in Galway, Ireland. Before grinding, the fractions which were difficult to mash (like bones, paper cups, etc.) and the non-biodegradable elements (such as plastics) in the fresh FW were removed manually. Then it underwent a grinding pre-treatment with a food processor to generate fine particles (< 2 mm in size) and subsequently thoroughly blended before the utilization (Jiang et al., 2018b). The major properties of inoculum and the substrates are depicted in Table 5-1.

Table 5-1. Physiochemical properties of anaerobic inoculum, PM, and FW *

No.	Parameters	Unit	Inoculum	Pig manure (solid fraction)	Food waste
1	pH	/	7.70 ± 0.03	7.80±0.04	4.20 ± 0.01
2	TS	%	19.30 ± 0.20	24.36 ± 0.18	44.90 ± 0.20
3	VS	%	12.70 ± 0.24	19.30 ± 0.02	43.10 ± 0.19
4	VS/TS	%	68.90 ± 0.56	79.40 ± 0.50	96.00 ± 0.04
5	sCOD	g/L	6.20 ± 0.56	40.60 ± 0.31	207.50 ± 0.78
6	tCOD	g/L	146.90 ± 8.16	215.10 ± 4.99	491.50 ± 27.83
7	TVFA	mg acetate /kg wet weight	0	22 593 ± 563	9 237 ± 124
8	FVFA calculated	mg acetate/kg wet weight	0.0	20.4±0.5	7 231.0±76.8
9	TAN	mg/L	1689.7 ± 56.9	4436.8 ± 269.5	183.2 ± 25.7
10	FAN calculated	mg/L	48.8 ± 3.6	93.6 ± 8.6	0.0

* PW, pig manure; FW, food waste; TS: total solid; VS, volatile solid; sCOD, soluble chemical oxygen demand; tCOD, total chemical oxygen demand; TVFA, total volatile fatty acid; FVFA, free volatile fatty acid; TAN, total ammonia nitrogen; FAN, free ammonia nitrogen.

5.2.2 Biochar

Three typical kinds of biochar, namely, bamboo biochar (BB), rice husk biochar (RHB), and pecan shell biochar (PS), which were derived from bamboo, rice husk and pecan shell respectively, were employed in the present study. These biochars were donated by Zhejiang Biochar Engineering Technology Research Center, a very experienced institute in biochar production and application. These biochars were produced via a slow pyrolysis process in the absence of oxygen, whose parameters employed were heating rate of 25°C/min, maintaining temperature of 550°C, and holding time of 1.5 hours. To obtain a similar size, all biochar was ground and sieved with a mesh size of 0.42-1.10 mm. Table 5-2 shows the physicochemical characteristics of the selected biochars.

Table 5-2. Physiochemical characteristics of the selected biochars.

Parameters	BB	RHB	PSB
pH	8.63±0.01	7.45±0.01	8.94±0.02
Particle size (mm)	0.42–1.10	0.42–1.10	0.42–1.10
Specific surface area (m^2/g)	17.90 ± 0.11	149.30 ± 0.37	6.14 ± 0.07
Electrical conductivity (mS/cm)	9.37 ± 0.35	5.76 ± 0.13	17.89 ± 0.83
Cation exchange capacity (c mol/kg)	14.70 ± 0.58	13.10 ± 0.50	42.00 ± 0.62
Pore size (nm)	3.35±0.06	2.57±0.04	8.68±0.08
Total pore volume (cm^3/g)	0.020	0.108	0.015

Note: BB, bamboo-derived biochar; RHB, rice husk-derived biochar; PSB, pecan shell-derived biochar.

5.2.3 Experimental setup

The batch AD assays were performed using a series of 2-L glass bottles with a working volume of 1.2 L. These glass bottles, served as bioreactors, were all equipped with a rubber stopper so as to create anaerobic conditions during microbial cultivation. Two ports were made in each rubber stopper: one was used for collecting biogas by inserting a hollow plastic tube, while the other was for temperature measurement by installing a probe rod. Besides, a sampling port with the diameter of 25 mm was set near the bottom in each reactor. The mixing ratio (VS basis) between PM and FW employed in present batch experiments was 25:25 with a Food to Microorganism (F/M) ratio of 50:50 (VS basis) in accordance with a former study by Jiang et al. (2018b). The mixture of substrates and inoculum was well prepared and then diluted to a TS content of 15% with tap water. Four experimental groups were established, consisting of three test groups amended with different biochars (BB, RHB, and PSB), which were referred to as RBB, RRH, and RPS, respectively; one control group without any biochar addition (referred to as Control). The dosage of biochar used in this study was 15 g/L, which was typically employed in previous studies (Ma et al., 2020; Wang et al., 2018a). Each group was performed in triplicate. Before start-up, each reactor was gassed with pure nitrogen gas for at least 3 minutes to remove the oxygen gas from the headspace. Afterwards, these reactors were transferred into a thermostatic incubator at 37.0 ± 1.0 ℃ for long-term cultivation, without any stirring system. The reactors were manually shaken once every day to improve the mixing conditions as possibly.

5.2.4 Sample preparation

Digestate (1 or 2 g) was sampled regularly from each reactor. After collection, the digestate sample was generally diluted 20 times with deionized water. The dilution rate was reduced to 10 or 5 when the VFAs concentrations dropped to a relatively low level (< 5g HAc/L). Afterwards, the diluted samples were separated by a centrifuge (Sigma 2-16P, Germany) at 20376 × g for 5 min and then the liquid part was filtered via a syringe microfilter with a pore size of 0.45 μm (Sarstedt Ltd., Germany). The obtained filtrate was used for the detection of sCOD, total VFA, and total ammonia nitrogen (TAN, consisting of NH_4^+-N and NH_3-N) concentrations.

5.2.5 Analytical methods

The collection of biogas produced in every digester was using a plastic gas bag. Periodically, the gas bag was taken off from each digester for the measurement of biogas volume which was normalized to STP (273.15 K, 100 kPa) volume when calculated the SMY. The components of biogas (mainly CH_4 and CO_2) were measured with the former method (Wang et al., 2020c), where a gas chromatography (GC 7890 A, USA), equipped with a thermal conductivity detector and a stainless-steel column (13803-U, USA) was used, with argon gas as the carrier gas. The pH values were recorded with a portable pH meter (WTW pH 3210, Germany). The total ammonia nitrogen (TAN, consisting of NH_4^+-N and NH_3-N) concentrations was measured using a Konelab nutrient analyzer (Thermo Clinical Labsystems, Finland) (Hu et al., 2021). COD was analyzed using a Hach DR 3900 spectrophotometer with commercial reagent kits (Hach Company, US). Other routine indicators (TS, VS) were determined according to the standard methods (APHA, 2012). To avoid the experimental error caused by the sampling process, the VS involved in samples taken from the digesters every time was considered when calculating the SMY.

5.2.6 Kinetic modelling

According to Wang et al. (2020c), CH_4 production was reckoned to be derived from two groups

of organics (readily-biodegradable organics, RBOs; and poorly- biodegradable organics, PBOs) with different utilization rates by microbes when anaerobically co-digesting PM and FW under high TS conditions. To obtain the maximum CH_4 production potential, production rate and lag phase, the superimposed model (Eq. (5-1)) newly developed by Wang et al. (2020c) was employed to descript the dry AD kinetics under the supplementation of different biochars.

$$M_{CH_4}(t) = M_{1max}[1 - \exp(-k \cdot t)] + M_{2max} \cdot exp\left\{-exp\left[\frac{R_m \cdot e}{M_{2max}} \cdot (\lambda - t) + 1\right]\right\} \quad (5\text{-}1)$$

where $M_{CH_4}(t)$, the accumulated SMY at a certain reaction time (t), mL/gVS_{added}; M_{1max}, the CH_4 production potential from the RBOs, mL/gVS_{added}; M_{2max}, the CH_4 production potential from the PBOs, mL/gVS_{added}; *t*, the reaction time, d; R_m, the maximum specific CH_4 production rate, mL/(gVS_{added}·d); λ, the lag phase time, days; *k* is the hydrolysis rate constant, d^{-1}; and *e*, the Euler's number, 2.71828.

