Effects of Nano-bubbles on Biogas Production from Lignocellulosic Biomass and Its Mechanism

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Abstract

The irregular climate change and energy crisis are considered to closely associate with excessive use of non-renewable fossil fuel, and biofuel may be a good solution for these issues. Biofuel generation from renewable resources can solve the energy shortage as well as the associated environmental problems. Agricultural wastes, such as crop straws have gained much attention due to their abundant distribution, carbon neutrality and ability to sidestep competition with food crops for land. They have also demonstrated significant potential to satisfy the feedstock targets for the advanced 2nd-generation biofuels.

Anaerobic digestion (AD) of lignocellulosic biomass is a promising approach for bioenergy production. AD of lignocellulosic biomass has been widely applied to make up the overuse of fossil fuels. However, the low energy conversion efficiency limits its application mainly due to the following two aspects: 1) the low metabolic activity of microbes, and 2) the rigidity and recalcitrant structure of lignocellulose. To cope with the above problems, various pretreatments including physical, chemical and biological methods have been attempted to enhance biogas production from AD of lignocellulosic biomass. However, the improvement of methane production is difficult to balance the additional energy consumption, high process cost and secondary pollution potentials due to the additives addition. Thus, environment-friendly methods are still urgently required to meet the sustainable development goals (SDGs).

Nanobubble water (NBW) is defined as the water containing gas nanobubbles (NBs) with diameter less than 1000 nm that have some special physicochemical properties (negative surface charge, rapid mass transfer rate and better stability). The chemical reactions at the gas–liquid interface can be enhanced by injecting NBW. The hydrophobic attractiveness force property of NBs enables them to adhere to solid surfaces, thus promoting the metabolism and chemical reactions. NBW has been proven to enhance methane production from AD of waste activated sludge. However, effect of NBW on CH_4 production from AD of lignocellulosic biomass has yet not been studied. In addition, mechanisms involved in the enhancement of AD remains unclear.

Therefore, my studies attempted to investigate the effects of NBW addition on AD of lignocellulosic biomass. The main objectives of this study were: 1) to elucidate the possible mechanisms involved in the NBW-supplemented AD process; 2) to explore the exact role of O_2 released by O_2-NBW on microorganisms and their metabolism; and 3) to realize the application

NBW-based AD technology without increase of reactor volume. Major results from this study can be summarized as follows:

(1) CH_4 production from AD of refractory cellulose was investigated at a high loading of 3.5 ($VS_{cellulose}/VS_{inoculum}$) under NBW addition. A longer proton spin-spin relaxation time (2611 - 2906 ms) of NBW during 35 days' storage reflected its high mobility and diffusion of water molecules. Higher volatile fatty acids were yielded at the hydrolysis-acidification stage under NBW addition. Methanogenesis tests showed that Air-NBW and CO_2-NBW supplementation accelerated the utilization of crystalline cellulose, achieving methane yields of 242 and 225 NmL/g-VS, increasing by 18% and 10% compared to deionized water addition (the control), respectively. In addition, under NBW addition the cellulose crystallinity reduction was enhanced by 14–20% with microbial community being enriched with hydrolytic and methanogenic bacteria.

(2) The effect of O_2-containing gas NBW for providing micro-oxygen environmental was explored on AD of cellulose for methane production. The cumulative methane yields from the reactors with Air-NBW (193 NmL/g-VS), N_2-NBW (196 NmL/g-VS) and O_2-NBW (233 NmL/g-VS) addition were increased by 8–30% in comparison to the control (with the same amount of deionized water (DW) addition) (179 NmL/g-VS). Under NBW addition, the reductions in cellulose content and cellulose crystallinity were respectively enhanced by 8–14% and 9–21% during AD, in which cellulase activity was elevated by 10–21%. The O_2-NBW reactor was found to have the highest electron transport system activity, increasing by 1.7 times compared to the control, most probably due to the collapse of O_2-nanobubbles and release of O_2 resulting in a micro-oxygen environment under the test condition. Besides, microbial community analysis suggests that the direct interspecies electron transfer could be quickly established with the addition of NBW.

(3) In order to avoid the increase of digester volume in the NBW-based AD system in practice, anaerobically digested sludge was first pre-augmented by N_2-NBW and O_2-NBW using corn straw as sole substrate for methane production with electron transfer activity being monitored. 20%, 33% and 38% of cellulose and 29%, 35% and 35% of hemicellulose were reduced respectively from the control, N_2-NBW and O_2-NBW pre-augmented sludge reactors. N_2-NBW and O_2-NBW pre-augmented sludge reactors achieved methane yields of 127 and 142 NmL/g-VS, about 10% and 22% higher than that from the control. Results show that use of NBW pre-

augmented anaerobically digested sludge as inoculum can remarkably enhance methane yield from corn straw.

Use of NBW can improve methane production from AD of lignocellulosic biomass. The NBW-based AD technology has low ecological risk with relatively low energy consumption. This thesis demonstrates the concept and fundamental of NBW-based AD systems, and the pre-augmentation of anaerobically digested sludge and then the improvement of methane production from corn straw can target no increase of AD reactor volume in practice and sustainable management of lignocellulosic biomass.

Keywords: Lignocellulosic biomass; Anaerobic digestion; Methane production; Miro-oxygen environmental; Pre-augmentation; Nanobubble water.

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Abbreviations and acronyms

AD	Anaerobic digestion
ANOVA	One-way analysis of variance
BD	Biodegradability
BMP	Biochemical methane potential
CBB	Coomassie Brilliant Blue
CHP	Combined Heat and Power
CrI	Crystallinity index
DNS	Dinitrosalicylic acid
DO	Dissolved oxygen
DW	Deionized water
ETS	Electron transport system
F/M	Feed-to-microorganisms
HAc	Acetic acid
HPr	Propionic acid
IEA	International Energy Agency
iso-HBu	iso-butyric acid
iso-HVa	iso-valeric acid
NBs	Nanobubbles
NBW	Nanobubble water
n-HBu	n-butyric acid
n-HVa	n-valeric acid
OLR	Organic loading rate
ORP	Oxidation-reduction potential
PCA	Principal component analysis
RA	Relative abundance
SD	Standard deviation
SDGs	Sustainable development goals
T_2	Proton spin-spin relaxation time
TMP	Theoretical methane production
TOC	Total organic carbon
TS	Total solids
VFAs	Volatile fatty acids
VS	Volatile solids
XRD	X-ray diffraction

Chapter 1 Introduction

1.1 The issues of energy crisis and environmental pollution

1.1.1 The current issues

The global total primary energy supply was reported to expand 1.6-fold from 1990 to 2017 (14 billion tons of oil equivalent in 2017) (IEA, 2020). Most of global energy supply relies on fossil fuels (e.g., oil, coal and natural gas), which was estimated to share 81% of global energy supply in 2017 (IEA, 2020). Namely, fossil fuels are the major energy supply, even though they are non-renewable energy resources. The irregular climate change and energy crisis are considered to closely associate with excessive use of non-renewable fossil fuel (Robertson *et al*., 2017). An increasing attention paid to environmental issues also promoted the development of various technologies and policies for resources recovery. Biofuel generation from renewable resources can alleviate fossil fuels demand as well as the associated environmental problems. Thus, in the 21st century overcoming renewable energy conservation is one of the most challenging subjects.

1.1.2 Alternative renewable energy

There is a great demand for seeking the clean or pollution free methods to gain the biofuel from renewable resources. Among renewable energy sources, including biomass, solar, wind, hydroelectric, geothermal, etc., biomass energy is not only the most widely used traditional energy but also the largest use of renewable energy on earth, ranking the fourth in current energy consumption (Hall, 1991). Sustainable and renewable energy and biofuels from lignocellulosic feedstocks are considered as promising alternatives to non-renewable resources (Baeyens *et al*., 2015). The huge amount of annual lignocellulosic biomass production may bring about environmental pollution and waste of resources if not being properly treated. In this context, anaerobic digestion (AD) is usually applied to treat lignocellulosic biomass for renewable energy (mainly CH_4) recovery. As outlined in the reported of International Energy Agency (IEA) Energy Technology Perspectives 2017, the requirements for sustainable bioenergy use, suggest that biomethane for transport should rise to 3.74 EJ by 2040 (IEA, 2020).

1.2 Lignocellulosic biomass: a renewable resource of biofuel

1.2.1 Lignocellulosic biomass

Lignocellulosic biomass is the most abundantly available raw material on the earth for the production of biofuels to make up the overuse of fossil fuels. It is estimated that 181.5 billion tons are produced annually (Dahmen *et al*., 2018). In USA, annually 80-90 billion gallons of biofuels are produced from 1.3 billons tons of lignocellulosic residues, which could replace 40% of the national fuel consumption (Fatma *et al*., 2018). Lignocellulosic biomass is primarily composed of plant cell wall containing three main components, i.e. cellulose (5-55%), hemicellulose (12-50%) and lignin (6-40%). Table 1-1 lists the potential lignocellulosic raw materials and their chemical compositions.

(1) Cellulose

Cellulose, a type of linear polysaccharides, is composed of several glucose units linked by β-1,4 glycosidic bonds. The saccharide chains are connected by hydrogen bonds and aggregated to form a three-dimensional structure of fibrils, which can be characterized by toughness and water insolubility (Wang and Zhang, 2013). Cellulose molecules have varying degrees of crystallinity throughout the structure. Cellulose consists of two regions: amorphous (low crystallinity) and crystalline (high crystallinity) regions (Atalla and VanderHart, 1984). The crystallinity of cellulose can be analyzed by the crystallinity index. Cellulose is one of the most abundant, cheapest and easily available resources in nature, accounting for 40–50% of lignocellulosic biomass (Cocero *et al*., 2018). Biogas from AD of cellulose is becoming more competitive due to the fact that the annual cellulose production is approximately 1.8×10^{11} tons (t) estimated from plants (Sundarraj and Ranganathan, 2018), thus cellulose is an important source of biomass energy.

(2) Hemicellulose

Hemicellulose is a kind of polysaccharides, composed of xyloglucans, mannan, xylans glucomannans, and glucans (Scheller and Ulvskov, 2010). The β-(1→3, 1→4)-glucans constitutes the chain structure of hemicellulose. The specific structure and abundance of hemicellulose has been noticed among different species (Scheller and Ulvskov, 2010). In contrast to cellulose, hemicellulose is more amorphous. The short and branched chains of hemicellulose contribute to

microfibers build a network and interact with lignin, rendering the cellulose-hemicellulos-lignin matrix extremely hard. Amorphous and branched-chain hemicellulose is highly susceptible to degradation by biological, thermal and chemical methods.

(3) Lignin

Lignin is a complex and large aromatic and hydrophobic amorphous heteropolymer, which is constructed of phenylpropane unites (e.g. coniferyl and sinapyl alcohol) (Hendriks and Zeeman, 2009). Lignin together with cellulose and hemicellulose can form a rigid three-dimensional structure of the cell wall as a cement. Thus, it is water insoluble and optically inert. Lignin can be dissolved in water at neutral pH, acid/alkaline conditions or high temperature (180 °C) depending on its precursors. Lignin is reported as the main obstacle to the utilization of lignocellulosic biomass in the process of biotransformation. The higher lignin content, the more difficulty of biomass to be degraded by biologically and chemically methods.

1.2.2 Lignocellulosic biomass conversion and bottleneck issues

AD of lignocellulosic biomass is a promising approach for bioenergy (mainly methane) production (Xu *et al*., 2019). AD has been implemented for the management of agricultural residues and organic wastes for years due to its environmental sustainability and low cost (Kahia *et al*., 2019). The economical driver of AD technology is the biogas (mainly CH_4 and CO_2) production, which can be converted into electricity and thermal energy by Combined Heat and Power units (CHP). Although AD of lignocellulosic biomass has been widely applied to achieve the sustainable management of agricultural wastes, the low energy conversion efficiency limits its application mainly due to the following two aspects: 1) the low metabolic activity of microbes, and 2) the rigidity and recalcitrant structure of lignocellulose (Huang *et al*., 2019).

1.3 Current methods used to improve lignocellulosic AD efficiency

To cope with the above problems, various methods have been attempted on lignocellulose to improve its CH_4 production efficiency. These methods are keen to disrupt the naturally recalcitrant carbohydrate-lignin shields that impair the accessibility of enzymes and microbe to cellulose and hemicellulose. The function of pretreatment for AD is to overcome the limiting step of substrate hydrolysis by enhancing the enzymatic degradability of lignocellulosic biomass. The

pretreatment methods for AD are reviewed as following 3 main ones: physical, chemical and biological process.

1.3.1 Physical pretreatment

Physical methods mean not use of chemicals or microorganisms during the pretreatment, such as comminution, extrusion, steam-explosion, and irradiation (microwave and ultrasound). Physical pretreatment can greatly enhance methane production from AD of lignocellulosic biomass (agricultural residuals). The particles size of lignocellulosic biomass must be reduced to 1-2 mm to alleviate the limitation of hydrolysis, which is a very expensive operation consuming about 33% of the total electricity demand of the whole process (Abraham *et al*., 2020). The comminution may cause an increase in the methane yield (12%) from AD of maize straw (Witaszek *et al*., 2020). Steam explosion pretreatment can decrease the hemicellulose content of corn straw by 28% and increase the neutral detergent solute by 24% (Shi *et al*., 2019). Severe steam explosion, excessive decomposition of hemicellulose and cellulose into AD inhibitory compounds (HMF and furfural), which may decrease the AD. In general, microwave has not been used individually for lignocellulosic biomass pretreatment. Bamboo shoot shell after microwave combined with fungal metabolism pretreatment can yield the increased methane of 163% compared to the control group, due to the increased dissolved products for microbial action under microwave irradiation (Fang *et al*., 2020).

1.3.2 Chemical pretreatment

Chemical methods refer to the use of chemicals (e.g. acids, alkalis, oxidation and ionic liquids) to change the physical and/or chemical properties of lignocellulosic biomass. Acids and alkalis are widely used in the pretreatment to improve CH_4 production from AD of lignocellulosic biomass (Monlau *et al.*, 2012). Phosphate addition could also accelerate the biogasification process of rice straw particles and total solids or volatile solids reduction to a certain extent (Lei *et al*., 2010). Ozone pretreatment can effectively degrade wheat straw lignin with resultant enhanced hydrogen production (158%) (Wu *et al*., 2013). Chemical pretreatment may leave chemical residues that affect the downstream AD process and secondary pollution to cause environmental pollution.

1.3.3 Biological pretreatment

The biological methods to increase biogas production mainly focus on fungal, microbial consortium and enzymatic pretreatment. Compared with physical and chemical pretreatment, biological methods usually require much lower energy input and no chemicals addition, which are conducted under milder environmental conditions, and almost no negative impact on AD. As reported, *Trichoderma reesei* addition effectively removed more lignin by 23% compared to the untreated rice straw (Mustafa *et al*., 2016). Fungal pretreatment can also accelerate methane production from AD of rice straw due to its enhancement effect on hydrolysis (Kainthola *et al*., 2019). The microbial consortium LTF-2 pretreatment on rice straw showed cellulose, hemicelluloses and lignin losses by 71.7%, 65.6% and 12.5%, respectively (Zheng *et al*., 2020). The conversion yield of cellulose to glucose reached 96.7% after 59 h of enzymatic hydrolysis from corncob residues (Liu *et al*., 2020b). However, the longer pretreatment time required by biological methods limits their use in commercial applications. In addition, compared with single pretreatment methods, combination pretreatment may be beneficial due to higher methane production and more complete biomass utilization. For instance, microwave assisted NaOH pretreatment of wheat straw greatly enhanced cellulose, hemicellulose and lignin reduction (Tsegaye *et al*., 2019). Microwaves and alkaline pretreatments on olive pomace properties achieved 13% methane production compared to control group (Elalami *et al*., 2020).

1.3.4 Other promising pretreatment for enhanced

The above methods may bring about secondary pollution, a large quantity of chemicals consumption and high operation cost. These pretreatment methods to a great extent make them difficult to be compensated by the increased CH_4 yield. Micro-oxygen technology reflect great potentials to improve the methane yield from AD of lignocellulosic biomass (Nguyen and Khanal, 2018). Liew *et al*. (2011) reported that improve the biological activity of the inoculum to promote methane production from AD of fallen leaves.

The oligosaccharides produced during high loading cellulose degradation cause the mass transfer resistance, and reduce the accessibility of water molecules to the surface of the cellulose particles (Vaquerizo *et al*., 2018), thus hindering cellulose degradation to produce methane.

Nanobubble water (NBW) has been reported to have high-diffusion water molecules, which may break down the oligosaccharide layer produced at high cellulose loadings. In addition, NBW has been proven to enhance methane production from AD of waste activated sludge (Wang *et al*., 2019a; Yang *et al*., 2019). Effect of NBW on CH_4 production from AD of lignocellulosic biomass has yet not been studied.

1.4 NBW technology and NBW-based AD

1.4.1 NB characterization

NBW contains 10^6–10^8 gas bubbles per milliliter with diameter smaller than 1000 nm and possesses unique properties. Among them, the most amazing property is its improvement effect on the physiological activity of living organisms (Ebina *et al*., 2013). NB can exist on surface (surface and interfacial NBs) (Theodoraki and Che, 2019) and disperse in a liquid phase (Oh and Kim, 2017). NBs denote two distinguished cases of nanoscale size bubbles, surface and free bubbles. Surface NB refers to a pocket-like space filled with gas (such as air, N_2, O_2, CO_2, etc.) forming at the interface with solid substrates (Theodoraki and Che, 2019). Free bubbles exist in the liquid along with Brownian motion (Kim *et al*., 2020). Surface NB instead of free bubbles has been applied in industrial, agricultural and medical fields (Theodoraki and Che, 2019) (Table 1-2).

(1) Stabilization of NBs

The diameter of bubbles is less than 5 μm, the virtual disappearance of buoyant force. Because its buoyant force is less than any current in the liquid and also submerged by the repulsive forces between other bubbles and other interaction forces (Zimmerman *et al*., 2011), bubbles with nanometer diameter can exist in liquid for a long time. NB could stay in the liquid for a long time, and the inside of NB with a higher pressure could fend off pressure from outside of NB. There are too few vapor atoms inside NBs, thus the inside of NBs would not be high enough to break through the force balance of NBs (Nagayama *et al*., 2006). As stated by Theodorakis and Che (2019), the pressure of the gas phase was estimated to be similar to the outside of NB, which might be the reason for the longer lifetime of NB in liquid. The internal pressure of NB calculated by curvature radius and bubble size determined by atomic force microscopy can further verify the above statement (Theodoraki and Che, 2019). In addition, NBs are more stable in alkaline

condition, due to the more adsorption of OH^- ions at the gas/water interface enhances the double-layer repulsive force, which can prevent bubble aggregation and coalescence (Hamamoto *et al*., 2018).

(2) Specific surface area of NB

NBs present a high specific surface area, which has been widely used for flotation in the mineral industry (Michailidi *et al*., 2019). The surface area increases with decreasing diameter, thus NBs reflects higher surface area comparing with micro-bubble or general bubble. The large specific surface area on NB surface results in a considerable capacity for water purification, which provides a very suitable space or site for microbe and contaminants (Yoshida *et al*., 2008). NB is able to remain in the suspension with the contaminants, increasing the probability of pollutant/adsorbent contact (Kyzas *et al*., 2019). Biological and chemical reactions can be enhanced on the surface of NB, providing a great potential for practical application. NB act as carries to transfer the small molecules of organic matters and trace elements (Kyzas *et al*., 2019). Zhang *et al*. (2008) evidenced that NB's high contact angle (low gas-side contact angle) could be attributed to the attractive forces between the solid-air and liquid-air interfaces. In addition, Hu and Xia (2018) pointed out that the smaller bubbles or their larger specific surface area may results in higher mass transfer efficiency.

(3) High mass transfer of NB

The mass transfer efficiency increases with decrease in the NB radius (Li *et al*., 2014). The high gas mass transfer efficiency of NBs dramatically improves dissolved oxygen supply for mitigating sediment anoxia and controlling nutrient release from sediments (Zhang *et al*., 2018b). Xiao and Xu (2020) confirmed that O_2-NBW improves 1.5 times higher O_2 transfer efficiency in aerated biofilm growth. Wang *et al*. (2020) elucidated that NBW greatly increase the photodegradation of oxytetracycline by its high mass transfer. Phan *et al*. (2020) presented that high mass transfer of NBW could improve seasoning boiled egg uniform impregnation efficiency. The low tendency to coalesce, the large interfacial area, and the low rise can obviously enhance the gas mass transfer efficiency even in the high viscosity liquid. In the field of biology, gas transfer property has a great positive impact on the AD process, especially on the growth and metabolism of microorganisms (Weber and Agblevor, 2005). Furthermore, compare with the traditional bubble aeration process, the oxygen utilization rate and volumetric mass transfer

coefficient of O_2-NBW is almost double in the synthetic wastewater treatment unit (Temesgen *et al*., 2017). The mass transfer rate of O_2 in the O_2-NBW is found 125 times faster than that of macro bubble water (Temesgen *et al*., 2017).

de Paula *et al*. (2019) stated that the mass transfer rate increased due to characteristic like low viscosity. The high viscosity of anaerobic digested liquor is responsible for the lower mass transfer efficiency (Weber and Agblevor, 2005). As these results, the presence of NBs may decrease the viscosity of liquid. However, the effect of the viscosity of various gas NBW on mass transfer efficiency during AD has not been reported. The higher viscosity means more power or operational costs to pump the liquid through the manufacturing equipment. NBs can reduce the viscosity of liquids, which become an enormous economic advantage for large-scale AD as the energy consumption for agitation can be minimized.

(4) Zeta potential

The Zeta potential of NBs in the suspended liquid is considered to be a physical property in a boundary layer (be called a sliding plane), which separated the double layer formed between the counter ions on the bubble interface and the bulk solution (Phan *et al*., 2020). Zeta potential as an important parameter to monitor the NB stability in aqueous solution (Parmar and Majumder, 2013). Ushikubo *et al*. (2010) proposed that NBs having absolute zeta potential more than 30 mV reflected more stable comparing with that of less than 30 mV. Zeta potential of the surface charge of NBs are generally negative over a wide range of pH (3–12) (Wang *et al*., 2019a). The negative charge could be explained by the excess of hydroxyl ions relative to hydrogen ions in the first molecular layers of water at the gas-liquid interface (Oh and Kim, 2017). It also confirmed that NBs is more stable in alkaline conditions. Intra and intermolecular interactions are formed on the surface of NB via chemical reactions of protonation and deprotonation, such as electrostatic repulsive (the same charge), electrostatic interactions (opposite charge) and hydrogen bonding interactions (neutral charge) (Michailidi *et al*., 2020). These interactions also affect the size and the zeta potential value of NBs, which also explain the higher conductivity of NBW and the ability to physically and chemically react on the surface of NBW.

The negative charge zeta potential also inhibits the coalescence of NBs in liquid due to the repulsion between the NBs, further explaining the long lifetime of NBs (over a few months)

(Michailidi *et al*., 2020). Absolute zeta potential depends on the NBW production time and gas introduced of NBW (Michailidi *et al*., 2020) and NB movement speed (Takahashi, 2005). For example, Air (34.0 eV) has a higher ionization energy than N_2 (14.5 eV) (Ahmed *et al*., 2018) with resultant N_2-NBW has higher zeta potential compared to Air-NBW. In addition, the ionization potential of O_2 has been found to correspond to 12.1 eV (Samson and Cairns, 1966). Numerous previous studies have recognized that O_2-NBW provides higher zeta potential values than other gas NBW (Michailidi *et al*., 2020). A relatively high zeta potential is observed during collapse of NBs (Agarwal *et al*., 2011). High zeta potential of NBW would enhance to absorb the positively charged ions. Clearly, different NBW exerts different mechanisms of biomass enhancement. For example, N_2 of N_2-NBW delivery govern factor in plant growth, while O_2 of O_2-NBW improve the activity of facultative bacteria (Ahmed *et al*., 2018).

(5) Free-radicals

Free radicals can be produced during the collapse of ultrasound-induced cavitation bubbles (Li *et al*., 2009). Liu *et al*. (2016c) experimentally determined the number of OH radicals generated as the order of 10^{14} per mL in oxygen NBW. One of the possible mechanisms is the high pressure and temperature inside the oxygen bubbles (Yasui *et al*., 2019). Many free radicals are produced by cavitation at their collapses (Krishnan *et al*., 2006). Free radicals can be generated at the gas-liquid interface, which has been proven by electron spin resonance spectroscopy experiments (Takahashi *et al*., 2007). It is well known that the lifetime of free radicals is in the order of 20 ns, which are unstable and difficult to store. Free radicals generated by nano-bubble water can be used for disinfection and sterilization (Yasui, 2018). The production of reactive oxygen species (ROS) by NBs would accelerate the metabolism of living organisms for lasting a very long time (Liu *et al*., 2016b). In addition, the type of gas inside NBs also influences the quantity of free radicals generated. NBs containing oxygen may favor the generation of ·OH compared to NBs without oxygen (Li *et al*., 2009).

1.4.2 Application of NBW technology

NBW technology has been widely applied in various field since last decade, such as industry (surfactant-free cleaning and mineral processing) (Lyu *et al*., 2019), biomedicine (enhanced drug

susceptibility, cancer cells, intracellular drug delivery) (Zhao *et al*., 2010) and wastewater or groundwater treatment (Hu and Xia, 2018). Table 1-2 summarizes the current studies of NBW applications in different areas. Up to the present, few reports are available on NBW-based AD systems.

1.5 Potentials and challenges of NBW-based AD system

NBW supplementation for AD enhancement does not bring about additional pollutants due to no chemical addition and easy operation at relatively low energy consumption, which is consistent with the sustainable development goals (SDGs). NBW can promote the mass transfer of organics from liquid phase to microbial cells, which may enhance the bioactivity of microorganisms, resulting improved decomposition of substrates with improved biogas production from AD of waste activated sludge (Wang *et al*., 2019a). The enhancement of NBW on biological activity can be mainly divided into two aspects, i.e. enzymatic activity and process itself. As Yang *et al*. (2019) reported, the activity of extracellular hydrolases (acid phosphatase, alkaline phosphatase, α-glucosidase and protease) was enhanced by 14-17% under NBW addition, promoting methane production from AD of waste activated sludge due to its enhanced hydrolysis. The NBW might promote the hydrolysis of oligosaccharide layer (produced by the decomposition of cellulose), providing abundant carbon and nutrient sources for the growth of microorganisms with resultant enriched microbial communities. In addition, Yang *et al*. (2019) found that NBW addition improved methane yield by enhancing not only the disintegration of high molecular weight compounds (proteins and polysaccharides) but also volatile fatty acids (VFAs) production during AD of waste activated sludge that usually contains a high lignocellulose content, accounting for 32–38% of the organic mass (Ruiken *et al*., 2013).

Most recently, the advancements in micro-oxygen technology reflect great potentials to address the low hydrolysis rate of highly recalcitrant feedstocks (like lignocellulosic biomass in this study) (Nguyen and Khanal, 2018). In general, oxidation-reduction potential (ORP) value in the range of 0 to −470 mV can be defined as micro-oxygen environment that could improve methane yield and volatile fatty acids (VFAs) production to a certain extent in AD process (Nguyen and Khanal, 2018; Nguyen et al., 2019). NBW can be relatively stably stored for three months (Michailidi *et al*., 2020), thus it is likely able to maintain a long lasting O_2 concentration

if being added into AD reactors. NBW may have great potential for lignocellulosic biomass degradation with high adaptability, especially O_2-NBW. It thus is speculated that NBW could probably enhance lignocellulosic biomass conversion during AD. Restated, little information is available regarding the impact of NBW addition on AD of refractory lignocellulosic biomass. In addition, mechanism involved in the enhancement of AD remains unclear.

1.6 Research objectives and thesis structure

To find out the cost-effective and environment-friendly process for the management of large amount of lignocellulosic biomass in practical application and minimize fossil fuels demand. To attained the above objective, this research firstly checked methane production from cellulose at high organic loading rate and analyzed its mechanism under different gas NBW addition; then explored the exact role of O_2 released by O_2-NBW on microorganisms and their metabolism; and finally resolved the increased reactor volume issue in the NBW-based AD system. This work will help to develop applicable NBW-based technologies for bioenergy production from lignocellulosic biomass. The thesis structure is presented in Fig. 1-1. The thesis was divided into the following 6 chapters:

(1) Chapter 1 Introduction

Firstly, the current situation and existing problems about energy crisis and environmental pollution was stated. Secondly, composition of lignocellulosic biomass and the state-of-art of lignocellulosic biomass conversion technologies were summarized in detail. Thirdly, characterization and application of NBW were demonstrated. Further, the novel NBW technology trends for biogas production were put forward. Finally, the objective of this study and the thesis structure were arrived.

(2) Chapter 2 Enhanced hydrolysis and acidification of cellulose at high loading for methane production via anaerobic digestion supplemented with high mobility nanobubble water

Chapter 2 explored increase in methane production from high loading cellulose through NBW addition were tried, and at the same time explored both high loading cellulose degradation mechanisms and the effect of NBW on methane production from cellulose via AD.

(3) Chapter 3 Supplementation of O_2-containing gas nanobubble water to enhance methane production from anaerobic digestion of cellulose

Chapter 3 examined the effect of micro-oxygen environmental created by O2 containing gas NBW on AD of cellulose achieveing no chemical addition and the SDGs for the enhanced AD process.

(4) Chapter 4 Improved methane production from corn straw using anaerobically digested sludge pre-augmented by nanobubble water

In Chapter 4, anaerobically digested sludge was firstly pre-augmented by N_2-NBW and O_2-NBW and then used as inoculum to produce methane from corn straw via AD process, resulting in enhanced methane production from AD of lignocellulosic materials with no increase of AD reactor volume.

(5) Chapter 5 Mechanisms analysis

The possible mechanisms involved in the novel NBW-based AD system for methane production were analyzed in this chapter, including the following three aspects: (1) function of NBW for AD; (2) key microbial under NBW-based AD system; and (3) key gas NBW for AD process.

(6) Chapter 6 Conclusion and future research

The major conclusions of the thesis were summarized with future research being prospected.

Table 1-1 The contents of cellulose, hemicellulose, and lignin in various lignocellulosic biomass

Lignocellulosic biomass	Cellulose (%TS)	Hemicellulose (%TS)	Lignin (%TS)	Reference
Corn straw	37	31-32	18	Saha *et al*., 2013
Wheat straw	46-50	27-30	6-7	Triolo *et al*., 2011
Rice straw	26-30	12-20	18-30	Sakdaronnarong *et al*., 2014
Sorghum	13-29	18-26	18-24	Monlau *et al*., 2012
Switch grass	5-20	30-50	10-40	Kang *et al*., 2014
Grasses	25-40	25-50	10-30	Saini *et al*., 2015
Cassava residues	24-25	18	12-13	Zhang *et al*., 2011
Softwood	27-30	35-40	25-30	Zabed *et al*., 2016

TS-total solid, VS-volatile solid.

Table 1-2 Summary of application of nanobubble technology in different areas.

Scientific application	Main findings
Agriculture	○ NBW irrigation directly affected soil microbial community structure and soil fertility, and promoted sugarcane (Zhou *et al*., 2020); ○ NBW stimulate chemical fertilizer to organic fertilizer for organic farming and thus tomato yield increased by 23% (Wu *et al*., 2019b); ○ NBW improved seeds germination rates by 6−25% (Ahmed *et al*., 2018).
Water and wastewater treatment	• O_2-NBW remediated anoxia by increasing dissolved O_2 (from 0 to 2.1 mg/L) (Ji *et al*., 2020); • O_2-NBW remediated sediment anoxia and reduce the internal P release in entrophic lakes (Zhang *et al*., 2020); • O_2-NBW promoted aeration biofilm formation in wastewater treatment (Xiao and Xu, 2020); • The water quality was significantly improved with 50% removal ratio of chemical oxygen demand and ammonia nitrogen (Wu *et al*., 2019a); • NBW accelerated the adsorption process by 366% (Kyzas *et al*., 2019).
Fishery	○ O_2-NBW can extend the survival time of zebrafish in a deoxygenated water system (Wang *et al*., 2018).
Anaerobic digestion	• NBW enhanced methane yield by 29% compared to the control group from anaerobic digested of waste activated sludge due to its augmentation on the hydrolysis of waste activated sludge (Yang *et al*., 2019).
Biomedicine	○ NBW has great potential to inhibit tumor growth through decreasing expression of hypoxia-inducible factor (Mahjour *et al*., 2019).
Architecture	• NBW improved the mechanical properties of concrete (e.g. increased compressive strength and tensile strength, and reduced water absorption and chloride ion permeability) (Khoshroo *et al*., 2018).
Cleaning	○ NBW dramatically cleaned the membrane due to the breaking down of organic matter by free radicals from collapse of NBs (Ghadimkhani *et al*., 2016).
Flotation	• NBs increased flotation recovery through enhancing the attachment of larger bubbles, almost 95% P_2O_5 recovery was reached during the first 1.5 min (Rosa and Rubio, 2018).

NBW-nanobubble water, NBs-nanobubbles.

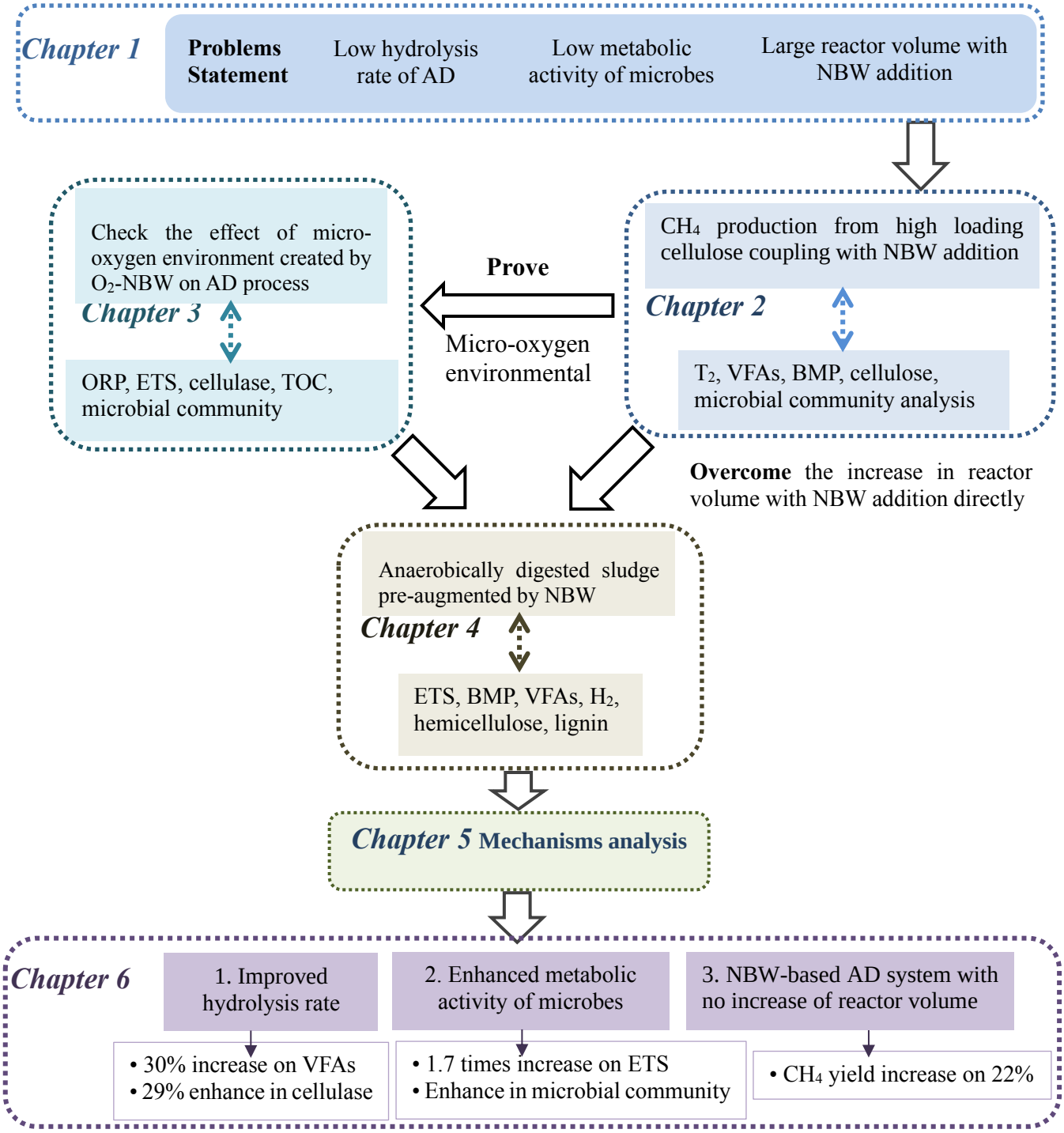

Fig. 1-1 The framework of the thesis. AD-anaerobic digestion, NBW-nanobubble water, BMP-biochemical methane potential, T_2-proton spin-spin relaxation time, VFAs-volatile fatty acids, ETS-electron transport system, ORP-oxidation reduction potential, TOC-total organic carbon.

Chapter 2 Enhanced hydrolysis and acidification of cellulose at high loading for methane production via anaerobic digestion supplemented with high mobility nanobubble water

2.1 Background

It is necessary to find out the cost-effective and environment-friendly process for the utilization of large amount of lignocellulosic biomass, in this study, the effects of Air-NBW and CO_2-NBW on the biological activity of anaerobic microorganisms were investigated with cellulose as the sole substrate. It is expected that Air-NBW or CO_2-NBW would enhance CH_4 yield from AD of cellulose, which may provide an effective means to sustainably manage the huge amount of lignocellulosic biomass and reduce the fossil fuel demand.