5.2.7 Calculations

Free ammonia (FAN) concentrations were calculated from the measured total ammonia (TAN) concentrations according to the following equation (Emerson et al., 1975):

$$\frac{TAN}{FAN} = \left(1 + \frac{10^{-pH}}{10^{-\left(0.09018 + \frac{2729.92}{T}\right)}}\right) \quad (5\text{-}2)$$

The fraction of unionized VFAs was calculated with the following equation (Watcharasukarn et al., 2009):

$$\frac{TVFA}{FVFA} = \left(1 + \frac{10^{pH}}{10^{pK_a}}\right) \quad (5\text{-}3)$$

where *FVFA*, the free volatile fatty acid concentration, mg/L; *TVFA*, total volatile fatty acid

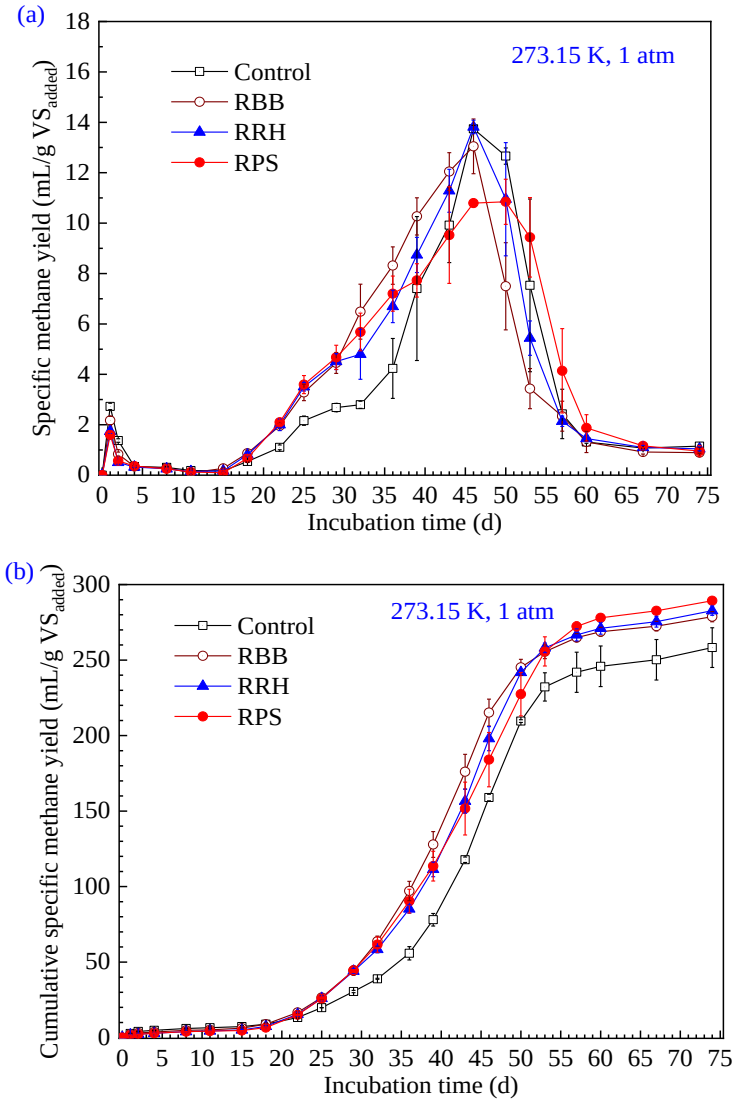
concentration, mg/L; and pK_a, the dissociation constant of individual VFAs, with values of 4.76, 4.87, 4.81, 4.84, 4.84 and 4.77 for acetic, propionic, butyric, isobutyric, valeric and isovaleric acids at 25 °C, respectively.

5.3 Results and discussion

5.3.1 Methane production of anaerobic digesters amended with different biochars

Figure 5-1 illustrates CH_4 production in the digesters amended with the three different biochars. Overall, profiles of the daily SMY in the Control, RBB, RHB and RPS digesters showed a similar trend. Two peaks of daily SMY over the incubation time were observed in all the digesters, although the peak time was slightly different (Figure 5-1a). Furthermore, the maximum CH_4 production rates of these digesters were almost at the identical level with the range of 13.1-13.8 NmL/g VS_{added}/d, except RPS which had a relatively lower rate (10.8 NmL/g VS_{added}/d) compared to the other three. It demonstrates that AD performance in terms of peak CH_4 production rate was not elevated along with the addition of selected biochars in this study.

The cumulative SMY of the BB, RHB and PSB amended systems were 278.7, 282.7 and 289.3 NmL/g VS_{added}, respectively, which were increased by 7.9%, 9.4% and 12.0% compared to the Control with non-biochar addition (258.3 NmL/g VS_{added}) (Figure 5-1b). It indicates that the biochar addition could make some positive effects on CH_4 production during dry AD of FW and PM, with RPS performing best among the three biochars assessed. The variation of CH_4 content in the different digesters is shown in Figure 5-1c. The result demonstrates that there is no significant difference in terms of variation of the CH_4 content observed among these digesters amended with different biochars. Additionally, the maximum CH_4 content (around 72%) in the Control (without any biochar addition) was at the same level as those amended with biochars.


(a)
273.15 K, 1 atm
Control
RBB
RRH
RPS
Specific methane yield (mL/g VS$_{added}$)
Incubation time (d)
(b)
273.15 K, 1 atm
Control
RBB
RRH
RPS
Cumulative specific methane yield (mL/g VS$_{added}$)
Incubation time (d)

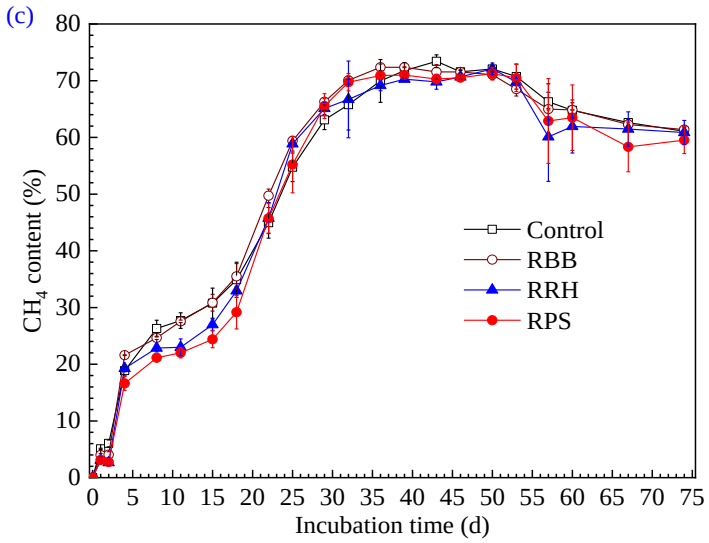


Figure 5-1. Methane production in digesters amended with different biochars: (a) Daily specific methane yield; (b) cumulative specific methane yield; (c) CH_4 content.

5.3.2 Kinetic modelling analysis

In this study, the measured CH_4 production data of the digesters amended with different biochars was analyzed with the newly established model (Eq. (5-1)) and the relevant results are shown in Table 5-3. The modelling results indicate that this model fitted the measured SMY data of these dry AD systems well. The parameters of M_{1max} and k had relations with the biodegradation of RBOs (Wang et al., 2020c). It is worth noting that M_{1max} was declined in these digesters amended with biochars as compared to the Control. This indicates that the biochar addition might exert some negative effects on the use of RBOs by microorganisms at the very beginning. It can be attributed to the fact that some components (such as polycyclic aromatic hydrocarbons) in the biochars had negative impacts on some microorganism (Oleszczuk et al., 2013) and microbes had to adapt themselves to the new surroundings with the existence of biochars. Nevertheless, the hydrolysis rate constant k was elevated with the addition of biochars. The k values in RBB, RHB and RPS were all observed around 2 folds of that in the control group (Table 5-3). However, there was no significant difference ($p>0.05$) for the k value among the digesters amended with the three different biochars. It means that a better hydrolysis rate of

readily biodegradable organics was achieved in AD systems amended with the BB, RHB, and PSB.