Most researches on methane production from AD were conducted at low F/M ratios (≤ 2 VS basis) (Xu *et al*., 2018; Díaz *et al*., 2011). The major reason is associated with the inhibition by fatty acids (propionic and valeric acid) accumulated during AD of excessive lignocellulosic biomass, which also makes properly mixing difficult to advance smoothly (Alzate *et al*., 2012). On the other hand, the oligosaccharides produced during high loading cellulose (F/M=3.5 (VS basis) in this study) degradation can create mass transfer resistance, and reduce the accessibility of water molecules to the surface of the cellulose particles (Vaquerizo *et al.*, 2018), thus hindering cellulose degradation to produce methane. NBW has been reported to have high-diffusion water molecules, which may break down the oligosaccharide layer produced at high cellulose loadings. In this study, we attempted to increase methane production from high loading cellulose through NBW addition, and at the same time explored both high loading cellulose degradation mechanisms and the effect of NBW on methane production from cellulose via AD.

2.2 Materials and methods

2.2.1 Materials

Anaerobically digested sludge was sampled from the Shimodate sewage treatment plant in Ibaraki, Japan, and stored in a refrigerator at 4°C before the experiments. The sludge was filtered through a 1-mm sieve before use and acclimated in an incubator at 35±1°C for 7 days to activate

microbial activity and decompose the remaining biodegradable organics. Cellulose used in this study was purchased from Sigma-Aldrich (Avicel® PH-101). The physicochemical properties of the cellulose and digested sludge are shown in Table 2-1. The preparation of NBW was described elsewhere (Wang *et al.*, 2019a). Briefly, 1 L DW in a 5 L plastic beaker was injected into the HACK FB11-UFB generator (HACK UFB Co., Ltd., Yamanashi, Japan) with different gases to obtain 'water containing NBs', namely NBW. Gas intake speed and DW circulation flow rate of the HACK FB11-UFB generator were controlled at 0.05 L/min and 3 L/min, respectively. The NBW generation process lasted for 20 min at room temperature (25±1°C). NBW was produced in a combination of pressurized mechanical cyclic and spiral liquid flow. One liter of air and CO_2 was used for NBW generation, respectively. The laboratory air was directly used for production of Air-NBW. CO_2 (purity ≥ 99.9995%) purchased from Taiyo Nippon Sanso Co., Ltd., Japan was used for CO_2-NBW production. DW was produced from a distillation unit (Elix, Merck Co., Ltd., Japan).

2.2.2 Batch AD tests

(1) Hydrolysis-acidification stage of AD of cellulose

Batch experiments were performed using 120 mL closed Schott Duran serum bottles. Each bottle was loaded with 30 mL of digested sludge mixed with 30 mL of CO_2-NBW, Air-NBW or DW. The addition of DW instead of NBW was deemed as the control. Previous researches on microbial lignocellulose degradation were conducted at F/M ratio ≤ 2 (VS based) (Xu *et al.*, 2018; Díaz *et al.*, 2011). In this study cellulose as the sole substrate was added into each AD reactor to achieve a higher F/M ratio of 3.5. To elucidate the effect of NBW on the hydrolysis-acidification stage of AD, 10 mM sodium 2-bromoethanesulfonate (BES) ($C_2H_4BrNaO_3S$, Tokyo Chemical Industry Co., Ltd, Japan), a coenzyme M analog, was added into each reactor to inhibit CH_4 production from methanogens. The reactors without cellulose addition were also prepared as blanks in order to exclude the VFAs production from the residual organics in the digested sludge from the total VFAs production under NBW or DW addition condition. Before starting the AD process, the initial pHs in all the reactors were adjusted to 7.00±0.05 with 2.0 M NaOH or HCl. Then all the reactors were sealed, evacuated and flushed with N_2 gas for 5 min to create anaerobic condition. The experiments were conducted in an incubator at 35±1°C for 16 days. The bottles

were shaken manually for 2 minutes right after the setup of the AD experiments and before each sampling time. All the experiments were performed in triplicate.

(2) Methanogenesis of cellulose

These AD tests were carried out as the same as 2.2.1 except the addition of BES. To estimate the methane production by the residual organics under NBW or DW addition condition, the blank reactors without cellulose addition were also considered by adding the same amount of NBW or DW. The biogas volume was directly read on the scale of 20 mL graduated glass syringe connecting to the headspace of each AD reactor. The triplicate experiments were conducted at 35±1°C for 50 days.

2.2.3 Calculation

Net CH_4 production (NmL/g-VS) from cellulose at all conditions was calculated according to Eq. 2-1 (He *et al.*, 2016).

$$CH_4\ (\text{NmL/g-VS}) = \frac{[CH_4\ (\text{NmL, cellulose+inoculum})] - [CH_4\ (\text{NmL, inoculum})]}{\text{Reduced biomass (g-VS)}} \quad 2\text{-}1$$

The biochemical methane potential (BMP) in this study was expressed as the volume of methane under standard conditions (1013 hPa, 273 K). A 20 mL graduated glass syringe directly connected to the headspace of each AD reactor was used to measure the biogas volume at lab condition (1013 hPa, 298 K). Then the methane volume (V) under standard condition was obtained by Eq. 2-2 (Lalak *et al*., 2016).

$$V = (273/T_{exp.}) \cdot (P_{exp.}/1013) \cdot V_{exp.} \quad 2\text{-}2$$

where $T_{exp.}$ is lab temperature (298 K in this study); P_{exp} is the atmospheric pressure in the laboratory at lab temperature (1013 hPa in this study); $V_{exp.}$ is the methane volume obtained from the lab test, which was calculated by methane content and biogas volume from the experiment.

Theoretical methane production (TMP) of organic matters can be estimated by using the stoichiometric equations (Tarvin and Buswell, 1934). In this study, the TMP of cellulose ($(C_6H_{10}O_5)_n$) was calculated according to Eqs. 2-3 and 2-4.

$$C_aH_bO_c + \left(a - \frac{1}{4}b - \frac{1}{2}c\right) H_2O \rightarrow \left(\frac{1}{2}a + \frac{1}{8}b - \frac{1}{4}c\right) CH_4 + \left(\frac{1}{2}a - \frac{1}{8}b + \frac{1}{4}c\right) CO_2 \quad 2\text{-}3$$

$$TMP = 22.4 \times \left(\frac{1}{2}a + \frac{1}{8}b - \frac{1}{4}c\right)/(12a + b + 16c) \quad 2\text{-}4$$

where a, b and c represent the molar fractions of C, H and O, respectively.

Anaerobic biodegradability (BD) of cellulose can be estimated by comparing the BMP (total CH_4 yield) with TMP (Raposo *et al.*, 2011) according to Eq. 2-5.

$$BD\ (\%) = 100 \times BMP/TMP \quad (2\text{-}5)$$

In this study, the methane production from cellulose was also fitted to the Gompertz model.

$$M(t) = P \times \exp\{-\exp[1 + R_{max} e(\lambda - t)/P]\} \quad (2\text{-}6)$$

where M(t) (NmL/g-VS) is the cumulative CH_4 production at time t; P (NmL/g-VS) is the simulated maximum CH_4 yield (NmL/g-VS); t (d) is the digestion duration; R_{max} (NmL/g-VS/day) is the maximum methane production rate; λ (d) is the lag phase time; and e is Euler's number (2.71828).

Cellulose content was determined as follows: the samples were dried at 60±2°C to constant weight and milled to powders (0.5 mm), then boiled with 5 mL 72% w/w H_2SO_4 solution for 4.5 h in order to hydrolyze the cellulose to glucose (Ververis *et al*., 2007). Glucose in liquid was determined with a dinitrosalicylic acid colorimetric method (Mendel *et al*., 1954). Cellulose content was calculated according to Eq. 2-7.

$$\text{Cellulose content (\%, w/w)} = 100 \times (0.9/0.96) \times (C_1 - C_0) \times V/M \quad (2\text{-}7)$$

where 0.9 is the coefficient produced by the molecular weight ratio of the polymer to the monomeric hexose; 0.96 is the saccharification yield; C_1 is the glucose concentration after hydrolysis (g/L); C_0 is the glucose concentration before drying (g/L) (the glucose produced during AD of cellulose); V is the total volume of the solution (L); and M is the dry weight of the sample (g).

2.2.4 Analytical methods for general properties of biomass and NBW

Total solids (TS) and VS of the mixture were determined according to Standard Methods (APHA, 2012). pH value was measured with a semi-solid pH meter (Testo 206, Germany). VFAs including acetic (HAc), propionic (HPr), iso-butyric (iso-HBu), n-butyric (n-HBu), iso-valeric (iso-HVa) and n-valeric acid (n-HVa) were quantified using a gas chromatography (GC-8A, SHIMADZU CO., Japan) equipped with a flame ionization detector (FID) and a Unisole F-200 30/60 column. The pressure of carrier gas N_2 was maintained at 200 KPa. The temperatures of column and detector/injector were fixed at 150°C and 180°C, respectively. Biogas composition

mainly including CH_4 and CO_2 was determined using a gas chromatography (GC) (GC-8A, SHIMADZU CO., Japan) packed with a thermal conductivity detector (TCD, 80°C) and a Porapak Q column (60°C). The method for crystallinity index (CrI) determination was described elsewhere (Park *et al.*, 2010). The solid residue of each sample was dried at 60°C for 72 h and grounded into powder for total organic carbon (TOC) analysis using a TOC analyzer (TOC-V_{CSN} with SSM-5000A, SHIMADZU CO., Japan). The digested sludge was centrifuged at 5000 rpm for 10 min with the supernatant being filtered through 0.22 μm filter membrane. Soluble organic carbon in the filtrate was determined by the same TOC analyzer packed with an ASI-V autosampler. Soluble proteins and carbohydrates were determined by using Coomassie Brilliant Blue (CBB) method and phenol-sulfuric method with bovine serum albumin and L-glucose as the standard, respectively. Cellulase activity was analyzed as described by Hua *et al*. (2014).

The numeric concentration of NBs was measured by Nanosight-LM10 (Malvern, UK) according to the nano-particle tracking analysis method. Zeta potential of NBW was analyzed by a zeta potential analyzer (Zetasizer Nano ZS, Malvern, UK). The proton spin-spin relaxation time (T_2) was determined by a pulsed NMR (JNM-MU25A, Nippon Denshi Co., Ltd., Japan) which was operated at 25 MHz and a constant temperature of 20°C. The Carr-Purcell-Meiboom-Gill (CPMG pulse sequence) technique was used to determine T_2.

2.2.5 Microbial community analysis

To analyze the archaeal and eubacterial communities, the samples were collected from each bottle after 50 days' batch tests for methane production. The total DNA was extracted from the wet sludge sample using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, US) according to manufacturer's instructions. DNA quality and quantity were checked by 2% agarose gel electrophoresis and NanoDrop 2000 UV-vis spectrophotometry (260 nm/280 nm ratio) (Thermo Scientific, Wilmington, USA). The V3-V4 region of bacterial 16S rDNA was amplified using the universal primer pair of 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The archaeal 16S rDNA was amplified with the primer pair of 524F10extF (5'-TGYCAGCCGCCGCGGTAA-3') and Arch958RmodR (5'-YCCGGCGTTGAVTCCAATT-3'). According to the standard protocol of Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China), the purified amplicons were combined on an Illumina

MiSeq platform (Illumina, San Diego, USA) with equimolar and paired end sequencing (2×300 bp).

2.2.6 Statistical analysis

All the experimental data were presented as mean ± standard deviation (SD) in this work. One-way analysis of variance (ANOVA) was applied to analyze the statistical difference among the same batch AD tests by using the Microsoft Office Excel 2018. All figures were plotted by using Origin 2018 (OriginLab Corporation, Northampton, MA, USA). Significant difference was assumed at $p < 0.05$.

2.3 Results and discussion

2.3.1 Change in T_2 during 35 days' storage of NBW

Proton-nuclear magnetic resonance relaxation time can be used to detect interactions between weak molecules such as spatial effects and molecular mobility (Liu *et al.*, 2013). It has been widely used to explore the mobility and diffusion of the water molecules in the biological and chemical fields. Fig. 2-1 shows the changes of T_2 of Air-NBW, CO_2-NBW and DW at 35±1°C during 35 days' storage. The longest T_2 of NBW was detected right after NBW generation for 20 minutes. As shown, NBW formed from pressurized mechanical circulation, rotating flow and cavitation is a kind of liquid with high water molecule activity. After one day storage, the T_2 of Air-NBW decreased from 2906.0 ms to 2711.8 ms, while the T_2 of CO_2-NBW decreased from 2768.0 ms to 2657.7 ms. After that, their T_2 values kept relatively stable till day 21. From day 21 to day 28, the T_2 values decreased again to 2631.2 ms for Air-NBW and to 2611.0 ms for CO_2-NBW, respectively. From day 28 onwards, the concentrations of NBs in Air-NBW and CO_2-NBW were also detected to decrease slightly. This phenomenon was positively correlated with the changes of NBs concentration in the corresponding NBW: the correlation coefficients between the NBs concentration and T_2 value were found to be 0.90 ($p = 0.002$) for Air-NBW and 0.74 ($p = 0.030$) for CO_2-NBW, respectively. Besides, the absolute values of zeta potentials of Air-NBW (-19.4±1.8 ~ -25.0±1.2 mV) and CO_2-NBW (-18.2±1.9 ~ 21.9±1.8 mV) were always much higher than those of DW (-1.6±0.23 mV) during the 35 days' storage. In addition, the relationship coefficients between the Zeta potential and NBs concentration were also found to be 0.77 ($p =$

0.025) for Air-NBW and 0.73 (p = 0.040) for CO_2-NBW, respectively. The high concentration of NBs may achieve T_2 and higher Zeta potential (absolute value). As stated by Takahashi (2005), a higher zeta potential (absolute value) may bring about a high moving speed, which might be the reason for the higher mobility of water molecules in NBW after being stabilized. In general, a longer T_2 time indicates a higher mobility and diffusion of water molecules (Liu *et al.*, 2013), which has been demonstrated to improve the penetration of water molecules into the cellulose matrix (Vaquerizo *et al.*, 2018). Thus, in this work, the longer T_2 of NBW (Fig. 2-1) is expected to have some enhancement effect on hydrolysis during the high cellulose loading AD process.

2.3.2 Effect of NBW addition on the hydrolysis-acidification stage of high cellulose loading AD

These batch tests were to identify whether NBW addition could contribute to methane production from AD at a high cellulose loading (F/M) of 3.5. Firstly, the effects of CO_2-NBW and Air-NBW on the hydrolysis-acidification stage of cellulose during AD were investigated by adding BES. VFAs yield and pH value were monitored during the 16 days' AD of cellulose and the results are shown in Fig. 2-2. As it can be seen, the VFAs yields (39. 7– 40.4 mg/g-cellulose$_{reduced}$) in all the reactors were detected to be significantly high during the initial 3 days. The reactors with Air-NBW addition achieved the highest VFAs yields, increasing by 30.3%, 17.8% and 17.3% compared to the control reactors with DW addition on day 6 (35.6 mg/g-cellulose$_{reduced}$), day 9 (33.6 mg/g-cellulose$_{reduced}$) and day 16 (16.9 mg/g-cellulose$_{reduced}$), respectively, followed by the reactors with CO_2-NBW addition, increasing about 11.4%, 13.0% and 16.2%. When compared to the reactors with CO_2-NBW addition, the reactors with Air-NBW addition increased by 17.0%, 17.8% and 0.9% on days 6, 9 and 16, respectively. Relatively low VFAs yields were detected in all the reactors on day 16, most probably due to the following two reasons: (1) after 16 days' AD test, the pH values decreased to 4.6–4.7 in all the reactors, which are not within the optimum range (5.5–6.5) for acidogenesis (Mao *et al.*, 2015); and (2) probably almost no hydrolyzable acidified cellulose was available. Generally, a higher VFAs production can yield a higher CH_4 production during the methanogenesis stage, thus the produced VFAs during the hydrolysis-acidification stage may function as a good indicator for the subsequent

methanogenesis process. This observation might be the major reason for the Air-NBW reactors to achieve the highest methane yield.

In these experiments, HAc yield was detected as the most dominant VFAs species in all the reactors. HAc yield increased along with the operation before day 9. Air-NBW addition achieved the highest HAc yield, 11.3 mg/g-cellulose$_{reduced}$, 14.5 mg/g-cellulose$_{reduced}$ and 18.0 mg/g-cellulose$_{reduced}$ on days 6, 9 and 16, respectively. HPr and HVa are usually regarded as the inhibition factors for CH_4 production during AD (Xu *et al.*, 2014), which were detected to be the lowest in the Air-NBW reactors, accounting for 32% of its final VFAs yield, 6.2% and 13.0% lower than those in the CO_2-NBW and DW reactors, respectively. As discussed in section 3.1, Air-NBW possesses the higher diffusion of water molecules compared with CO_2-NBW, suggesting that the enhanced hydrolysis-acidification could achieve a higher VFAs yield at a high cellulose loading (F/M) of 3.5. In addition, the CO_2-NBW and Air-NBW reactors had higher cellulase activities (averagely 0.20 U/(mL·min) and 0.22 U/(mL·min), respectively) than the DW reactors (averagely 0.17 U/(mL·min)) during the hydrolytic-acidification stage, which is in agreement with the result of VFAs yield (Fig. 2-2). Interestingly, Air-NBW addition achieved lower inhibition factors during the hydrolysis-acidification stage of cellulose (Fig. 2-2). Restated, the promotion effect of NBW on the metabolism of microorganisms during the hydrolysis-acidification stage of high cellulose loading AD is most probably resulted from its high mobility of water molecules. Therefore, NBW addition can favor the microbial decomposition of refractory cellulose to VFAs, which are then available to methanogens for more methane production.

2.3.3 Methane production

(1) Effect of NBW on CH_4 production from cellulose

The effects of NBW addition on cumulative CH_4 production during AD are illustrated in Fig. 2-3a. The CH_4 production increased rapidly before day 40; after day 40, it reached a plateau. The CH_4 yields from the DW reactors (Control) were 204.5±0.1 NmL/g-VS after 50 days' AD of cellulose. As expected, the highest CH_4 yields, 241.5±10.1 NmL/g-VS ($p < 0.05$) were achieved in the Air-NBW reactors during the 50 days' AD, followed by the CO_2-NBW reactors (225.4±5.5 NmL/g-VS), increasing by 18% and 10% when compared to the control. This observation was probably and mainly contributed by the enhanced hydrolysis-acidification of cellulose under

NBW addition. As for the two kinds of NBWs, Air-NBW showed better enhancement effect on CH_4 production than CO_2-NBW, most probably owing to the micro-oxygen environment created by Air-NBW addition, favoring the growth of facultative anaerobes which may have synergetic utilization of cellulose (Wushke *et al*., 2015) and then contribute to the enhanced hydrolysis-acidification process. As recently reported, microaeration pretreatment by a pure bacterial strain achieved 17% increase in methane yield from corn straw (Xu *et al.*, 2018). Micro-oxygen has also been claimed by Fu *et al.* (2016) to be responsible for the enhanced methanogenesis of corn straw. During the hydrolysis-acidification stage, the produced HPr and HVa might be consumed by the facultative anaerobes under Air-NBW addition, thus enhancing CH_4 production from the subsequent methanogenesis stage of high cellulose loading AD.

(2) Kinetic analysis by the modified Gompertz model

The modified Gompertz model was applied to simulate the kinetic patterns of CH_4 production during the high cellulose loading AD with NBW addition. Table 2-2 lists the results of TMP, BMP, biodegradability, the maximum CH_4 production rate (R_{max}) and lag phase time (λ) for all the reactors, which are helpful to better understand the methanogenesis process of cellulose. According to Eq. 2-4, the TMP of cellulose used in this work was calculated as 414.8 NmL/g-VS. The experimental CH_4 productions from the reactors with CO_2-NBW and Air-NBW addition were 54% and 59% of TMP, respectively according to Eq. 2-5 (Table 2-2) in comparison to 49% of TMP in the DW-reactors. This observation also suggests that NBW addition can enhance the CH_4 production from high cellulose loading AD. According to the results simulated from the modified Gompertz model, the λ values under NBW addition conditions were shortened to 0.3–0.8 day from 1.0 day with DW addition, implying that the high cellulose loading AD process took a shorter start-up period when NBW was supplemented to the AD system. A shorter lag phase duration is critical to improve the economic benefits of the process since the resultant shorter AD duration can bring about a smaller AD reactor system and thus lower operation cost. Moreover, the R_{max} was determined as 12.2±0.8 NmL/g-VS/day with the addition of Air-NBW, followed by 9.4±0.5 NmL/g-VS/day and 9.2±0.5 NmL/g-VS/day with the addition of CO_2-NBW and DW (Control), respectively. These results indicate that NBW addition could shorten the lag phase period and accelerate CH_4 production from the refractory cellulose. In addition, the simulated

maximum cumulative CH_4 production (P) was quite similar with the experimental CH_4 yield, indicating the good fitness of the modified Gompertz equation to the experimental data (Table 2-2). This observation might be attributable to no or few inhibitory factors involved in the high cellulose loading AD process in this study, which is further evidenced by the high R^2 values (0.990–0.994) under the test conditions.

2.3.4 Changes in cellulose content and crystallinity degree

The results of refractory cellulose reduction during the methanogenesis stage are shown in Fig. 2-3b. Clearly, the highest cellulose reduction was detected in the Air-NBW reactors ($p < 0.05$). The cellulose contents in the Air-NBW and CO_2-NBW reactors were found to decrease sharply to 25.2±1.1% and 28.1±0.9% after 50 days' AD, resulting in 65% and 60% of cellulose reduction, respectively, about 29% and 21% higher than those in the DW reactors. It is also observed that a higher cellulose reduction corresponded to a higher CH_4 production under the test conditions. Since cellulose was the sole substrate in these experiments, TOC removal was also correlated with the cellulose reduction as shown in Table 2-2. The TOC removals by the Air-NBW and CO_2-NBW reactors were 43.1±6.4% and 39.6±4.3%, about 12% and 3% higher than those by the DW reactors, respectively. In addition, VS reductions as shown in Table 2-2 also reflect the similar trend: A higher VS reduction (54–58%) was achieved in the NBW supplemented reactors in comparison to those with DW addition (45%). The above results imply that NBW addition enhanced cellulose reduction or degradation for methane production.

On the other hand, cellulose structure can be divided into two regions, namely the amorphous region that is easily digested by microorganisms and enzymes, and the crystalline region that is difficult to be digested (Gupta *et al.*, 2016). Table 2-2 also shows the crystallinity reduction of the samples after 50 days' AD. The X-ray diffraction (XRD) diffraction data indicate that the crystallinity reductions were 80.7±2.1% and 76.9±0.2% in the Air-NBW and CO_2-NBW reactors, increasing by 20% and 14% compared to the DW reactors, respectively. This observation implies that NBW addition could enhance the reduction of cellulose crystallinity, probably through improving the metabolic activity of hydrolytic bacteria or facultative anaerobes in the AD process. Crystallinity of cellulose is one of the limiting factors affecting its hydrolysis (Chávez-Guerrero *et al.*, 2019). Thus, the reduction of crystallinity might be the major reason for the promoted

hydrolysis-acidification of cellulose under NBW addition and then increased VFAs production for methanogenesis.

2.3.5 Changes in microbial communities under NBW addition

All the above enhancements brought about by NBW addition may have some impact on the bacterial and archaeal communities in the AD process. Recently, NBW has been demonstrated to stimulate the functional microbes to have enhanced metabolic activities and degrade organic efficiently (Sun *et al.*, 2018).

(1) Diversity of microbial communities

The Shannon Index and the ACE/Chao1 estimate are ecological indicators which can be used to indicate microbial species richness and diversity, respectively. Larger bacterial sequence, OTU and ecological indicators were obtained in the samples collected in this study, suggesting the much higher bacterial diversity and abundance than archaea. The coverage of bacteria and archaea exceeded 0.995, indicating that most of the sequences were detected. The digestate with NBW addition showed higher Shannon, ACE and Chao index of archaea, suggesting that the addition of NBW might increase the abundance of archaea during AD. One possible explanation for this observation may be that the higher water mobility in NBW could accelerate microbial metabolism and promote archaeal growth. In general, a higher microbial diversity may enhance its ecological stability (Wrighton *et al*., 2008), which can also bring about a high digestibility and biogas production (Lu *et al*., 2012). As for the two kinds of NBW, the reactors with Air-NBW addition were found to have higher numbers of OTU, Chao1 and ACE of bacterial and archaeal communities than those reactors with CO_2-NBW addition, implying that different gas NBW also has some effect on the species richness of both bacteria and archaea.

(2) Bacterial phyla

Fig. 2-4a shows the bacterial community at phylum level under the test conditions. The results indicate that *Aminicenantes, Spirochaetae, Parcubacteria, Bacteroidetes,* and *Chloroflexi* were the most dominant phyla in all the reactors, which are considered to be actively involved in AD process. *Aminicenantes* was found to be the dominant species in all the reactors, accounting for 8.3%, 20.5% and 21.9% in the DW, CO_2-NBW, Air-NBW reactors, respectively. *Aminicenantes* is claimed to be related to the production of VFAs (Robbins *et al*., 2016), which

has been proven in this study that the reactors with NBW addition yielded higher VFAs during the hydrolysis-acidification stage of cellulose. *Spirochaetae* was found in all the samples, while the relative abundance (RA) of this phylum was rather high in the NBW supplemented reactors (7.4%, 11.0% and 5.7% for the CO_2-NBW, Air-NBW, and DW reactors, respectively). This phylum participates in the decomposition of lignocellulose to simple compounds (Zhao *et al.*, 2018). *Parcubacteria* is also considered to contribute to CH_4 yield from cellulose (Dai *et al.*, 2016). Besides, *Bacteroidetes* can play an important role in the degradation of complex polymers, residual stubborn substances and high molecular weight organic compounds (Liu *et al.*, 2016a). The metabolic products are beneficial for enhanced biogas production from high cellulose loading AD. The enrichment of all these bacteria implies their important contributions to AD of cellulose, especially under Air-NBW addition condition which may be partially contributed by its micro-oxygen environment. A higher RA of *Chloroflexi* was noticed in the NBW reactors, which can primarily utilize various carbohydrates during the initial stage of AD, i.e. hydrolysis and acidification (Ruan *et al*., 2019).

(3) Archaeal genera

As it is known, archaea play a critical role in AD process. The major archaea at genus level in all the reactors are shown in Fig. 2-4b. The RA of *Methanosaeta* in the Air-NBW (73.4%) and CO_2-NBW (52.7%) reactors were 63.8% and 17.6% higher than that in the DW reactors (44.8%), respectively. *Methanosaeta,* belonging to the order *Methanosarcinales,* is an obligate aceticlastic methanogen which can produce CH_4 through direct electron transfer (DIET) (Rotaru *et al.*, 2014). *Methanosaeta* can efficiently utilize HAc to produce CH_4 (Chen *et al.*, 2016), which might explain why little accumulation of HAc was detected in the methanogenesis at high cellulose loading in this study (data not shown). In the DW reactors, no *Methanospirillum* was observed; however, it appeared in the Air-NBW and CO_2-NBW reactors with RA of 0.04% and 0.09%, respectively. *Methanospirillum,* a genus of strictly mesophilic hydrogenotrophic methanogens, can utilize H_2 or formate to produce CH_4 (Jiang *et al.*, 2018). This genus belongs to the family of hydrogenotrophic methanogen and is involved in the interspecies hydrogen transfer (Lin *et al.*, 2019). The enrichment of *Methanomassiliicoccus* in the NBW reactors may also contribute to enhanced CH_4 production by using hydrogen as the substrate (Li *et al.*, 2018c). These observations may explain why almost no H_2 was detected throughout the whole methanogenesis

of cellulose. It was found that the Air-NBW reactors had the highest RA (1.01%) of *Methanobacterium* among the test reactors. Fu *et al.* (2016) reported that the RA of *Methanobacterium* was doubled under microaerobic condition. Thus the micro-oxygen environment created by Air-NBW addition might contribute to the shift of microbial community and promote the CH_4 formation. In addition, *Methanolinea* can utilize CO_2 to produce CH_4 (Cayetano *et al.*, 2019), which offers a reasonable explanation for the reactors with CO_2-NBW addition resulting in the highest RA of *Methanolinea*, most probably due to CO_2 could be collapsed and generated from CO_2-NBW and then used for CH_4 production. Taking all the results together, it can be denoted that the above-mentioned bacteria and archaea are fundamentally important for the high cellulose loading AD process in this study. Meanwhile, it can also be inferred that high mobility of water molecule in NBW may promote the functional metabolism of microorganisms.

2.4 Summary

This study demonstrated the positive effects of high-mobility NBW on the AD of refractory cellulose. VFAs yields were increased by 11–30% during the hydrolysis-acidification stage of cellulose under NBW addition. When Air-NBW was supplemented, 18% increase in CH_4 yield (263.6 NmL/g-VS) and 20% enhancement in cellulose crystallinity reduction (81%) were achieved in comparison to the control. These enhancements are attributable to the greatly enhanced hydrolysis-acidification stage of high cellulose loading AD, most probably due to the micro-oxygen environment created by Air-NBW addition. These findings can provide valuable insights into the mechanisms involved in the NBW-supplemented AD process.

Table 2-1 Physicochemical characteristics of cellulose and inoculum used in this work.

Items	Unit	Inoculum	Cellulose
pH	-	6.8±0.1	ND
TS	%, w.w.	1.77±0.04	98.90±0.2
VS, of TS	%, w.w.	72.88±3.2	99.98±0.3
Total carbon	%, d.w.	28.1±2.1	43.5±0.4
Total nitrogen	%, d.w.	2.9±0.6	ND
TVFAs	mg/L	46.3±1.4	ND
Soluble total organic carbon	mg/L	126.8±2.3	ND
Soluble carbohydrates	mg/L	69.3±1.2	ND
Soluble proteins	mg/L	8.2±0.3	ND
Cellulose	%, d.w.	15.8±1.2	98.6±2.3
Cellulose crystallinity	%	46.80±0.6	80.79±2.0

d.w.-dry weight basis, ND-not detected, TS-total solid, TVFAs-total volatile fatty acids, VS-volatile solid, w.w.-wet weight basis.

Table 2-2 Performance of methane production and crystallinity, TOC and VS reductions during anaerobic digestion with DW or NBW addition.

Water	TMP (NmL/g-VS)	BMP (NmL/g-VS)	BD (%)	Modified Gompertz model				Crystallinity reduction (%)	TOC reduction (%)	VS reduction (%)
				P	R_{max}	λ	R^2			
DW		204.5±0.1	49.3	203.3±3.0	9.2±0.5	1.0	0.994	67.4±3.8	38.6±0.2	44.7±0.3
CO_2-NBW	414.8	225.4±5.5	54.3	224.1±3.7	9.4±0.5	0.8	0.994	76.9±0.2	39.6±4.3	53.6±1.3
Air-NBW		241.5±10.1	58.2	237.7±3.9	12.2±0.8	0.3	0.990	80.7±2.1	43.1±6.4	58.0±3.4

BD-biodegradability, DW-deionized water, BMP-biochemical methane production, NBW-Nano-bubble water, P-simulated maximum methane yield (NmL/g-VS), TMP-theoretical methane production, TOC-total organic carbon, R^2-correlation coefficient, R_{max}-maximum methane production rate (NmL/g-VS/day), λ-lag phase time (day).

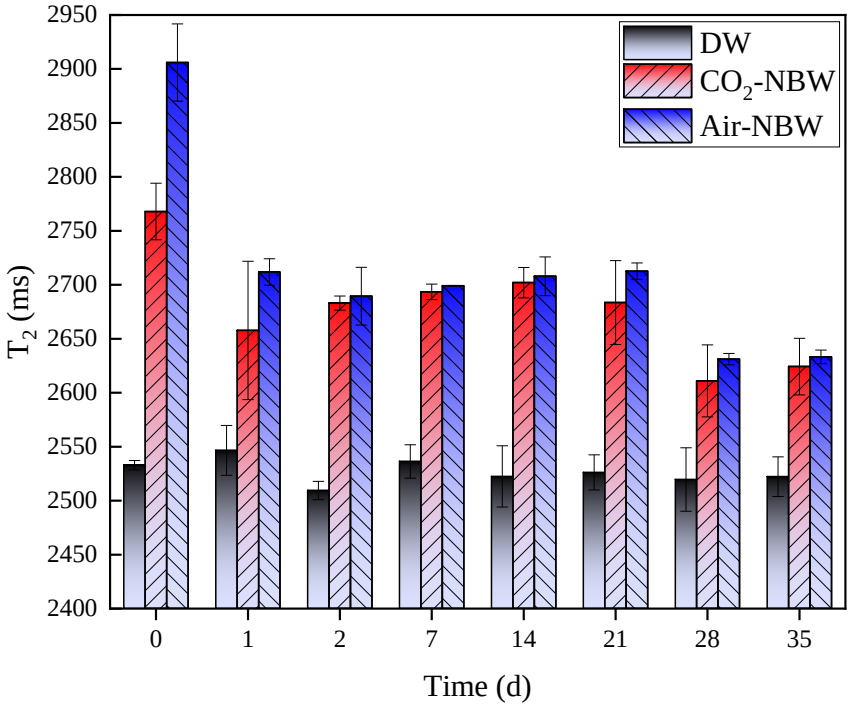

Fig. 2-1 Variations of spin-spin relaxation times (T_2) of Air-NBW, CO_2-NBW and DW at 35±1°C during 35 days' storage. DW-deionized water, NBW-nano-bubble water.

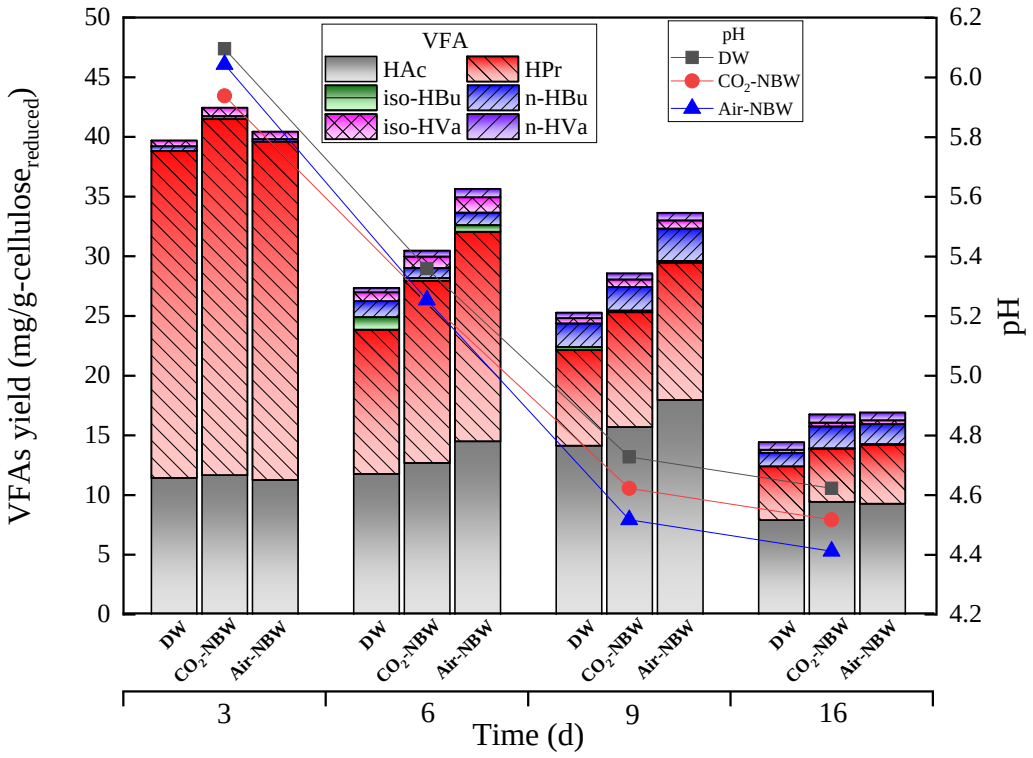

Fig. 2-2 Variations of VFAs yield and pH value during the hydrolysis-acidification stage of AD of cellulose after DW or NBW addition. AD-anaerobic digestion, DW-deionized water, NBW-nano-bubble water, HAc-acetic acid, HPr-propionic acid, iso-HBu-iso-butyric acid, n-HBu-n-butyric acid, iso-HVa-iso-valeric acid, n-HVa-n-valeric acid.