The biodegradation of PBOs in dry AD was linked to these parameters of M_{2max}, R_m and λ (Wang et al., 2020c). M_{2max} in RBB, RHB, and RPS digesters was increased in comparison to digesters without any biochar addition. Additionally, the lag phase λ for degradation of PBOs in RBB, RHB, and RPS digesters was shortened by 17.2%, 13.0%, and 17.8%, respectively. It indicates that the addition of the three biochars to dry AD facilitated the degradation of PBOs. However, the maximum CH_4 production rates R_m for the biodegradation of PBOs in these digesters were almost at the same level with the range of 10.2-12.5 NmL/g VS_{added}/d.

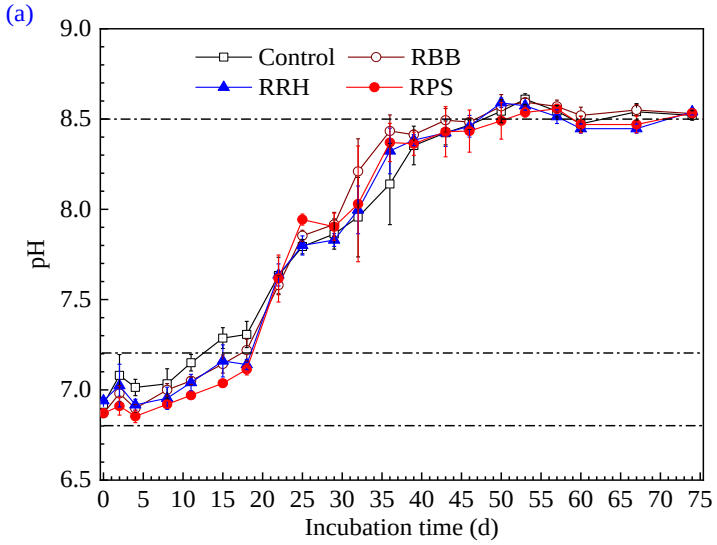
Table 5-3. Modelled kinetic parameters in digesters amended with biochars

Parameters	**Control**	**RBB**	**RRH**	**RPS**
M_{max} (NmL/g VS_{added})	306.5	292.3	305.5	317.7
M_{1max} (NmL/g VS_{added})	90.2	37.0	49.8	30.5
k (1/d)	0.011	0.027	0.021	0.028
M_{2max} (NmL/g VS_{added})	216.3	255.3	256.7	287.2
R_m (NmL/g VS_{added}/d)	12.5	11.8	11.5	10.2
λ (d)	35.4	29.3	30.8	29.1
R^2	0.995	0.997	0.995	0.996
Reduced Chi-Sqr	53.0	35.7	63.7	51.8

5.3.3 Evaluation of process stability

During the 74 days of the batch experiment, all these anaerobic digesters produced methane-rich biogas, without any acidification or failure in fermentation. Globally, there is no significant difference ($p>0.05$) in pH variation between digesters amended with different biochars and the Control without any biochar addition. After keeping around 6.9 in the first few days, it gradually increased to around 8.5 and then levelled off (Figure 5-2a). At the steady state, the pH values were maintained at a similar level in the Control, RBB, RRH, and RPS, with a pH of 8.61, 8.57, 8.53 and 8.51, respectively. The results agree with our previous finding that PM could provide sufficient buffering capacity to prevent pH decrease (Wang et al., 2020c), which would drop

substantially during the buildup of VFAs (Figure 5-3), especially under high TS condition. On the other hand, with the degradation of proteins, the ammonia concentration kept increasing (Figure 5-2b), which led to a slight rise of pH values even during the period of VFAs accumulation. The results manifest that the introduction of biochars to dry co-AD of PM and FW had no significant effect ($p>0.05$) on the pH regulation, since PM could effectively prevent acidification, and the pH values were all within the admissible pH spectrum (6.5-8.5) for anaerobic microbes (Yi et al., 2014) already throughout the AD process. The TAN concentration in all digesters gradually increased along with the incubation time, and then reached a relatively stable plateau (Figure 5-2b). At the end of reaction, the TAN concentrations reached a similar level in the Control, RBB, RRH, and RPS, with a concentration of 3028.8, 2826.9, 2805.0 and 2840.1 mg N/kg wet weight, respectively.



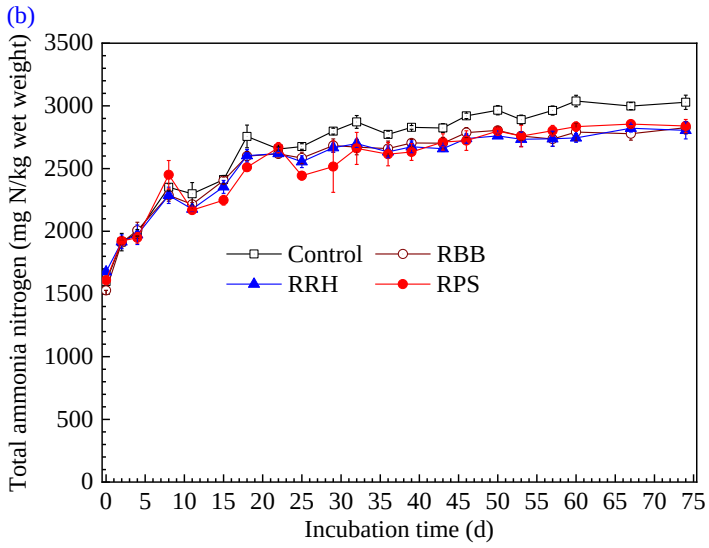
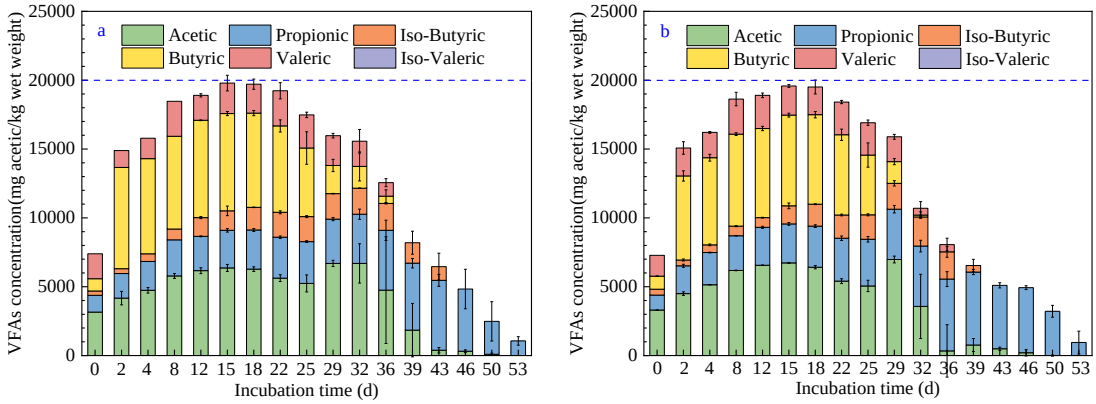


Figure 5-2. Evolution of pH (a) and total ammonia nitrogen (b) under biochar supplementation. (BB: bamboo-derived biochar; RH: rice husk-derived biochar; PS: pecan shell derived biochar)

VFAs, the crucial metabolic intermediates in AD, are considered as one of the most significant and accessible criteria for the evaluation of AD process stability (Wang et al., 2020c). VFA accumulation is caused by imbalanced reaction rates between fermentation and methanogenesis, which often results in AD instability or even failure. The variation and distribution of VFAs in the digesters amended with different biochars is shown in Figure 5-3. Overall, no significant difference ($p>0.05$) in the variation and distribution of VFAs was displayed between the biochar amended digesters and the control group. Only one TVFA peak was observed in the Control, as well as in RBB, RRH and RPS. Also, the corresponding peak in each group was achieved after 18 d. Furthermore, the variation of VFAs in all the digesters was almost identical, with the maximum TVFA concentration of around 20 g HAc equivalent/kg wet weight. TVFA gradually accumulated during the period of days 0-28 and then decreased after reaching the maximum value, along with active CH_4 production. After Day 53, TVFA in all the digesters became almost undetectable, though the CH_4 production was still active. These results indicate that biochar addition did not exert significant effects on the variation and distribution of VFAs in dry AD systems.