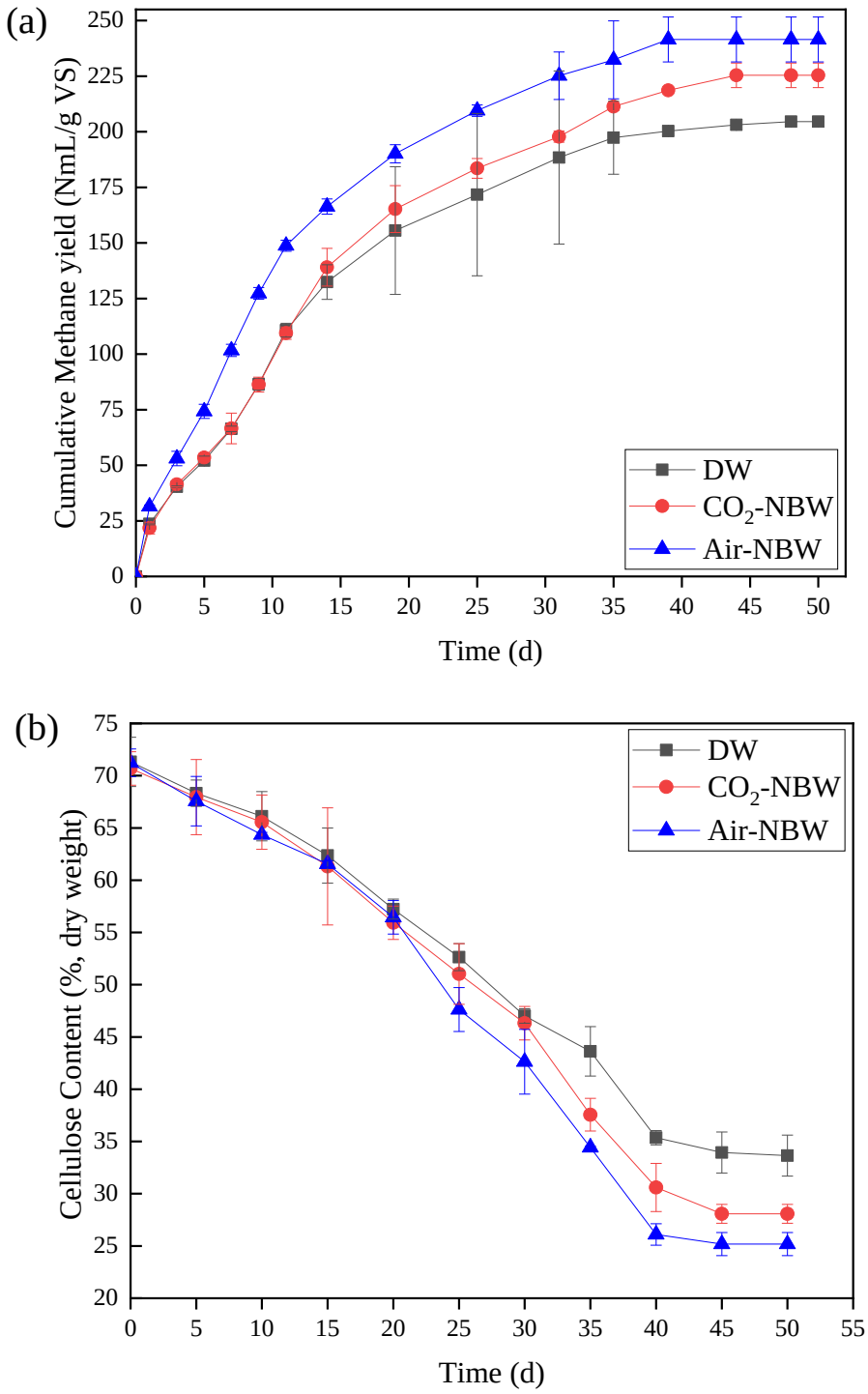

Fig. 2-3 Changes in cumulative methane production (a) and cellulose content (b) during AD of cellulose after NBW addition. DW-deionized water, NBW-nano-bubble water.

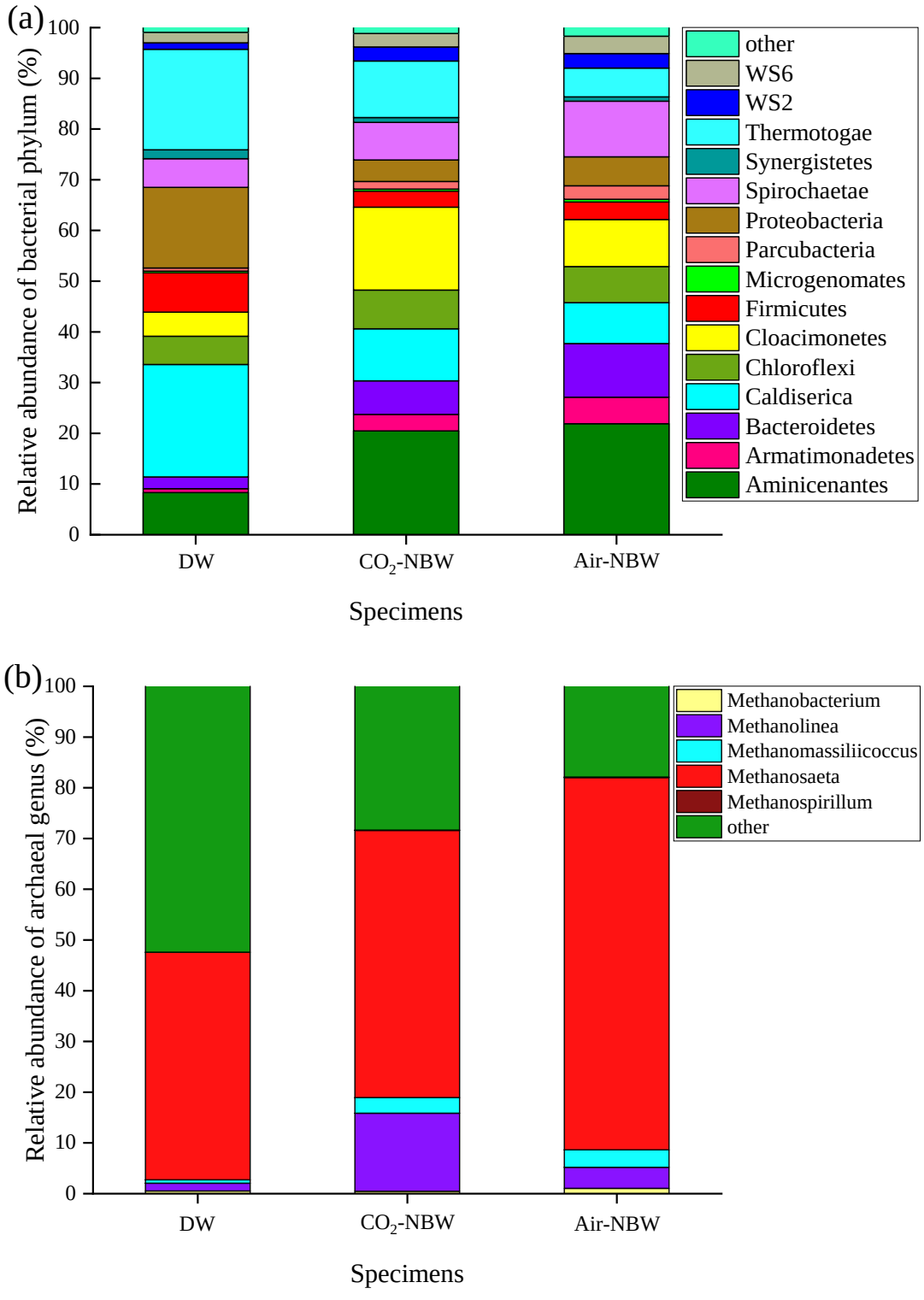

Fig. 2-4 Bacterial (a) and archaeal (b) community changes in the samples taken at the end of AD of cellulose with NBW addition using high-throughput 16S rDNA pyrosequencing. AD-anaerobic digestion, DW-deionized water, NBW-nanobubble water.

Chapter 3 Supplementation of O_2-containing gas nanobubble water to enhance methane production from anaerobic digestion of cellulose

3.1 Background

The advances in micro-oxygen technology have potential to address the low hydrolysis rate of highly recalcitrant feedstocks (lignocellulosic biomass) (Nguyen and Khanal, 2018) and low metabolic activity of microbes. Micro-oxygen environment could improve methane yield and VFA production to a certain extent in AD process (Nguyen and Khanal, 2018). The underlying rationale is augmentation in diversity and activity of microorganism with resultant enriched hydrolysis of substrate (Nguyen *et al*., 2019). The conventional systems mechanical aerators or diffusers are utilized, which require high electricity costs and maintenance of mechanical part (El-Zahaby and El-Gendy, 2016). Some chemical oxidants (H_2O_2 and calcium peroxide) release peroxide as reacting with water to improve O_2 levels, but also kill the microbial and pollute waters ecology (Wang *et al*., 2019b). All these works indicate that micro-oxygen environmental technology required to meet nontoxicity, no chemical addition and the SDGs for an alternative enhancement method on AD process.

In chapter 2, Air-NBW was presumed to created micro-oxygen environment that greatly improved hydrolysis-acidification stage of high cellulose loading AD. An intermittent micro-aeration system can also accelerate VFAs conversion by facultative bacteria and improve methane yield by hydrogenotrophic methanogens, respectively. Hydrolysis is the rate-limiting AD step of refractory substrates such as lignocellulosic biomass (Shrestha *et al*., 2017). The underlying rationale of an enhanced AD process is usually relating to the augmentation of microbial diversity and activity (facultative heterotrophs) resulting in improved hydrolysis of substrate (Nguyen *et al*., 2019). For example, the micro-aerobic AD system was detected to have a higher proportion of Firmicutes phylum associated with a higher substrate hydrolysis rate (Nguyen and Khanal, 2018). In order to achieve the micro-aeration purpose, high costs are required for electricity and maintenance of the mechanical parts when conventional mechanical aerator or diffuser systems are applied (El-Zahaby and El-Gendy, 2016). All these works indicate that the micro-aeration biotechnology would be more promising if it is non-toxic with

no chemical addition, especially for the enhancement of AD process. Thus, in this study, N_2-NWB and O_2-NBW were chosen. In addition, the CO_2-NBW has lower stability, due to the lower pressure and higher solubility of CO_2 in liquid comprised to N_2 and O_2 (Zhang *et al*., 2008). The high stability of O_2-NBW make them continually potent O_2 suppliers in liquid (Ebina *et al*., 2013). In addition, the mass transfer rate of O_2 to the liquid phase in O_2-NBW was claimed to be 125 times faster than that of bulk bubble (diameter > 1000 nm) (Temesgen *et al*., 2017). O_2-NBW was trialed to control methane emission from algae bloom waters (Shi *et al*., 2018). Seen from the above works, O_2-containing gas NBW has great potentials for enhanced AD of lignocellulosic biomass. However, whether O_2-containing gas NBW can create a micro-oxygen environment and its underlying mechanism for methane yield from AD of lignocellulosic biomass still remain ambiguous so far. Specifically, what is the exact role of O_2 released by O_2-NBW on microorganisms and their metabolism in a micro-oxygen environment? What is the effect of O_2 content in NBW on methane yield?

To address these unknowns, the present work is thus to investigate the effects of different O_2-containing gas NBWs (Air-NBW, N_2-NBW, O_2-NBW, and artificial N_2/O_2 gas mixture with different mixing ratios) addition on AD of cellulose were investigated. The main objectives of this study were: 1) to investigate the effects of NBW on electron transport system (ETS) and enzyme activity during AD of cellulose under the addition of different NBWs; 2) to quantify the cellulose content and crystallinity reduction efficiency; 3) to verify O_2-NBW for BMP by the different N_2 and O_2 gas mixture derived NBW; and 4) to elucidate the possible mechanisms by analyzing bacterial and archaeal communities for a better understanding of methane production from cellulose.

3.2 Materials and methods

3.2.1 Materials

The anaerobically digested sludge and cellulose were as the same in Chapter 2 (2.2.1).

3.2.2 Preparation of NBW

NBW was prepared as the same as in Chapter 2 (2.2.1). The N_2/O_2 gas mixture was produced by using a wet gas meter (W-NK-0.5, Shinagawa Co., Japan).

3.2.3 BMP tests

The BMP test was used to assess the biodegradability of cellulose with NBW and DW addition, which has been shown in Chapter 2 (2.2.2). Cellulose at a concentration of 5 g/L, achieving F/M ratio of 1 (VS basis). with no addition of other substances like macro- and/or micro-nutrients. As reported, slightly alkaline condition can enhance the hydrolysis of cellulose (Crutchik *et al*., 2018), the limiting step of AD. In our previous studies (Yang *et al*., 2019), under the addition of NBW, VFAs accumulation usually occur during the initial stage of AD of waste activated sludge and cellulose, which may lead to an acidic environment influencing the BMP tests. Thus, in this study, after inoculation, the initial pH of the mixture was adjusted to 8.0 using 2.0 M NaOH or HCl to avoid (or alleviate) the above-mentioned VFAs inhibition effects on AD process. Then, the AD reactors were subjected to 5 min of N_2 flushing to fill the headspace with N_2. All the AD experiments were conducted for 18 days in a temperature-controlled incubator at 35±1^{o}C, and all the tests were carried out in triplicate. Six types of NBW derived from different gases (air, N_2, O_2, and the N_2/O_2 gas mixture at ratios of 4:1, 1:1 and 1:3) were tested to investigate BMP from cellulose. The reactors were labelled as Air-NBW, N_2-NBW, O_2-NBW, N_2/O_2 (4:1)-NBW, N_2/O_2 (1:1)-NBW, N_2/O_2 (1:3)-NBW, respectively. The BMP in this study was expressed as the volume of methane under standard conditions (1013 hPa, 273 K) as described in Chapter 2 (2.2.3).

3.2.4 Analytical methods

(1) General parameters

The analytical methods were as the same as in Chapter 2 (2.2.4). Dissolved oxygen (DO) was determined by a HQ40d Portable Meter (Hach, USA). Oxidation-reduction potential (ORP) was measured using a YK-23RP ORP meter (Lutron electronic enterprise Co., Ltd., Taiwan). Conductivity was determined using an AS710 conductivity meter (Mechworth Co. Ltd., Thailand).

(2) Analysis of electron transport

ETS activity of the methanogenic communities was measured using the INT (2-(p-iodophenyl)-3-(pnitrophenyl)-5-phenyltetrazolium chloride) reduction method according to Zhang *et al*. (2018a). Firstly, 0.5 mL sample was taken from each reactor and added into a 10 mL

centrifuge tube. After addition of 0.1 mL 0.2% INT solution, the tube was incubated under dark condition and shaken in a water bath oscillator at 100 rpm and 37±1°C for 30 min. Then, 1 mL methanol (37%) was added to terminate the reaction. The supernatant was discarded after centrifugation at 10,000 rpm and 4°C for 5 min. Afterward, 5 mL methanol was added into the sediment and the sample was again shaken in dark in the water bath oscillator (100 rpm and 37±1°C) for 10 min. The supernatant obtained after centrifugation (10,000 rpm, 5 min) was measured at 485 nm by a spectrophotometer. The sediment was dried at 105±1°C to a constant weight and weighed. ETS activity was calculated according to Eq. 3-1.

$$U=\frac{A_{485}\times V}{K_T\times W\times t} \quad 3\text{-}1$$

where U is ETS activity ($mg\cdot(g\cdot h)^{-1}$); A_{485} is absorbance at 485 nm; V is the volume of methanol (mL); K_T is the slope of calibration curve; W is the dry weight of the sediment (mg); and t is reaction time (0.5 h).

(3) Analysis of bacterial and archaeal communities

The sludge samples were collected at the end of experiment for bacterial and archaeal community analysis, respectively. For the identification of the archaea, 109F (5′-ACTGCTCAGTAACACGT-3) and 519R (5′-CGTATTACCGCGG CTGCTGG-3') were used as the primer pair for amplification. The method has been described in Chapter 2 (2.2.5).

3.3 Results and discussion

3.3.1 Micro-oxygen environment created by O_2-containing gas NBW

Micro-oxygen environment has also been reported to have enhancement effect on methane yield from AD of lignocellulosic biomass (Fu *et al*., 2016). In this regard, the oxygen contained in NBs is difficult to be detected by the standard DO meter or Winkler oxygen titration method (Alheshibri *et al*., 2016). Since ORP has a linear relationship with the logarithm of oxygen concentration, and a DO of 0.1 mg/L is equivalent to an ORP of -50 mV in the aqueous phase (Nguyen *et al*., 2019). ORP value in the range of 0 to -470 mV defined as a micro-oxygen environment (Nguyen and Khanal, 2018; Nguyen *et al*., 2019). Although the DO value of O_2-NBW was slightly higher than Air-NBW and N_2-NBW after reaching stable (Fig. 3-1a). The highest ORP value was detected in the O_2-NBW reactor (-412 mV), followed by Air-NBW (-475

mV), N_2-NBW (-480 mV) and DW (-489 mV) reactors under the test conditions (Fig. 3-1b). There is no significant difference (p=0.2 > 0.05) in the ORP between Air-NBW and N_2-NBW, likely because the O_2 content in the laboratory air is not high enough to create a micro-oxygen environment during NBW generation. Thus in the O_2-NBW reactor the oxygen content was calculated as 4 μL higher than that in the DW reactor. In addition, the optimal ORP for methanogens is lower than -350 mV (Khanal and Hunag, 2003), indicating that the micro-oxygen environment brought about by O_2-NBW addition may not inhibit the AD of cellulose to produce methane. From the result of Nguyen *et al*. (2019), a lower ORP is more beneficial for methane production. The observations on DO and ORP changes in this study are similar with the O_2-NBW applications in the remediation of anaerobic environment by Wang *et al*. (2018). In previous study, O_2-NBW was claimed to prolong the survival time of aerobic zebrafish. These observations may indicate that the O_2-containing NBW can provide a micro-oxygen environment, which would enhance methane production from AD of lignocellulosic biomass.

3.3.2 Changes in TOC removal and cellulose content

TOC was measured to determine the effect of NBW on the reduction of organic matter during AD. Fig. 3-2a shows the initial and final TOC concentrations and TOC removals during AD. After 18 days of AD, TOC was significantly reduced in all the reactors. The TOC removal reached to 31.8%, 34.1%, 34.6%, and 34.9% in the DW, Air-NBW, N_2-NBW and O_2-NBW reactors, respectively. The results indicated that NBW addition enhanced the utilization efficiency of cellulosic compounds. The highest TOC removal was achieved in the O_2-NBW reactor, increasing by 9.7% (p=0.03 < 0.05) compared to that of the DW reactor. Cellulose reduction was detected in all the reactors (Fig. 3-2b). As shown, the cellulose content gradually decreased along with the 18 days' AD. In the O_2-NBW reactor, cellulose reduction reached to 96.5%, about 13.6% higher compared to the DW reactor (84.9%), followed by N_2-NBW (94.8%) and Air-NBW (92.1%) reactors, respectively. The hydrolysis of cellulose particles can form an oligosaccharides layer, which may hinder the further degradation of cellulose particles (Vaquerizo *et al*., 2018). However, that report did not clarify the method of degrading the oligosaccharide layer. The addition of NBW may enhance the decomposition of the oligosaccharide layer due to the high mass transfer efficiency and miniaturization of NBs as well as high mobility of water molecules in NBW. Thus,

NBW could increase availability of small molecules to methanogens, resulting in the enhanced cellulose degradation by microbial during AD (Fig. 3-2b and Fig. 3-7). Furthermore, the degradation of cellulose mainly depends on cellulase activity.

3.3.3 Cellulase activity

Cellulase activity can be used to indicate the crystalline cellulose degradation ability of microbial consortia. Cellulase can break down the β-(1,4)-glycosidic linkages to degrade cellulose, with the concomitant release of glucose monomers (Chávez-Guerrero *et al*., 2019). In this study, cellulase was analyzed as a representative enzyme involved in the hydrolysis of crystalline cellulose. As shown in Fig. 3-3a, the cellulase activity was relatively low during the initial two days, which was apparently enhanced from day 2 to day 4, and then decreased from day 6 to day 12. This result is to some extent similar with the change of cellulase activity in the AD of corn straw and cow dung (Yu *et al*., 2016). The highest cellulase activity (0.246 U/(mL·min)) was detected in the Air-NBW reactor, about 37.9% higher than that of the DW reactor, which maintained for 2–3 more days compared with other reactors. While during the last stage (12–18 days), the N_2-NBW reactor exhibited the highest cellulase activity (0.212 U/(mL·min)), about 12.6% higher than that of the DW reactor. In addition, the cellulase activity in the Air-NBW and O_2-NBW reactors showed no significant difference (p=0.3 >> 0.05) on day 18, which were 10.1% higher than that in the DW reactor. These results suggest that NBW addition could enhance the cellulase activity, thus promoting crystalline cellulose hydrolysis during AD. However, these results cannot explain the highest methane production from the O_2-NBW reactor. The AD of cellulose basically follows four stages, i.e. hydrolysis, acidification, acetogenesis and methanogenesis. Cellulase hydrolysis only occurs during the hydrolysis stage. Therefore, it could be preliminarily inferred that the methane yield was affected by other three stages with NBW addition, especially trance O_2 in the O_2-NBW reactor may be exert significant effect on methane production.

To further clarify the crystalline cellulose hydrolysis during AD, Table 3-1 shows the cellulose crystallinity reduction in the test reactors. The highest reduction in cellulose crystallinity (38.6%) was detected in the O_2-NBW reactor, about 21.0% increase compared to the DW reactor (31.9%). The cellulose crystallinity reduction was enhanced by 8.4–17.5% under other NBW

additions. Reduction in cellulose crystallinity can increase its surface area and accessibility for better enzymatic hydrolysis/microbial degradation. In Chapter 2 we found that NBW enhanced the reduction of cellulose crystallinity, probably improving the metabolic activity of microbial. However, there is no direct evidence for metabolic activity by adding NBW.

3.3.4 INT-electron transport system activities

The microbial bioactivity during the 18 days' AD with NBW addition were assessed by monitoring the electron transport rate during the microbial metabolism. It is correlated with intracellular ETS through measuring the rate of INT reducing 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride formazan by providing a super electron donor (NADH and succinic acid) (Zhang *et al*., 2018a). In this study, as shown in Fig. 3-3b, initially the ETS activity increased rapidly in all the reactors, which was consistent with cellulase activity changes in the system. The highest ETS activity was detected in the O_2-NBW reactor on day 4, reaching to 247.2 mg/(g·h), which was approximately 1.7 times that of the DW reactor, followed by N_2-NBW (1.6 times) and Air-NBW (1.1 times) reactors (Fig. 3-3b). It has been confirmed that the ETS activity is closely associated with the methanogenic efficiency (Fig. 3-6a). ETS generally occurs in the cell membrane of bacteria (Szabó, 2003). It can transfer electrons from the coenzymes produced by the Szent-Györgyi-Krebs cycle (NADH, NADPH) to the terminal electron acceptor in the oxidative phosphorylation (electron transport) pathway. The surface of NBs has negative charges (Kyzas *et al*., 2019), which could attract NAD^+; NBs also have higher speed of motion, favoring the movement of NAD^+ that is used for reduction reaction to generate NADH. This might be the reason why NBW addition promoted the ETS in the reactors (Fig. 3-7). In addition, the correlation coefficients of ETS and cellulase activity in DW, Air-NBW, N_2-NBW and O_2-NBW was 0.95 ($p < 0.001$), 0.67 ($p < 0.05$), 0.94 ($p < 0.001$) and 0.84 ($p < 0.05$), respectively (Table 3-2) (Pearson > 0, $p < 0.05$ show signification relationship). This suggests that NBW increased the cellulase activity by enhancing the microbial metabolism, which is a result of the hydrolysis of cellulose. Ahmed *et al*. (2018) found that NBW could contribute to seed germination and plant growth, possibly due to the promotion effect of NBs on ETS activity.

O_2 can increase ETS activity because O_2 may easily act as an electron acceptor in ETS (Szabó, 2003). A significant amount of energy can be released by the reduction of O_2 with NADH, which

then produces a proton gradient that drives the synthesis of ATP and the transport of other metabolites (Fig. 3-7). These deductions also suggest that the ETS in the O_2-NBW reactor created micro-oxygen environment should be higher than other reactors, which might be one major reason for the enhanced methane production. Still, future research is demanding on more direct evidence for synthesis of ATP and the transport of other metabolites by adding NBW. The positive effect of NBW addition on ETS activity indicates that NBW could increase the bioactivity of microbial by accelerating the electron transport, and then contribute to the enriched microbial community.

3.3.5 Microbial community analysis

(1) Bacterial phyla

Fig. 3-4a shows the RA of bacterial community at phylum level in the DW, Air-NBW, N_2-NBW, O_2-NBW, N_2/O_2 (4:1)-NBW, N_2/O_2 (1:1)-NBW, and N_2/O_2 (1:3)-NBW reactors. The RA of *Thermotoga*, *Chloroflexi*, *Bacteroidetes*, *Spirochaetae*, *Synergistetes* and *Firmicutes* in the NBW reactors were higher than those of the DW reactor. Most of them are known phyla of hydrolytic and acidogenetic bacteria. *Thermotogae* possesses high capability to degrade lignocellulosic biomass by producing hydrolytic enzymes (Benedetti *et al*., 2019). *Chloroflexi* may accelerate the cellulose hydrolysis as it is regarded as a multicellular filamentous microorganism that hydrolyzes carbohydrates (Ruan *et al*., 2019). The abundant bacterial phylum in all the reactors was *Bacteroidetes* that are responsible for hydrolysis of lignocellulosic biomass to produce acetate, butyrate and propionaye (Xing *et al*., 2020). The RA of *Bacteroidetes* was the highest in the O_2-NBW reactor, which may contribute to the increased efficiency on cellulose degradation. *Spirochaetae* belongs to acetogens, and is capable of acetogenesis and may accelerate the transfer of VFAs to acetate (Zou *et al*., 2018). *Synergistetes* can reduce the accumulation of propionic acid and butyric acid due to its efficient syntrophic oxidation capability (Deng *et al*., 2018), which may contribute to the relatively stable pH during the whole AD. *Firmicutes* can produce extracellular enzymes such as protease, lipase and cellulase, and then involve in the degradation of organic compounds and the formation of VFAs (Zou *et al*., 2018). The results from Fig. 3-3a can explain the increased activity of cellulase in the NBW reactors. As shown, the RA of *Firmicutes* reached the highest in the O_2-NBW (3.6%) reactor, followed by the N_2/O_2 (1:3)-NBW (2.2%), N_2/O_2 (1:1)-NBW (2.1%), Air-NBW (2.0%), N_2/O_2 (4:1)-NBW (1.9%)

and DW (1.8%) reactors, showing a decrease trend as the oxygen content decreased. The above observation to some extent agrees with Fu *et al*. (2016) who found that the RA of *Firmicutes* after micro-aeration treatment was 6% higher than that of the control group. As expected, bacterial phyla analysis showed that the micro-oxygen environment created by NBW addition might promote the enrichment of hydrolytic bacteria in the AD reactors.

(2) Archaeal genus

The RA of archaeal community at the genus level is shown in Fig. 3-4b. The methanogenic archaea were dominant, accounting for 37.4%, 55.1%, 64.6%, 70.7%, 60.7%, 34.8% and 65.5% of archaeal 16S rRNA gene sequences recovered from the sludge samples in the DW, Air-NBW, N_2-NBW, O_2-NBW, N_2/O_2 (4:1)-NBW, N_2/O_2 (1:1)-NBW, and N_2/O_2 (1:3)-NBW reactors, respectively. As seen, *Methanobacterium*, which is highly related to hydrogenotrophic methanogenesis and can enhance interspecies H_2 transfer during AD (Zhang *et al*., 2019), was enriched in all the NBW reactors, especially in the O_2-NBW reactor. The RA of *Methanobacterium* was 10.1% in the O_2-NBW reactor, while it was only 1.2% in the DW reactor. Fu *et al*. (2016) suggested that the RA of *Mathanobacterium* was doubled under microaerobic condition. These observations further clarified the micro-oxygen condition provided by O_2-NBW.

In the Air-NBW and O_2-NBW reactors, the RA of *Methanomassiliicoccus* was relatively high, about 2.9% and 5.3%, respectively in comparison to 1.0% in the DW reactor. *Methanomassiliicoccus* can utilize H_2 and methanol or methylamines to produce methane (Kröninger *et al*., 2016). A higher RA of *Methanospirillum* was detected in the NBW reactors than that in the DW reactor. *Methanospirillum* is considered as the major contributor to methane production from cellulose (Li *et al*., 2018b). Besides, the RA of *Methanolinea* was higher in the O_2-NBW and N_2-NBW reactors, which can use H_2 for growth and methane production (Cayetano *et al*., 2019). These results might be the major reason why almost no H_2 was detected throughout the whole AD process. In addition, interspecies H_2 transfer can contribute to the reduction of pressure-H_2, which may enhance the hydrolysis of organic matter and produce methane (Huang *et al*., 2016). A high pressure-H_2 may have a persistent inhibition effect on the hydrolytic and acetogenic microbial activities, which then inhibits the production or secretion of extracellular enzymes by retroinhibition, reducing the physiological activity of the microorganisms (Cazier *et al*., 2019).

The genus level identification showed that *Methanosaeta* was the dominant genus, which is a strict aceticlastic methanogen genus and can produce methane using acetic acid and CO_2 through DIET via directly accepting electrons from *Geobacter* (Zhao *et al*, 2017; Rotaru *et al*., 2014). *Methanosaeta* usually plays an important role in the methanogenic process, contributing to about 70% of methane produced. NBW has a higher zeta potential and a higher conductivity, especially the O_2-NBW (Fig. 3-5). Li *et al*. (2016) proposed that the surface conductivity of particles may be responsible for the zeta potential. So, it is inferred that the high conductivity of O_2-NBW might be attributable to its high zeta potential. Conductive materials can strengthen the DIET in the complex organic waste (Li *et al*., 2018a). Therefore, the increase in RA of *Methanosaeta* suggests the potential increase of DIET after NBW addition.

3.3.6 Methane production

(1) Methane yield from AD of cellulose under NBW addition

The daily methane production peak appeared on day 4 in the DW, N_2-NBW and N_2/O_2 (1:1)-NBW reactors, while it was on day 6 in the Air-NBW, O_2-NBW, N_2/O_2 (4:1)-NBW and N_2/O_2 (1:3)-NBW reactors; then their daily methane productions decreased. After day 16, the daily methane productions gradually decreased to 0 in all the reactors.

Fig. 3-6a shows that the cumulative methane yields from cellulose in the DW, Air-NBW, N_2-NBW and O_2-NBW reactors varied during the 18 days' batch BMP tests. In the first two days, little methane production from the NBW addition reactors was detected, which is similar with that of the DW reactor. After that, their methane productions increased remarkably until day 14 when their cumulative methane yields became level off. During the 18 days' BMP, the cumulative methane yields from cellulose in the O_2-NBW, N_2-NBW and Air-NBW reactors reached 233.0±6.2, 196.1±1.4, and 193.3±4.5 NmL/g-VS, increasing by 29.9%, 9.3% and 7.7% compared with that from the DW reactor (179.4±2.4 NmL/g-VS). In this study, the highest cumulative methane yield was achieved from the O_2-NBW reactor, most probably attributable to the enriched microbial community (as noted in 3.5), thus enhanced decomposition of cellulose. To clarify the effect of O_2 content in NBW on BMP, the N_2 and O_2 gas mixture NBW was examined through the BMP of cellulose at different mixing ratios (Fig. 3-6b). As expected, the highest methane yield was achieved from the N_2/O_2 (1:3)-NBW reactor, followed by N_2/O_2 (1:1)-NBW and N_2/O_2 (4:1)-

NBW reactors. Thus, the highest O_2 content of NBW has the best performance in the methane production from AD of cellulose.

(2) Analysis based on the modified Gompertz model

The simulated maximum cumulative methane yields (P) are very similar to the experimental methane yields, indicating that the AD of cellulose can be perfectly fitted by the modified Gompertz equation model-based best-fit evaluation of kinetic parameters. The maximum methane production rate (R_{max}) increased in the reactors with NBW addition compared with that in the DW reactor, indicating their enhanced AD efficiency. However, AD with NBW addition showed a longer lag phase time (λ) than the DW reactor, possibly due to that the anaerobic microorganisms need a short adaption period to accommodate the new environment created in the reactors by NBW addition. As shown table 3-1, the theoretical methane productions (TMPs) were calculated based on the chemical formula of cellulose ($(C_6H_{10}O_5)_n$); and BD represents the biodegradability of cellulose during the AD process, which was calculated by the ratio of BMP to TMP. The BD value can be also used to predict the overall enhanced energy recovery and to better assess the degradation of organic matter during AD process. A maximum BD value of 56.2% was achieved in the O_2-NBW reactor, followed by N_2/O_2 (1:3)-NBW (51.2%), N_2/O_2 (1:1)-NBW (49.1%), N_2/O_2 (4:1)-NBW (48.6%), N_2-NBW (47.3%), Air-NBW (46.6%) and DW (43.2%) reactors (Table 3-1), respectively. The results reflect that methane production from cellulose was enhanced with the addition of NBW.

3.4 Summary

The present study provided a promising approach to increase methane yield from AD of cellulose through micro-oxygen environment provided by O_2-NBW. In agreement with the hypothesis, NBW supplementation improved methane production from cellulose during AD. 8%–30% increase of methane yield and 8%–14% increase of cellulose reduction were achieved from AD of cellulose with NBW addition compared to the control (DW addition). The cellulase activity, cellulose crystallinity reduction and ETS activity were also enhanced under NBW addition. O_2-containing gas NBW enriched facultative bacteria and hydrogenotrophic methanogens. In this study, we first confirmed O_2-NBW could provide micro-oxygen environment and then check its

effects on methane production from cellulose in lab-scale tests, which is also great significant to better comprehend the fundamentals involved. O_2-containing gas NBW created micro-oxygen environment could contribute to the methane production with no chemicals addition and/or addition energy consumption.

Table 3-1 Main parameters relating to the modified Gompertz model and cellulose crystallinity.

Reactors	TMP	BMP	BD	Modified Gompertz Model				Cellulose crystallinity
	(NmL/g-VS)	(NmL/g-VS)	(%)	P	R_{max}	λ	R^2	reduction (%)
DW		179.4±2.4 ^{e}	43.2	181.2±3.1	27.3±1.9	2.1	0.996	31.9±0.6 ^{e}
Air-NBW		193.3±4.5 ^{d}	46.6	193.3±1.2	32.1±1.2	2.4	0.999	36.0±0.5 ^{bc}
N_2-NBW		196.1±1.4 ^{cd}	47.3	194.0±2.1	33.8±1.8	2.2	0.998	37.5±0.2 ^{b}
O_2-NBW	414.8	233.0±6.2 ^{a}	56.2	232.1±0.7	50.0±1.0	2.5	0.999	38.6±0.4 ^{a}
N_2/O_2 (4:1)-NBW		201.5±1.3 ^{c}	48.6	209.4±5.0	26.4±2.0	2.6	0.995	34.6±0.8 ^{d}
N_2/O_2 (1:1)-NBW		203.6±2.4 ^{bc}	49.1	206.1±3.2	34.8±2.6	2.2	0.996	37.3±0.6 ^{b}
N_2/O_2 (1:3)-NBW		212.5±3.6 ^{b}	51.2	215.7±2.0	35.4±1.4	2.6	0.998	35.0±0.4 ^{c}

BD-biodegradability, DW-deionized water, NBW-nanobubble water, BMP-Biochemical methane potential, R_{max}-maximum methane production rate (NmL/g-VS/day), P-simulated maximum cumulative methane yield (NmL/g-VS), R^2-correlation coefficient, TMP-theoretical methane production, λ-lag phase time (day). Data are expressed as mean ± SD, and the data with different superscript letters are significantly different at $p < 0.05$ according to statistical analysis.

Table 3-2 Correlation coefficients of ETS activity and cellulase activity in DW, Air-NBW, N_2-NBW and O_2-NBW. DW-deionized water, NBW-nanobubble water.

Reactors	DW	Air-NBW	N_2-NBW	O_2-NBW
Pearson	0.95305	0.66950	0.94208	0.83987
P-value	2.0×10^{-5}	3.4×10^{-2}	4.6×10^{-5}	2.4×10^{-3}

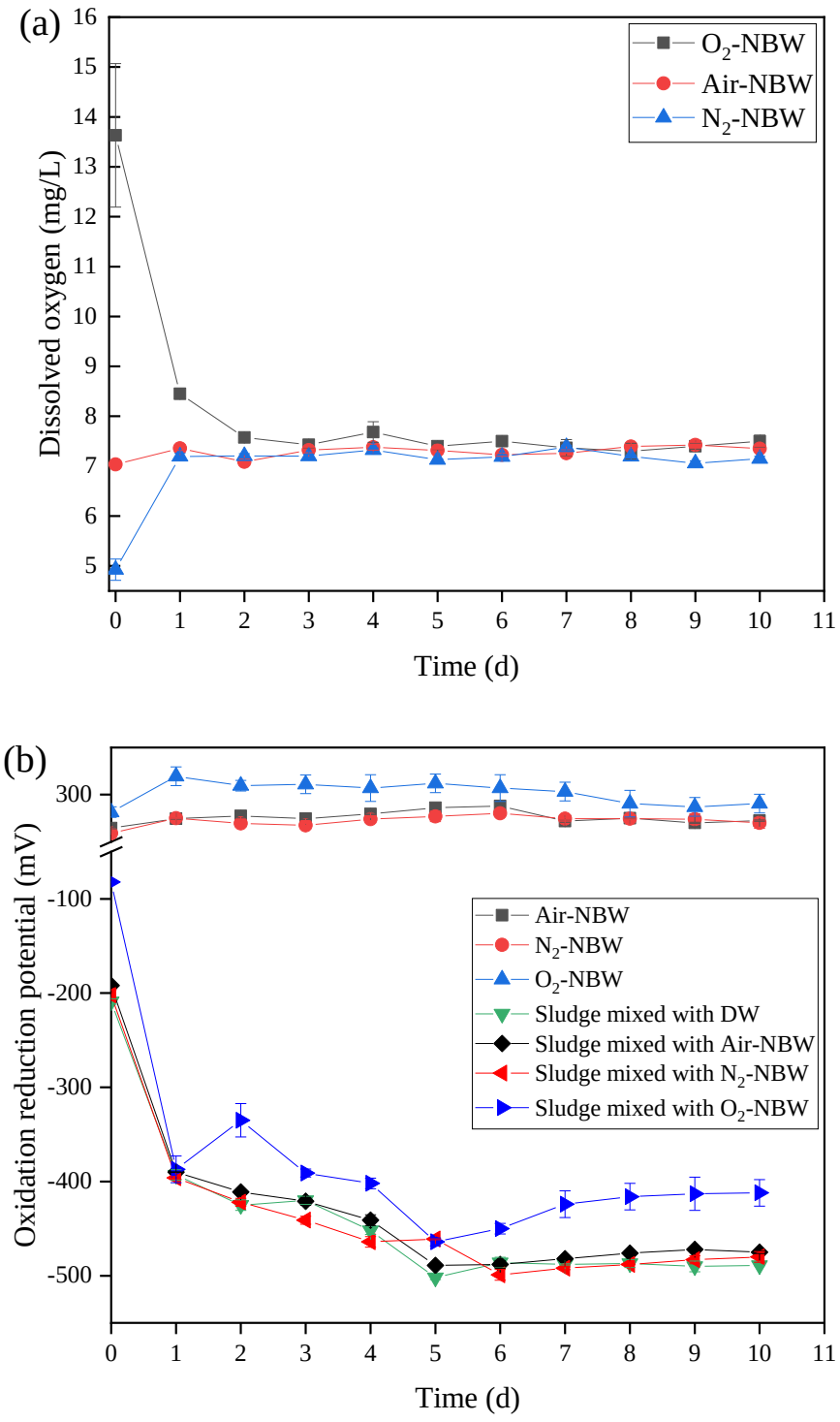

Fig. 3-1 Changes in dissolved oxygen (a) and oxidation reduction potential (b) during 10 days' storage at 35±1°C. NBW-nanobubble water, DW-deionized water.