As to the variation of specific VAFs, the propionic acid seemed to be more persistent in these digesters compared to the others (Figure 5-3). After the commencement of the AD, the propionic acid increasingly accumulated until to a high level (around 5 g HAc equivalent/kg wet weight) before decreasing only after most of the other VFAs had been depleted (Figure 5-3). Towards the completion of the experiment, the propionic acids in all digesters were almost exhausted by methanogens. Since the anaerobic biodegradation of propionic acid is thermodynamically unfavorable ($\triangle G_0' = +76.1$ kJ/mol) (Wang et al., 2020c), a buildup of propionic acid during AD is not rarely seen. It is reported that the biochar supplementation could promote the degradation of propionic acids (Ma et al., 2019; Wang et al., 2018a). Ma et al. (2019) found that the propionic acid concentration was significantly reduced by 57% in the presence of 5% fruitwood biochar (based on TS) in the AD of dry chicken manure at the organic loading rate of 6.25 g VS/L/d. However, this effect has not been observed in the current study. As shown in Figure 5-3, the propionic acids profiles were similar in all the digesters. The results indicate that the effect of biochar on VFA degradation is case-specific and could be affected by many factors, which is subject to further studies.



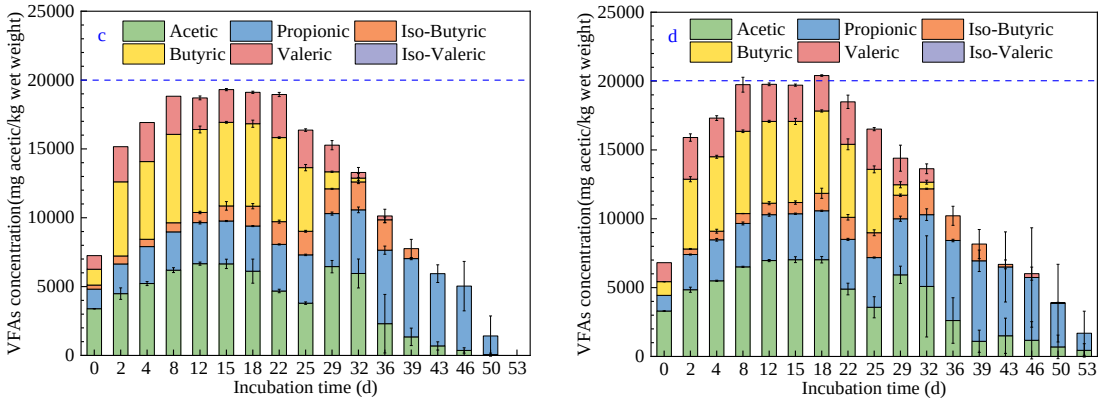


Figure 5-3. The variation and distribution of VFAs in the digesters amended with different biochars (a: Control; b: RBB; c: RRH; d: RPS)

5.3.4 Discussion

In this study, the application of biochar to dry co-digestion of PM and FW led to moderate promoting effects on CH_4 production performance under mesophilic conditions. The pecan shell derived biochar (PSB) performed best among the three biochars assessed, with a SMY increased by 12%. However, no significant effect on the peak CH_4 production rate was observed along with the individual addition of the selected biochars. The effects of the selected biochars on AD performance in this study are compared with previous studies in Table 5-4. In short, the stimulatory effects of biochar on CH_4 production in this study are not as significant as in previous studies. For example, Inthapanya et al. (2012) achieved a 31% increase of CH_4 yield in the wet AD of cattle manure by the supplementation of rice husk derived biochar (RHB), a much better performance than that (9.4%) obtained in the current study. Also, it was reported that the maximum CH_4 production rate could be enhanced by supplementing bamboo and rice husk derived biochar (Guo et al., 2018; Ma et al., 2019), which has not been observed in this study.

According to previous studies, biochar can enhance CH_4 production mainly through three mechanisms, i.e. pH buffering, providing supporting surface and enhancing electron transfer (Codignole Luz et al., 2018; Jang et al., 2018). It is often reported that the alkaline nature of

biochar could effectively improve CH_4 production by maintaining suitable pH ranges in AD through the bicarbonate buffer system (i.e., the balance of CO_2, HCO_3, and CO_3^{2-}) (Mumme et al., 2014; Wang et al., 2017a). In this study, the pH values of the assessed biochars (BB, RHB, and PSB) were all above 7. It means that the alkalinity of the AD systems would increase with the introduction of biochar, thereby improving the pH buffering capacity. However, owning to the high alkalinity of pig manure, the co-AD systems in current study could have possessed adequate buffering capacity already, as indicated by the stable pH and good performance of the Control (Figure 5-2). Therefore, the pH buffering function of biochar could be less significant in co-digestion, especially in a co-AD system receiving substrates with high alkalinity, like pig manure in this study.

Biochar was reported to be an excellent material to provide large supporting surfaces for microbes in AD systems owing to its physical features (in particular, porous structure and high specific surface area), which could facilitate biofilm formation, enhance microbial activity and strengthen microbes' resistance to various inhibitory compounds (Lü et al., 2016; Luo et al., 2015). Among the biochars adopted in this present study, RHB possessed the largest specific surface area (SSA, 149.30 ± 0.37 m^2/g) and total pore volume (0.108 cm^3/g), which are 1-2 orders of magnitude higher than that of the other two biochars (Table 5-2). However, the SMY of the RHB amended digesters was not the highest. Specifically, the highest SMY was obtained with the PSB which had the lowest SSA (6.14 m^2/g). The SMY of BB is only marginally lower than that of RHB, although its SSA is an order of magnitude lower than RHB. These results indicated that the SSA (and total pore volume) of biochars did not act a pivotal role in promoting methanogenesis in dry co-AD.

Another mechanism for biochar to enhance CH_4 production is to promote electron transfer. Using corn stover biochar, Lu¨et al. (2020) achieved a 46.9% enhancement in CH_4 yield in the AD of excess activated sludge. They attributed the improvement to the redox cycling of functional groups on the biochar surface to deliver electrons for methanogenesis. Yuan et al. (2018) also reported that the charging and discharging capacity of functional groups present on the biochar surface played a pivotal part in the electron-transport process. In this study, the

conductivity and cation exchange capacity of PSB were much higher than the two others, which is in line with the CH_4 yield in digesters amended with different biochar. Hence, the conductivity and cation exchange capacity of biochar might play an important part in stimulating methanogenesis in the dry AD systems.

Application of other biochars (derived from fruitwood, dairy manure, and cow manure) in high-solid AD systems was also assessed in few recent studies (Jang et al., 2018; Pan et al., 2019; Sun et al., 2019). The stimulatory effect observed in those studies is more remarkable than that in the current study. In those studies, all the dry AD systems were fed with the sole substrate, while co-substrates (PM and FW) were employed in the current study. Co-digestion of PM and FW can provide more balanced nutrients and suitable pH conditions for anaerobic microbes, thus mitigating the VFAs accumulation (Wang et al., 2020c). Thus, it can be inferred that the stimulating effects of biochar could be more significant in mono digestion than in co-digestion.

Table 5-4. Comparison of effects of biochar on AD performance between this study with the former studies

Biochar			**Conditions of AD***			**Improvements**					**Reference**
						Specific Methane yield (Nml CH_4/VS added)			**Increased maximum CH_4 production rate**	**Shortened lag phase time**	
Source	**Dosage (g/L)**	**Particle size (mm)**	**Feedstock**	**Total solid**	**Operating Model**	**Control group**	**Test group**	**Increase**			
Bamboo	16	0.075-0.15	Landfill leachate	< 5%	Batch	-	-	None	64%	21.4%	(Guo et al., 2018)
Rice husk	15	-	Sweet sorghum	< 5%	Batch	221–228	221–228	None	25 %	44 %	(Ma et al., 2020)
Rice husk	1:1**	1.7–2.0	Citrus peel waste	< 5%	Batch	165.9	172.1	3.7%	22%	4.4%	(Fagbohungbe et al., 2016)
Rice husk	1%**	< 1	Cattle manure	5%	Batch	-	-	31%	-	-	(Inthapanya et al., 2012)
Rice husk	1%*	< 1	Cattle manure	5%	Semi continuous	-	-	4-5%	-	-	(Inthapanya & Preston, 2013)
Cow manure	10	0.5-1.0	Beer lees	25%	Batch	210.5	378.1	82.6%	-	28.2%	(Sun et al., 2019)
Dairy manure	10	0.42-0.60	Dairy manure	>15%	Batch	380.0***	474.6***	24.9%	32.3%	26.9%	(Jang et al., 2018)
Fruitwood	5%	< 2	Chicken manure	12%	Batch	174	226-294	30-69%	8.6-31.4%	None	(Pan et al., 2019)
Bamboo	15	0.46-1.10	PM and FW	15%	Batch	258.3	278.7	7.4%	None	17.2%	This study
Rice husk	15	0.46-1.10	PM and FW	15%	Batch	258.3	282.7	9.4%	None	13.0%	This study
Pecan shell	15	0.46-1.10	PM and FW	15%	Batch	258.3	289.3	12.0%	None	17.8%	This study

* Operated under mesophilic condition. ** Biochar to substrate ratio based on dry mass. *** ml CH_4/VS removal.

5.4 Summary

In this study, the effects of commonly used biochars (bamboo, rice husk, and pecan shell) on dry co-AD were investigated under mesophilic conditions. The results showed that biochar addition moderately promoted the SMY in dry co-AD. In comparison, the supplementation of the selected biochars did not significantly affect the maximum CH_4 production rate in dry co-AD. The stimulatory effect of biochar on CH_4 production in dry co-AD is not as remarkable as reported in previous studies (mostly under mono digestion condition). Among the three mechanisms of enhancing methanogenesis by biochar (buffering, providing supporting surface and enhancing electron transfer), the first two mechanisms did not function significantly in dry co-AD, while the third mechanism (i.e., enhancing electron transfer) might play an important part in dry AD. It is recommended that the utilization of biochar for the enhancement of biomethanation in dry AD should be more focused on mono digestion in future studies.

Chapter 6
Conclusions and Recommendations

6.1 Overview

In this PhD research, several batch experiments under mesophilic conditions were conducted to: (1) investigate the effects of the TS content on the performance of anaerobic co-digestion of PM and FW, and the microbial (bacterial and archaeal) community structure, and metabolic characteristics (particularly methanogenic pathways); (2) explore the impact of different ammonia levels on methanogenesis and the mechanism of the alteration in the methanogenesis pathways; and (3) examine the stimulatory effects on methane production performance of dry AD of PM and FW by the supplementing the commonly used biochars (bamboo, rice husk, and pecan shell).

6.2 Main conclusions

6.2.1 Digestion performance, system stability and methanogenic pathways during the co-digestion of FW and PM under different TS content

The effects of the TS content on the performance of anaerobic co-digestion of PM and FW, the microbial (bacterial and archaeal) community structure and metabolic characteristics were assessed in Chapters 3.

(1) The specific methane yield had no significant difference with the increase of TS contents from 5% to 15% (278.8-291.7 NmL/g VS_{added}), while it was reduced at a 20% TS content (259.8 NmL/g VS_{added}). Moreover, the VS reduction decreased with the elevating TS content.
(2) Two peaks of total VFAs and daily methane production were observed in the high-solid digesters (TS above 10%). A new kinetics model was developed to describe the two-peak methane production behavior at high TS contents.

(3) The microbial community structure clearly showed the different evolutions of methanogenic pathways in low and high solids content systems, with a general shifting from the acetoclastic pathway to the mixotrophic pathway and the hydrogenotrophic pathway in dry AD (20%TS).

6.2.2 Distinguishing responses of acetoclastic and hydrogenotrophic methanogens to ammonia stress

The impact of different ammonia levels on methanogenic activities and the mechanism of the alteration in the methanogenesis pathways were analysed and discussed in Chapter 4.

(1) AM was proved to be much more sensitive to ammonia exposure compared to HM. The tolerance level of HMs to free ammonia (FAN, IC_{50}=1345 mg N/L) and NH_4^+ (IC_{50}=6050 mg N/L) was nearly 11 times and 3 times as those of AMs (NH_3, IC_{50}=123 mg N/L; NH_4^+, IC_{50}=2133 mg N/L), respectively.
(2) A low TAN concentration (1.0-4.0 g N/L) can cause irreversible inhibition of the acetoclastic methanogenic activity, while the inhibitory effects of ammonia under a certain level (TAN≤10.0 g N/L) on the activity of HMs were reversible.
(3) Differential tolerance of AMs and HMs to ammonia exposure might be associated with the cell morphology, multiple energy-converting systems, and Gibbs free energy from substrate-level phosphorylation.

6.2.3 Stimulatory effects on methane production performance of dry AD of PM and FW with the supplementation of biochar

The stimulatory effects on methane production performance of dry AD of PM and FW by the supplementing the commonly used biochars (bamboo, rice husk, and pecan shell) and possible mechanism were examined and discussed in Chapters 5.

(1) The specific methane yield was elevated with the supplementation of biochars by 7.9%, 9.4% and 12.0% for bamboo, rice husk and pecan shell derived biochar additions, respectively.
(2) Biochar did facilitate the degradation of poorly biodegradable organics and shortened the lag phase in dry AD. However, there was no significant effect on the peak methane production rate by the supplementation of the selected biochars.
(3) Among the three mechanisms of enhancing methanogenesis by biochar (buffering, providing supporting surface, and enhancing electron transfer), the third mechanism (i.e., enhancing electron transfer) might play an important part in dry AD process.

6.3 Significance of findings

6.3.1 Clarification of the TS effects on the performance of PM/FW co-AD

The detailed study of co-digestion of PM and FW under different TS conditions at lab-batch scale clarified the TS effects on the performance of PM/FW co-AD under mesophilic conditions, and it provided detailed information on methane yields, kinetics, and AD process stability. These information on methane yields at different TS contents, can provide significant references for practical applications of AD, such as economic analysis of on-farm dry co-digestion of PM and FW in Ireland, and identification of optimal TS contents in the design and operation of PM/FW AD. The data of the methane yield under different TS conditions achieved in this study can be used as a basis for economic analysis of on-farm dry co-digestion of PM and FW.

6.3.2 Establishment of a new mathematical model for two-peak methane production behaviour

In this study, a new superimposed model was developed by coupling the first-order kinetic model with the modified Gompertz model, based on the two-peak methane production at high TS contents, resulting from the different bioavailability of organic compounds. This model can be applied to predict the methane yield of AD with a two-peak methane production behavior at high TS contents. In addition, the parameters in this model are linked to the degradation of the readily and poor biodegradable organic compounds, which can provide detailed information on the degradation kinetics of organic matters. This newly developed model provides an alternative to commonly used kinetic models (the first order and the modified Gompertz models), especially for predicting methane production from AD under high TS conditions.