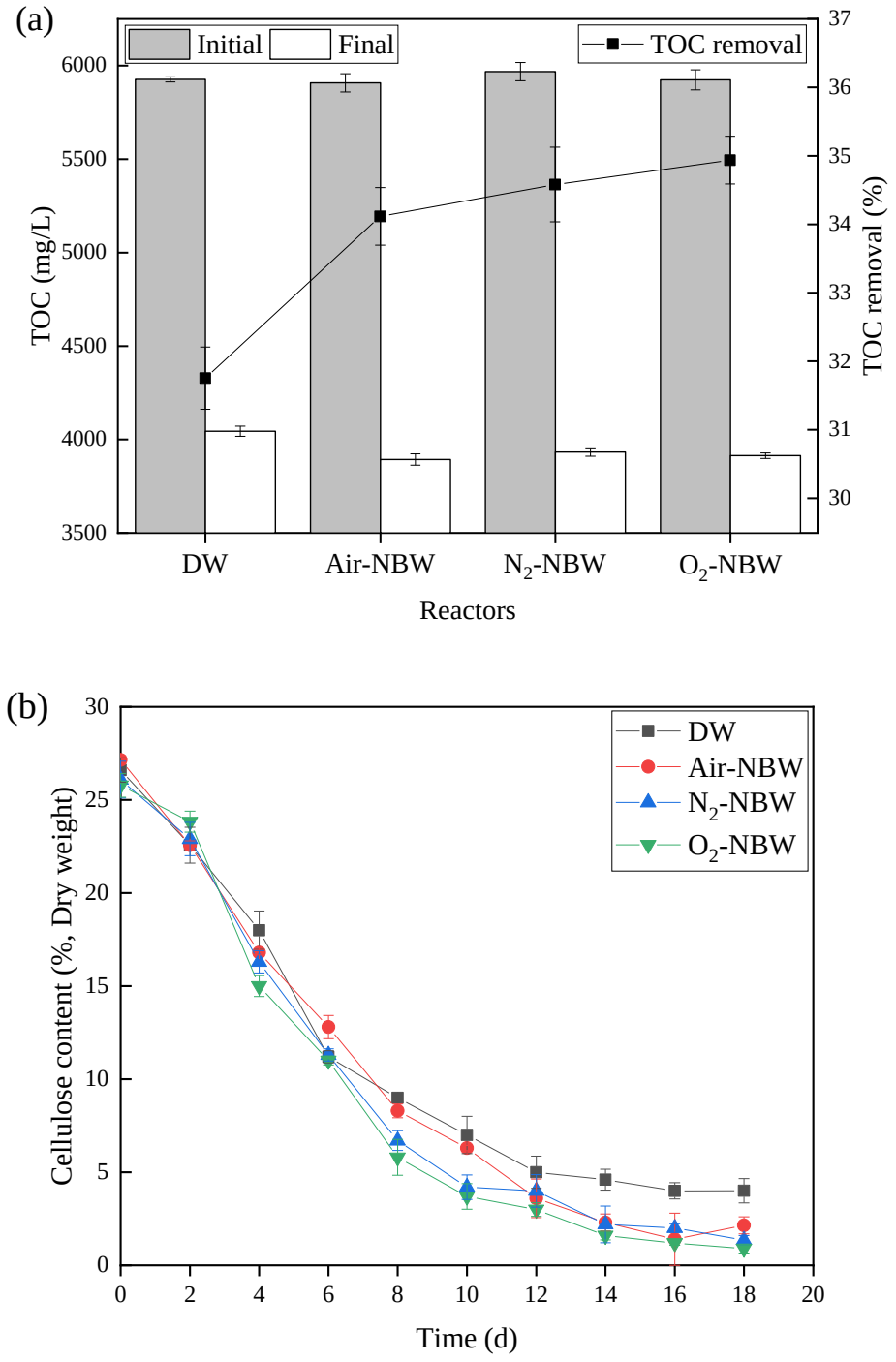

Fig. 3-2 Changes in total organic carbon (TOC) of the initial and finial samples and TOC removals (a), and reduction in cellulose content (b) during anaerobic digestion of cellulose under DW and different NBW addition. DW-deionized water, NBW-nanobubble water.

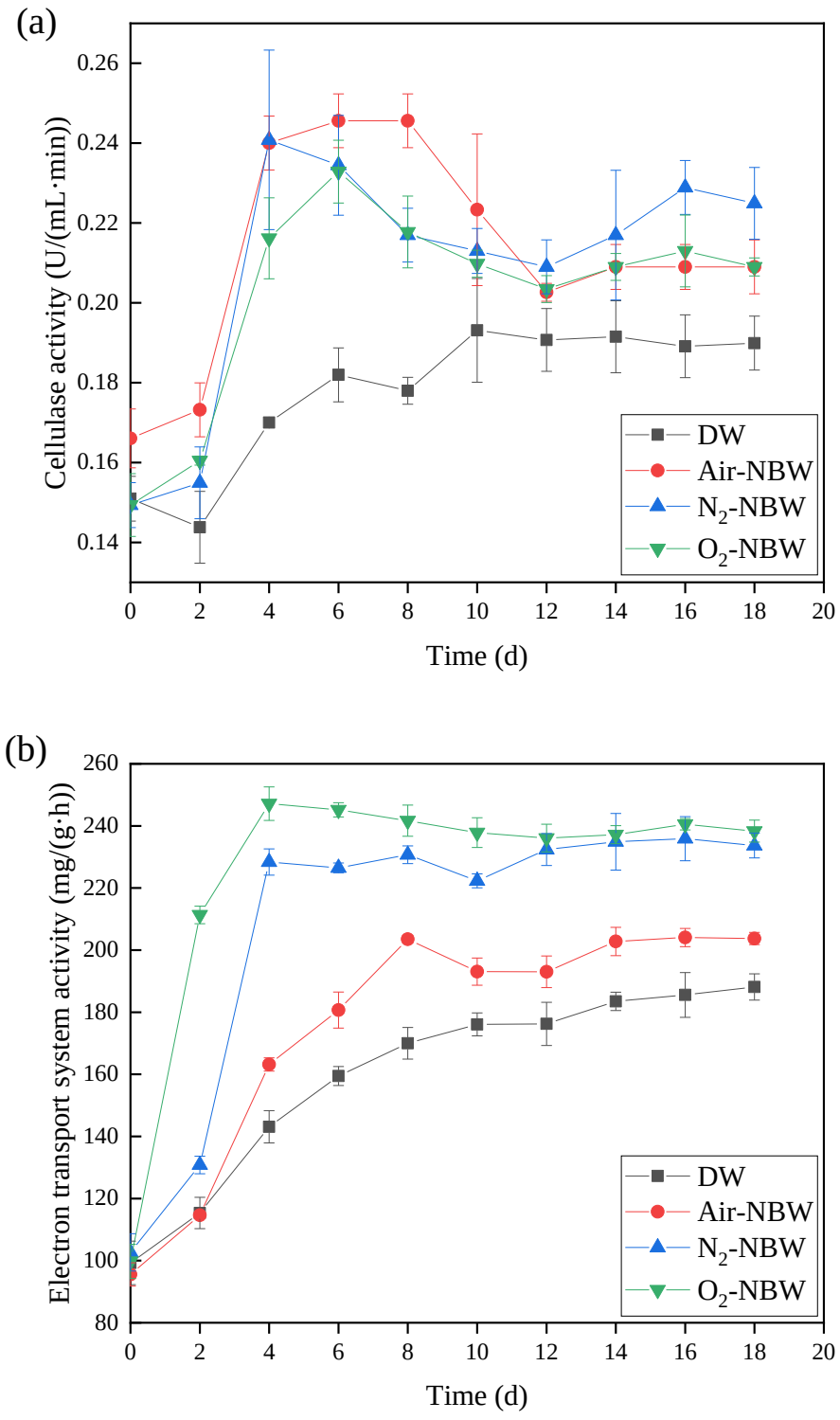

Fig. 3-3 Changes in cellulase activity (a) and electron transport system activity (b) during 18 days' AD under DW, Air-NBW, N_2-NBW and O_2-NBW addition. DW-deionized water, NBW-nanobubble water.

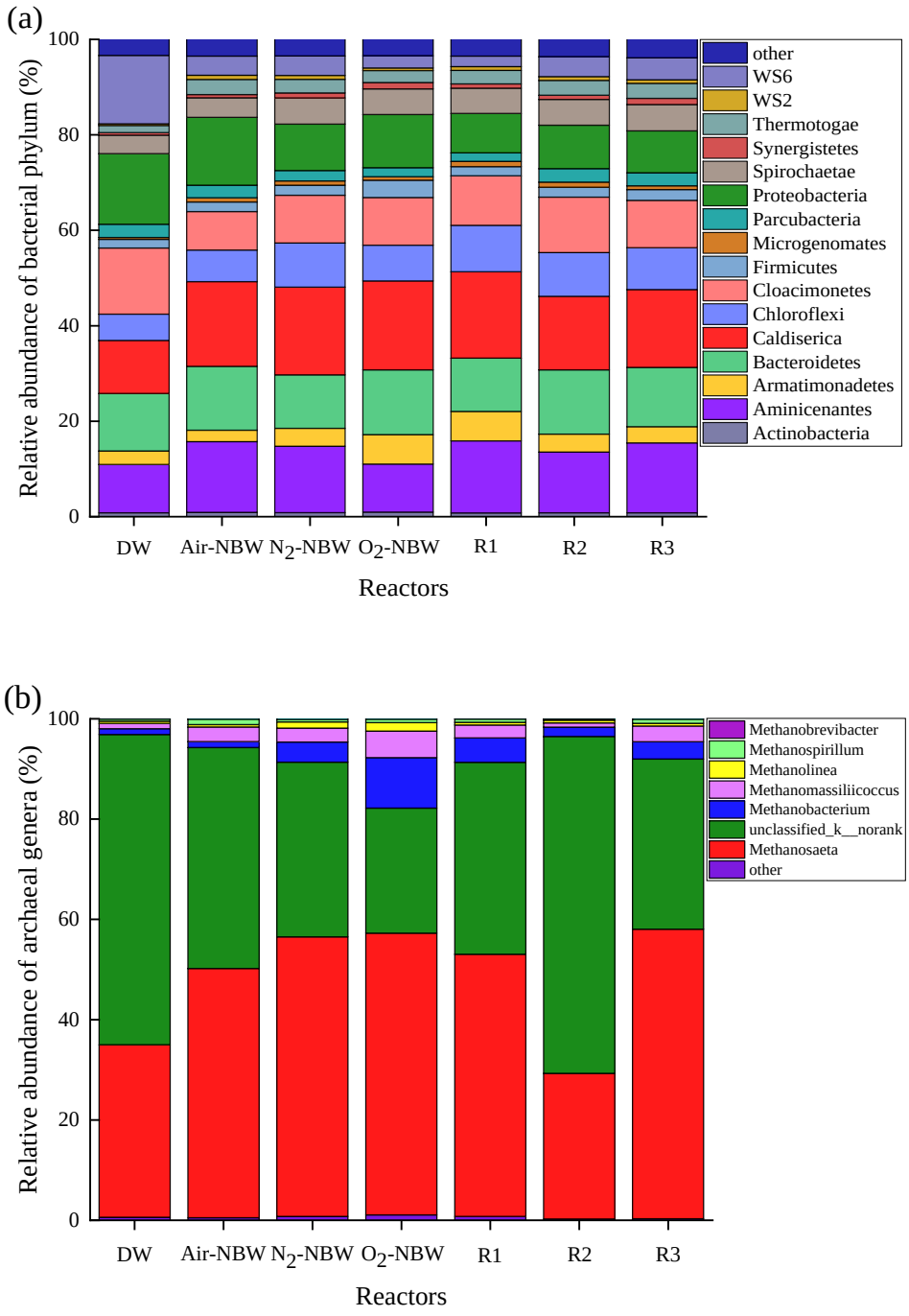

Fig. 3-4 The relative abundance of prevalent bacterial phylum (a) and detected archaeal genera (b). DW-deionized water, NBW-nanobubble water. N_2/O_2 (4:1)-NBW, N_2/O_2 (1:1)-NBW, and N_2/O_2 (1:3)-NBW reactors are denoted as R1, R2 and R3, respectively.

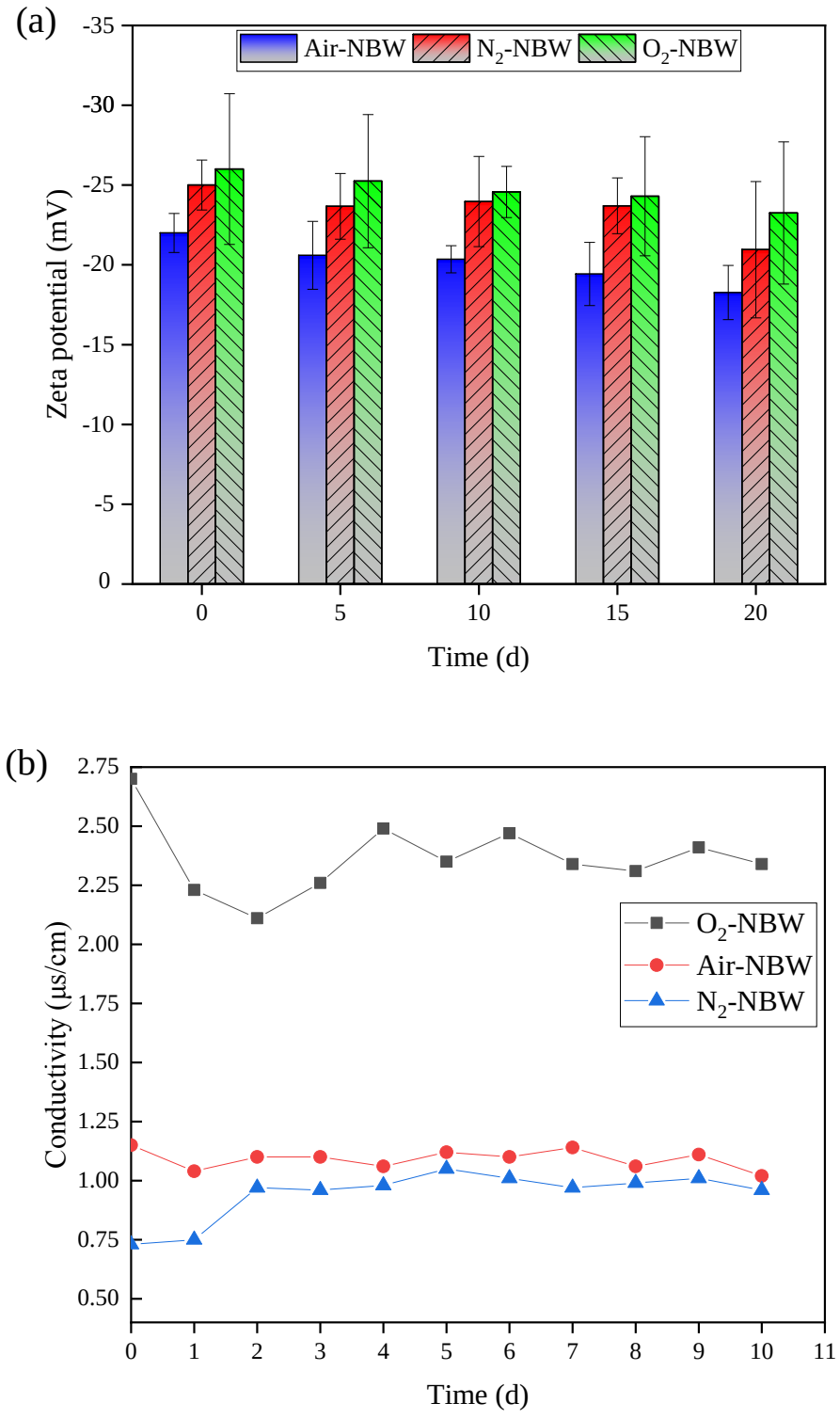

Fig. 3-5 Changes in zeta (a) and conductivity (b) of O_2-NBW, Air-NBW and N_2-NBW storage at 35±1°C. NBW-nanobubble water.