6.3.3 Identification of evolutions of the methanogenic pathway in wet/dry AD

The lab-scale batch experiment provided information on different evolutions of methanogenic pathway in wet and dry AD. The insightful view of the impacts of TS content on the methanogenic pathway can contribute to an in-depth understanding of dry AD. Additionally, the identification of shifts in archaeal community organisation towards hydrogenotrophic methanogenesis in dry AD may be useful for the development of strategies for improving dry AD performance, such as adding syntrophic acetate-oxidizing bacterium into dry AD systems. Besides, there is a potential to upgrade biogas by controlling the domination of hydrogenotrophic methanogenesis in AD systems.

6.3.4 Filling knowledge gap concerning the differential ammonia tolerance of acetoclastic and hydrogenotrophic methanogens

The consensus from different studies is that HMs are much more resistant to ammonia inhibition than AMs, but it is inferred from microbial community changes or metabolic

shifts under ammonia stress in most studies. In this study, direct comparisons of the ammonia tolerance levels of these two types of methanogens were carried out. In addition, the ammonia tolerance levels of AMs and HMs were quantitatively compared and the viability and recoverability of HMs and AMs after acute ammonia inhibition were assessed. The study provides direct evidence for supporting the general belief that the AMs were more impacted by FAN. Furthermore, these findings in this study could contribute to a better understanding of the different responses of AMs and HMs to ammonia stress and provide useful information for developing tailored operating strategies to mitigate ammonia inhibition in practical applications. For example, lowering pH would be less effective in relieving ammonia inhibition for the HMs-dominated AD system, because the inhibition by NH_4^+ would be more serious when relieving the FAN impact by lowering pH.

6.3.5 Provision of a feasible method to improve dry AD performance

Dry AD often suffers from low methane production and VS reduction. It is necessary to enhance the methane production performance of dry AD. These findings in this study verified that the addition of biochar is a potential strategy for the enhancement of dry AD. Additionally, carbon-rich biochar is a cost-effective material, and biochar blended in digestate after digestion could directly be used as soil amendments without any environmental threats. Therefore, biochar is a feasible method to improve dry AD performance in practice. The findings offer useful information on the practical application of biochar in dry AD process.

6.4 Summary

Through co-digesting of PM with FW at different TS contents, the digesters could achieve stable methane production. However, the elevated TS content (above 15%) led to the decline of methane yield and the VS reduction. A newly developed kinetics model satisfactorily described the two-peak methane production behavior at high TS contents.

Besides, the different evolutions of methanogenic pathways in low and high solids content systems were observed, with a general shifting from the acetoclastic pathway to mixotrophic pathway and hydrogenotrophic pathway in dry AD (20%TS). The distinguishing responses of acetoclastic and hydrogenotrophic methanogens to ammonia stress were observed in the mixed cultures, and a low TAN concentration can cause irreversible inhibition of the acetoclastic methanogenic activity, while the inhibitory effects of ammonia under a certain level on the activity of HMs were reversible. These could contribute to the alteration in the methanogenesis pathways under high ammonia stress. Additionally, the methane production performance of dry AD of PM and FW could be stimulated by appropriately supplementing the biochar, which might enhance electron transfer in dry AD process. Therefore, these findings could contribute to an in-depth understanding of wet and dry AD and provide an effective approach to improve the performance of dry AD.

6.5 Recommendations for future research

Several recommendations regarding future research directions are made based on the findings of this study, as follows.

(1) *Exploration of the metabolic pathway in dry co-digestion of FW and PM*. Chapter 3 showed a distinct shifting of methanogenesis pathways in dry AD. Several studies reported the biodegradation of acetate gradually shifted from acetoclastic methanogenesis to the syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis (SAO-HM) pathway in mesophilic AD reactors under high ammonia stress, but there is no direct evidence showing that the SAO-HM pathway dominates in dry AD of FW and PM. The microbiological tools (like metagenomic sequencing) can be employed in the future studies. Understanding the metabolic pathway in dry co-digestion of FW and PM can provide an in-depth understanding of dry AD, which

will provide a foundation for developing efficient approaches to improve dry AD performance.

(2) *The effects of elevating ammonia concentration on the semi-continuous dry co-digestion of FW and PM.* Chapter 4 showed the distinguishing responses of acetoclastic and hydrogenotrophic methanogens to ammonia stress. These responses to ammonia stress are based on the short-term exposure. There is no idea about the effects of elevating ammonia concentration on dry AD on a long-term operation. The ammonia inhibition on the semi-continuous dry AD process should be further studied.

(3) *Upscaling semi-continuous dry co-digestion of FW and PM.* The performance and stability of dry co-digestion systems should be assessed using a semi-continuous operation model in the long term to provide reliable data for the design, construction, and operation of the dry AD systems in industrial practices.

(4) *Technical-economical analysis on dry co-digestion of FW and PM.* The technical-economical studies of dry co-digestion systems should be conducted based on a typical farm in Ireland to assess the feasibility in practice.

(5) *Taking advantage of the dominating the SAO-HM pathway in AD under high ammonia stress*. An AD system with the dominating SAO-HM pathway should be active in hydrogenotrophic methanogenesis preferably using hydrogen as the substrate. With sufficient hydrogenotrophic methanogens in this anaerobic system, a biotechnology will be developed for upgrading biogas to biomethane. This anaerobic system should be equipped with a gas recirculation system and fed with hydrogen as the electron donor to reduce CO_2 to methane.

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Appendix A
Chapter 3 supplementary information

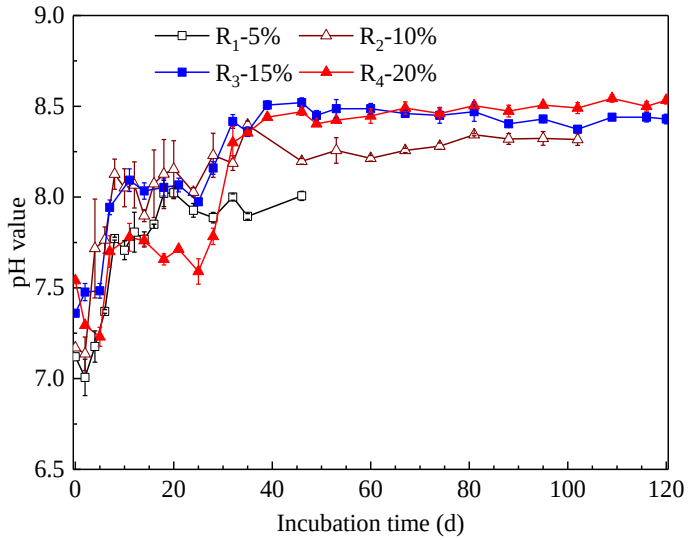

Figure A-1. Evolution of pH in digesters operated at different initial TS contents.

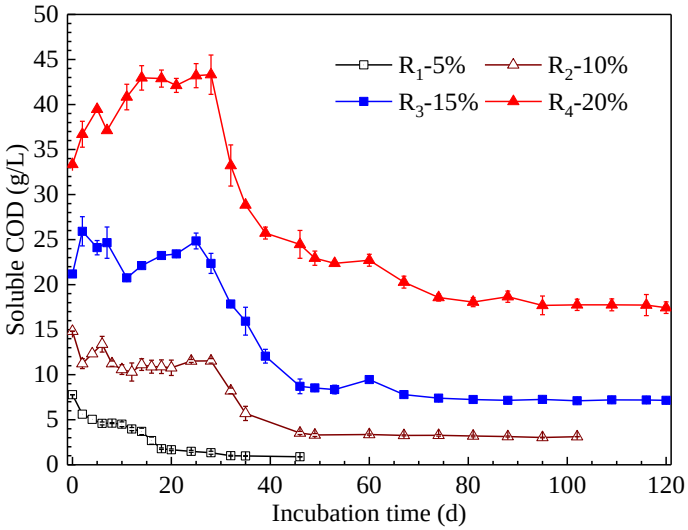

Figure A-2. Evolution of soluble COD in digesters operated at different initial TS contents. COD: chemical oxygen demand.

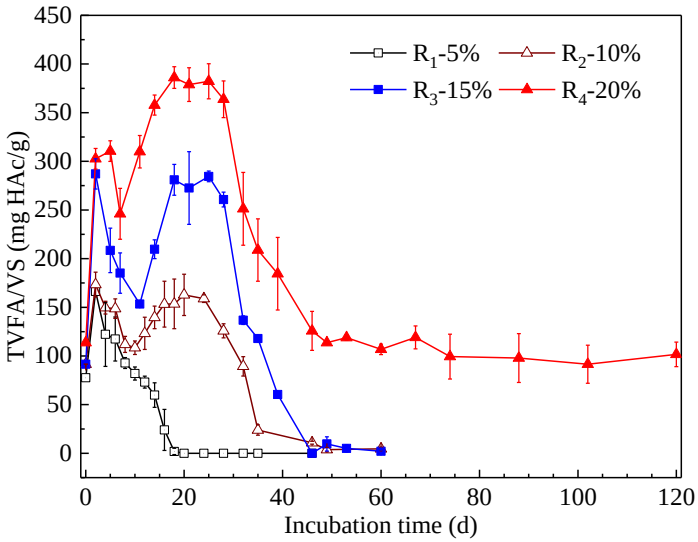

Figure A-3. Evolution of TVFA/VS ratio in digesters operated at different initial TS contents. TVFA: total volatile fatty acid; VS: volatile solid; TS: total solid.

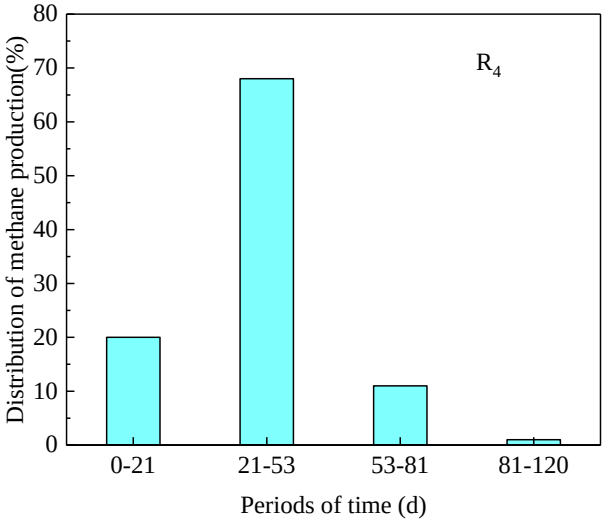

Figure A-4. Distribution of methane production of dry anaerobic digesters (TS 20%) over different periods of time.

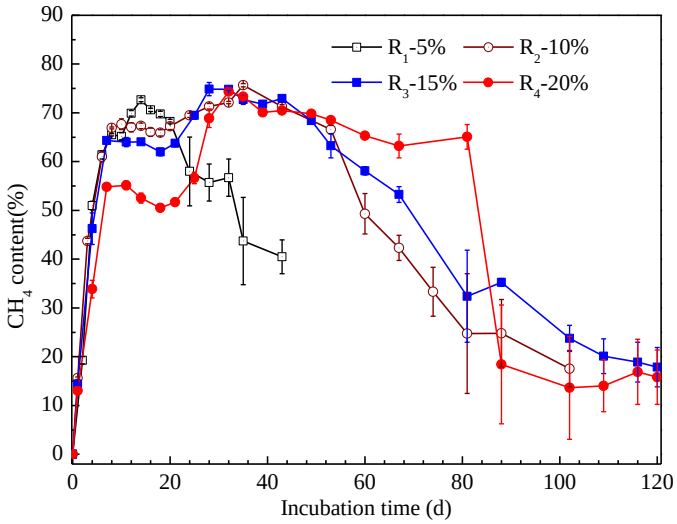

Figure A-5. Evolution of CH_4 content in digesters operated at different initial TS contents.

Table A-1. Kinetic parameters of the first-order kinetic model and the modified Gompertz model *

Reactors	First order model			Modified Gompertz model			
	P_{max} mL/g VS	k d^{-1}	Adj. R-Square	P_{max} mL/g VS	R_m mL/g VS/d	λ d	Adj. R-Square
R_1(5% TS)	320.1	0.07	0.9611	282.1	18.1	1.9	0.9952
R_2(10% TS)	300.1	0.05	0.9949	289.7	10.2	0	0.9935
R_3(15% TS)	308.2	0.03	0.9582	294.6	7.4	1.3	0.9830
R_4(20% TS)	325.9	0.02	0.9163	267.6	6.2	11.1	0.9774

* TS: total solid; VS: volatile solid.

Appendix B
Chapter 4 supplementary information

B.1 Living cell staining

The LIVE/DEAD® BacLightTM cell viability kit (L-7012, Molecular Probes, USA) was employed to measure the number of living microorganisms during the batch inhibition experiments. To make sure that the cells were placed in a monolayer on the glass slide as much as possible, the samples collected from serum bottles were dispersed using ultrasonic waves. Prior to ultrasonication, each sludge sample (5 mL) was centrifuged and washed with PBS solutions and was then diluted with the deionized water (as much as 62.5 times for AMs and 50 times for HMs). Ultrasonic waves (30 kHz) were applied to the diluted sample for 45-60 seconds with using an ultrasonic disrupter (VWR USC100T). Afterwards, the prepared samples were stained with using the cell staining kit according to the manufacture's protocol. After 20-30 minutes, 8 µL of the labelled sample was placed on the glass slide with a micropipette for the microscopic observation, using a fluorescence microscope (ECLIPSE 90i, Nikon, Japan; DS-Fi1, Nikon, Japan) equipped with a bandpass filter cube. A binarized image analyzing software (NIS-Elements BR 3.2 64-bit, with 1280×960 pixels) was used to measure the fluorescent cell area in a microscopic field at a 100-fold magnification (10×20). At the magnification the software covered 0.568 mm2 of the coverslip area (pixel resolution = 0.68 µm).

B.2 Supplementary curves

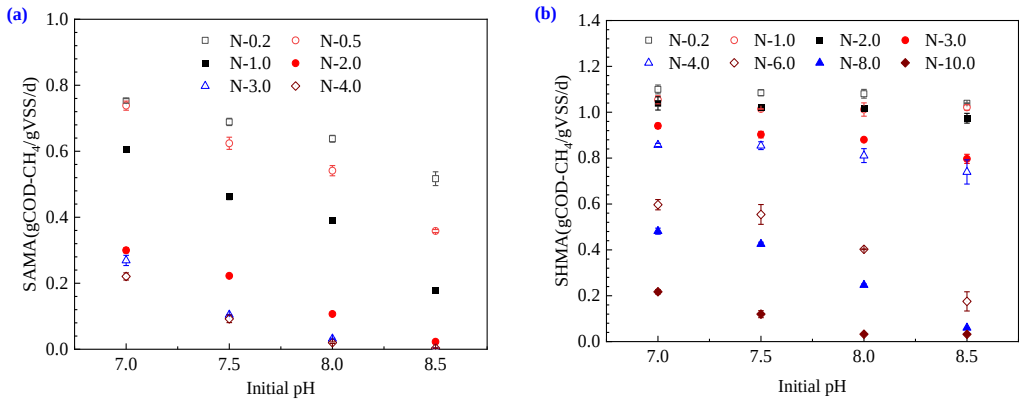

Figure B-1. Inhibitory effects of pH on the activity of AMs (a) and HMs (b). AMs refers to as acetoclastic methanogens; HMs refers to as hydrogenotrophic methanogens.

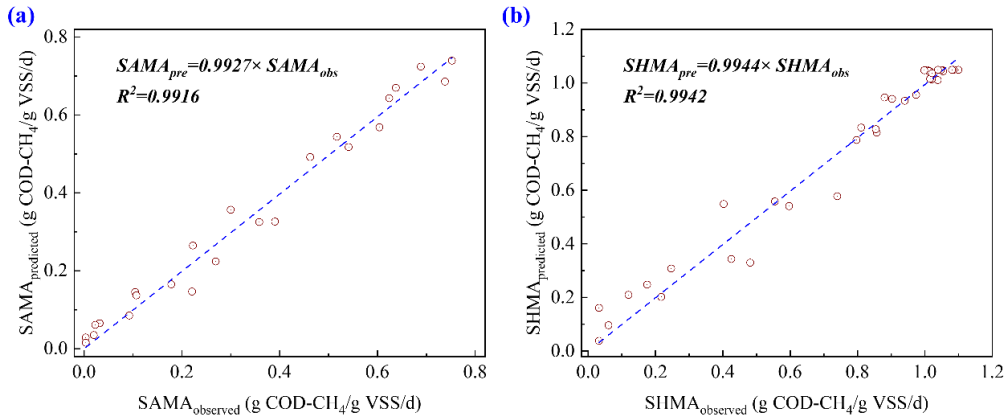

Figure B-2. Fitting with the modified Monod inhibition model for the inhibitory effects on AMs (a) and HMs (b) in terms of FAN and NH_4^+ concentration.

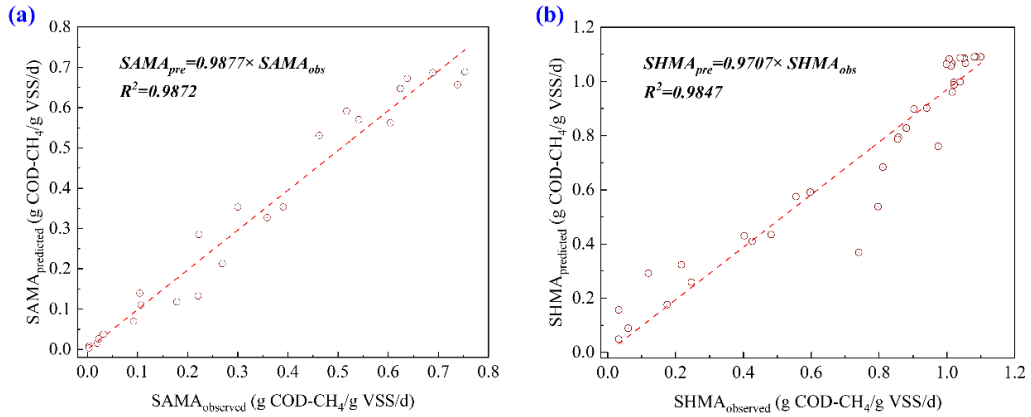

Figure B-3 Fitting with the simple Monod inhibition model for the inhibitory effects on AMs (a) and HMs (b) in terms of FAN and NH_4^+ concentration.

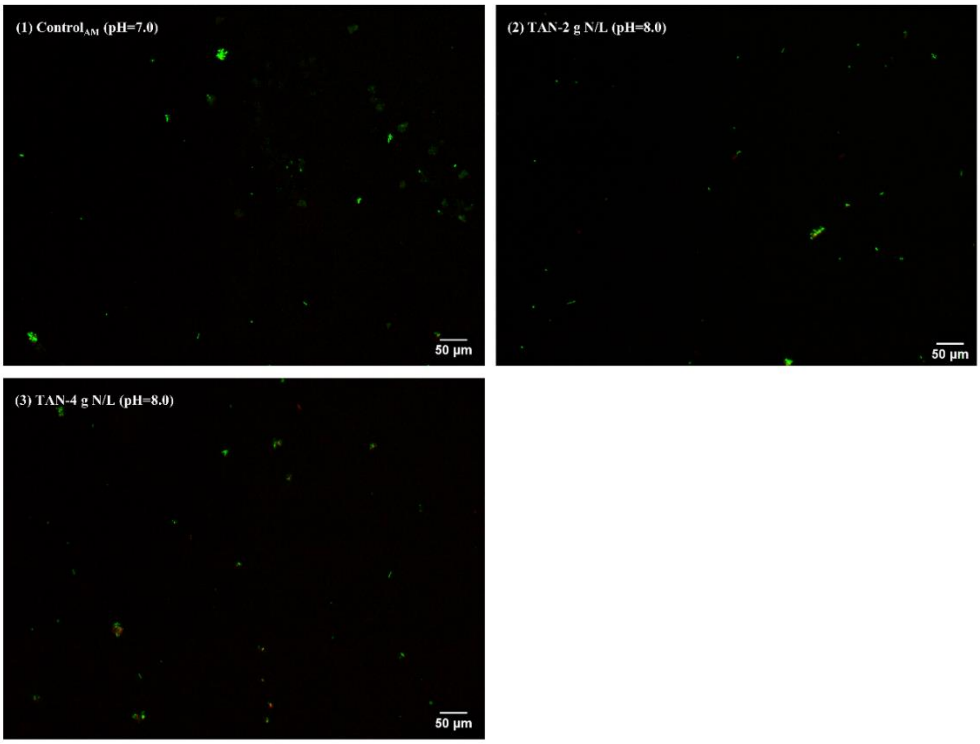

Figure B-4 Fluorescent stained living and dead cell for AMs under different TAN concentrations. (100 magnification with 62.5-time sample dilution)

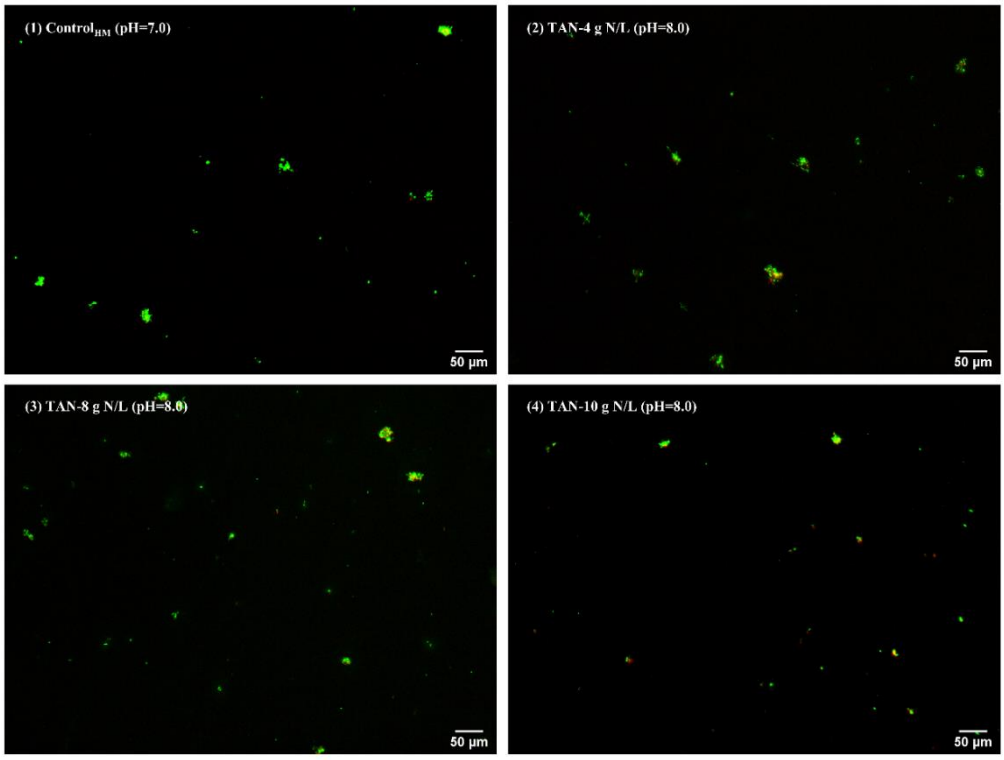

Figure B-5 Fluorescent stained living and dead cell for HMs under different TAN concentrations. (100 magnification with 50-time sample dilution)