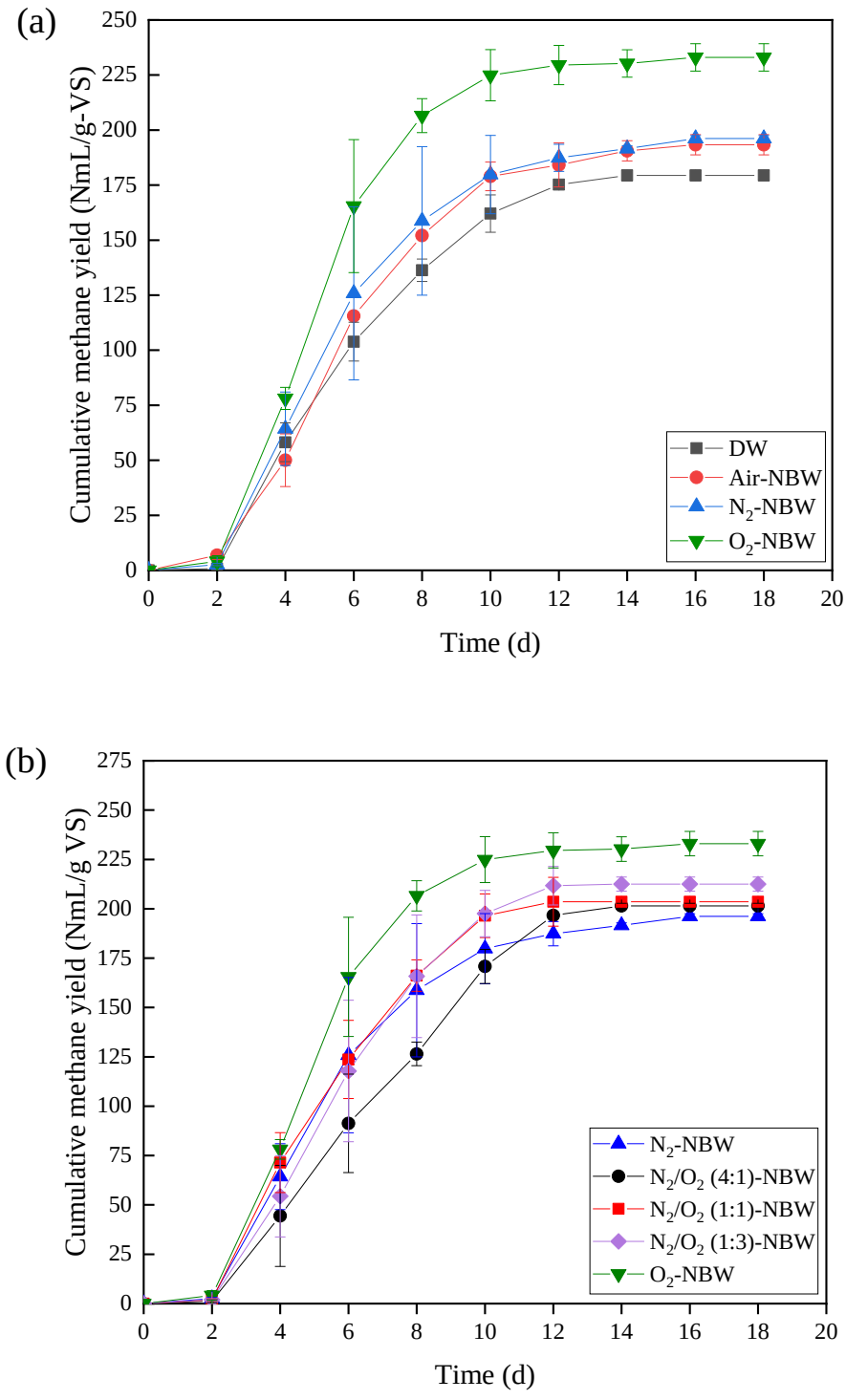

Fig. 3-6 Cumulative methane yields from the DW, Air-NBW, N_2-NBW and O_2-NBW reactors (a), and the different ratios of N_2/O_2 gas mixture-NBW reactors (b) during 18 days' AD of cellulose. DW-deionized water, NBW-nanobubble water.

Character 4 Improved methane production from corn straw using anaerobically digested sludge pre-augmented by nanobubble water

4.1 Background

Corn straw is a typical agricultural waste that is produced in large quantities globally for food, feed and industrial applications. Corn productions in the US and China account for 40% and 20% of the world, respectively (Veljković *et al.*, 2018). In China, the annual production of corn straw is about 216 million tons, which shows high potential for methane production (Wei *et al.*, 2020). Although AD of lignocellulosic biomass has been widely applied to achieve the sustainable management of agricultural wastes, the low energy conversion efficiency limits its application mainly due to the following two aspects: 1) the low metabolic activity of microbes, and 2) the rigidity and recalcitrant structure of lignocellulose (Huang *et al.*, 2019).

In chapters 2 and 3, NBW has been proven to enhance methane production from AD of waste activated sludge and lignocellulosic biomass. Obviously, however, the shortcomings of the NBW-based AD technology are relating to its large reactor volume and then high construction cost. Therefore, the NBW-based AD systems need further improvements for its practical application. In this study, in order to increase the methane production from AD of lignocellulosic materials using NBW while at the same time with no increase in reactor volume, anaerobically digested sludge was firstly pre-augmented by N_2-NBW and O_2-NBW and then used as inoculum to produce methane from corn straw via AD process.

4.2 Materials and methods

4.2.1 Raw materials

Corn straw was collected from Japan Agricultural Co-operatives, Ibaraki, Japan. The harvested corn straw was washed with DW and dried at 60±1^{o}C to constant weight, then passed through a cutting mill (WDL-1, Osaka Chemical Co., Ltd., Japan). The crushed biomass was sieved through a 0.6 mm pore size sieve and stored at room temperature in vacuum bags, which was used as the sole substrate for AD in this study.

The anaerobically digested sludge used in this study was the same as in Chapter 2 (2.2.1). The characteristics of corn straw is listed in Table 4-1. NBW was prepared as same as Chapter 2 (2.2.1). The other methods have been shown in Chapter 2 (2.2.4).

4.2.2 Pre-augmentation of anaerobically digested sludge by DW, N_2-NBW and O_2-NBW

The interaction between the digested sludge and NBW was explored during the long-term AD of corn straw. In this study, N_2-NBW and O_2-NBW were selected to pre-augment the anaerobically digested sludge. The total effective volume of the pre-augmentation reactors (600 mL serum bottles) was about 400 mL, which composed of 200 mL of anaerobically digested sludge and 200 mL of NBW or DW. Corn straw was added into the bottles to achieve a F/M ratio of 1.5 (VS basis) at the beginning of each pre-augmentation with DW or NBW addition. The initial pH of the pre-augmentation reactor was adjusted to 7.00±0.05 by use of 2.0 M NaOH or HCl. Afterwards, all the pre-augmentation bottles were sealed using rubber stoppers and purged with nitrogen gas for 5 min to create anaerobic condition and pressure balance with the atmosphere, which were then placed into a temperature-controlled incubator (35±1°C). The reaction residue was centrifuged at 5000 rpm for 10 min to remove the supernatant when methane production was less than 1% of the accumulated volume of methane during three consecutive days (Holliger *et al*., 2016), which was then used as the inoculum for the next step of pre-augmentation. Namely, the equal amounts of DW or NBW and corn straw were added to repeat the above steps for 6 times, totally about 214 days for this pre-augmentation duration. All the experiments were conducted in triplicate. In the process of DW or NBW pre-augmentation, 2 mL of reaction liquid was sampled from each bioreactor during each augmentation cycle (about 30 days) for the determination of ETS activity.

4.2.3 BMP tests

According to the protocol proposed by Angelidaki *et al*. (2009), BMP tests were conducted to assess the biodegradability of corn straw and methane production by using the above pre-augmented anaerobically digested sludge as inoculum. All the tests were performed in triplicate in closed Schott Duran serum bottles (120 mL). In these bottles, the added mass weight of pre-augmented anaerobically digested sludge as inoculum was 35 g, with an appropriate mass weight

of corn straw to obtain an F/M of 1.5 (VS basis). In addition, to avoid detection error, the effective volume of each bottle was controlled at 60 mL by adding DW. The BMP tests were performed under the same condition as the AD in section 2.2. Blank treatments were also carried out to eliminate the methane production from the inoculum only, which was anaerobically digested with DW, N_2-NBW and O_2-NBW, respectively (Holliger *et al*., 2016). All the bottles were manually shaken twice at the fixed time points every day. Samples from every bottle were collected to analyze the total volatile fatty acids (TVFAs), pH, crystallinity degree, cellulose, hemicellulose and lignin contents, etc.

4.2.4 Major polymers quantification and structural characterization

All the samples from the reactors were respectively dried at 105±2°C to constant weight followed by milling, and used for the determination of lignin, cellulose, hemicellulose contents and elemental content (C, H, O and N). After being hydrolyzed with 72% H_2SO_4 (w/w), lignin content was determined by gravimetric method. The liquid after hydrolysis was used to analyze glucose and reducing sugars, which were utilized for the estimation of cellulose and hemicellulose contents (Ververis *et al.*, 2007). Glucose was measured by a colorimetric micro-method (Mendel *et al.*, 1954), and reducing sugar was quantified with a dinitrosalicylic acid (DNS) colorimetric method (Miller, 1959). Elemental analysis of corn straw was performed on a CHN elemental analyzer (Perkin-Elmer 2400 II, USA).

4.3 Results and discussion

4.3.1 Bioactivity of anaerobically digested sludge during pre-augmentation with DW or NBW

The increase in reactor volume due to NBW addition hinders the applications of NBW-based AD technology for methane production from waste lignocellulosic biomass. Thus, in this study, NBW was firstly used to pre-augment anaerobically digested sludge with corn straw as the sole substrate in order to avoid the volume increase of the real AD reactors if being applied in practice. It is of the first importance to evaluate the activity of microorganisms during the pre-augmentation with NBW or DW addition. Results show that N_2-NBW or O_2-NBW stored at 35±1°C for 30 days can maintain its NBs concentration and zeta potential with no obvious difference. This

phenomenon is similar with the result from Ghadimkhani *et al*. (2016). Thus, in order to utilize NBW to continuously enhance the biological activity of digested sludge during 214 days of augmentation, DW, N_2-NBW and O_2-NBW were supplied to the reactors approximately every 30 days by taking NBs stability into consideration.

ETS is an effective indicator for the respiration of microorganisms and the potential performance of substrate removal in sludge (Wang *et al.*, 2016). As shown in Fig. 4-1, during the entire NBW or DW pre-augmentation of anaerobically digested sludge, the ETS activities in all the reactors were enhanced along with the pre-augmentation times. Till the last pre-augmentation (the 6^{th} pre-augmentation), the ETS activity in the reactors with DW, N_2-NBW and O_2-NBW addition increased by 13.8%, 11.8% and 21.0% when compared to the 1^{st} pre-augmentation. The highest ETS activity was achieved in the O_2-NBW reactor (155.2±2.6 mg/(g·h)) after the 6^{th} pre-augmentation, followed by N_2-NBW (149.1±3.5 mg/(g·h)) and DW (134.8±3.1 mg/(g·h)) reactors. This observation suggests that the electron transport efficiency of microbial metabolisms was facilitated with the addition of NBW. O_2 can act as an electron acceptor to increase the ETS activity (Gui *et al.*, 2017), which might be the reason for the highest ETS activity in the O_2-NBW pre-augmented anaerobically digested sludge. This observation indicates that NBW could increase the ETS activity of anaerobically digested sludge and enhance the microbial metabolism. In contrast, copper oxide nanoparticles treatment can greatly reduce ETS activity by 24-48% due to its toxic effects on microorganisms (Zhao *et al*., 2020). Silver nanoparticles may also decrease ETS activity, showing toxicity to microbial glucose metabolism (Liu *et al*., 2020a). Results from this work suggest that the contaminant-free NBW is advantageous for the respiration of microorganisms.

Under DW or NBW pre-augmentation condition, the methane yield was also observed to increase along with the pre-augmentation times. In the 1^{st} pre-augmentation, the methane yields from the DW, N_2-NBW and O_2-NBW reactors showed no significant difference ($p > 0.60$), while the yields increased along with the pre-augmentation operation. During the last or 6^{th} pre-augmentation, the O_2-NBW and N_2-NBW reactors produced more methane from corn straw, about 19.5% and 8.5% respectively higher than the DW reactor (the control group) after 214 days' pre-augmentation of anaerobically digested sludge. The changes in ETS activity were in agreement with the methane yields obtained in this study. Results from this work indicate that the microbial

activity of anaerobically digested sludge was enhanced by NBW pre-augmentation, which can overcome the low metabolic activity of microbes. Still, future research is demanding on more direct evidence for changes in microbial community during the augmentation process by adding NBW. The enhanced metabolic activities of anaerobic microbes pre-augmented by NBW on corn straw degradation for methanogenesis were confirmed in the BMP tests by methane production and cellulose, hemicellulose and lignin reductions in the following sections 3.2.2 and 3.4.1, respectively.

4.3.2 Changes in crystallinity degree and digestibility of corn straw during BMP tests

(1) Crystallinity degree

Crystallinity degree, an indicator for the digestibility of lignocellulosic biomass, can be used to reveal the relative extent of cellulose crystallinity of corn straw (Perrone *et al.*, 2016). Highly crystalline cellulose can be degraded by microorganisms during the hydrolysis stage, resulting in the decrease in crystallinity degree of biomass (Gupta *et al.*, 2016). It's necessary to investigate the effect of NBW pre-augmented anaerobically digested sludge on the decrease of corn straw crystallinity during BMP tests. In this study, the crystallinity degree of corn straw was determined by XRD (Table 4-2). After AD of corn straw, the largest reduction in cellulose crystallinity (21.1%) ($p < 0.01$) was detected in the O_2-NBW pre-augmented sludge reactors, followed by N_2-NBW (20.2%) ($p = 0.02$) and DW (17.4%) pre-augmented sludge reactors. There is no significant difference ($p = 0.14$) in the reduction of cellulose crystallinity between the O_2-NBW and N_2-NBW reactors. A higher crystallinity reduction of corn straw reveals more crystalline cellulose was degraded during the AD process (Xu *et al.*, 2018). The decrease in crystallinity of corn straw could destroy the cellulosic crystalline areas and inner surface areas, subsequently improving the hydrolysis of corn straw. This observation might explain why more cellulose decomposition occurred in the reactors with NBW pre-augmented anaerobically digested sludge. Thus, use of NBW pre-augmented sludge as inoculum could enhance the degradation of corn straw.

(2) Reductions of cellulose, hemicellulose and lignin

In order to further understand the improvement of corn straw digestibility and the utilization of its major organic constituents, cellulose, hemicellulose and lignin contents were quantified during BMP tests with their reductions shown in Fig. 4-2. The DW or NBW pre-augmented

anaerobically sludge can achieve 20.4-38.4%, 28.8-35.4% and 8.4-9.4% of cellulose, hemicellulose, and lignin reductions, respectively during these tests. The observation also reflects that generally the NBW pre-augmented anaerobically sludge had a better performance on the degradation of corn straw. Specifically, all the BMP tests showed no significant difference in the reduction of lignin ($p > 0.05$), most probably attributable to the more recalcitrant nature of lignin than cellulose and hemicellulose (Li *et al.*, 2018b). Compared to the DW test group (control), the reductions in cellulose and hemicellulose were enhanced by 60.3-88.2% and 21.5-22.9%, respectively in the reactors with the NBW pre-augmented anaerobically digested sludge. As expected, the largest cellulose reduction was detected in the reactors with the O_2-NBW pre-augmented sludge ($p < 0.05$). These results imply that the anaerobically digested sludge augmented by O_2-NBW was enriched with microbial consortium that possess higher biodegradation capability of corn straw. The rigidity and recalcitrant structure of the lignocellulose can be destroyed by the NBW pre-augmented digested sludge. More corn straw can be decomposed and converted into soluble monomers. Thus the reductions of cellulose, hemicellulose and lignin can promote the degradation of lignocellulose and then improve intermediate products (VFAs and hydrogen) production for methanogenesis.

4.3.3 Changes in TVFAs and hydrogen during BMP tests

(1) TVFAs

During a typical AD process, conversion of macromolecular matters to soluble substrates is the first step, namely, the hydrolysis-acidification stage, which is also the limiting step of the AD of corn straw (Janke *et al.*, 2017). As the important intermediate products from the hydrolysis-acidification, TVFAs were detected as shown in Fig. 4-3a. Initially, the TVFAs increased rapidly in all the reactors, and the highest TVFAs were detected in the DW reactors on day 6 (2337.3 mg/L), followed by N_2-NBW (1971.7 mg/L) and O_2-NBW (1747.2 mg/L) reactors on day 2. On day 18, almost no TVFAs production was detected in the O_2-NBW reactors, and a very small amount of TVFAs in the N_2-NBW and DW reactors; from then onwards, very little TVFAs were detected in all the reactors. The trace amount of oxygen in AD might enhance the proliferation of hydrolytic bacteria, strengthening the hydrolysis and acidification to produce more TVFAs (Ruan *et al.*, 2019). Also, in Chapter 2 we reported that micro-oxygen environment could enhance VFAs

yield during the hydrolysis and acidification of cellulose under Air-NBW addition. It thus was expected that the trace amounts of oxygen in O_2-NBW could improve the production of VFAs during the hydrolysis of corn straw. However, lower TVFAs concentrations were detected in the O_2-NBW reactors, likely attributable to the higher consumption rate of TVFAs by the O_2-NBW pre-augmented sludge than TVFAs production rate from hydrolysis of corn straw. This observation implies that NBW pre-augmentation may enrich the methanogens in the anaerobically digested sludge, thus enhancing the methanogenic stage of AD. As a result, the O_2-NBW pre-augmented sludge reactors gained the highest methane yield, followed by the N_2-NBW and control groups. Further discussion could be found in section 3.4.

Besides, the oxidation of propionic acid and butyric acid can generate electrons that are transferred to protons to produce hydrogen (Tian *et al.*, 2019), providing a reasonable explanation for the reactors with O_2-NBW pre-augmented sludge to have a rapid consumption of VFAs. This is most probably due to the highest ETS activity gained by the O_2-NBW reactors that promoted the oxidation of VFAs to hydrogen. Results from this work show that anaerobically digested sludge pre-augmented by NBW could enhance VFAs production during the hydrolysis-acidification stage and then the consumption of VFAs to produce hydrogen for methanogenesis.

(2) Hydrogen

Hydrogen as the major intermediate gaseous product during the BMP tests was monitored as shown in Fig. 4-3b. Hydrogen and carbon dioxide can be converted into methane by hydrogenotrophic methanogens (Amani *et al.*, 2010). As it can be seen, the hydrogen content was relatively high (2.7%, 4.1% and 4.8% in the DW, N_2-NBW and O_2-NBW reactors, respectively) during the initial stage of AD; then its content gradually decreased to zero in all the reactors. NB has been claimed to generate reactive oxygen species (ROSs) when NB collapses, a cost-effective and non-chemical approach, which include singlet oxygen ($^1O_2$), hydroxyl radicals (·OH), and super-oxide anion radicals (O_2^-) (Lyu *et al.*, 2019). All of them have been proven to contribute the improved hydrogen yield from AD of organic compounds (Wang *et al.*, 2019b). In addition, O_2-NB favors the formation of free radicals compared to N_2-NB (Li *et al.*, 2009). These observations may explain why the highest hydrogen content was detected in the O_2-NBW reactors in this study. Generally, the activity of hydrogenotrophic methanogen can be enhanced under high hydrogen content condition, resulting in the accelerated consumption of hydrogen to produce

methane. Therefore, a rapid consumption of hydrogen can be an indicative of a faster methane production during the subsequent methanogenesis (Ambuchi *et al.*, 2017).

4.3.4 Methane production during BMP tests

(1) Methane yield from AD of corn straw with anaerobically digested sludge pre-augmented by NBW or DW

The cumulative methane yields from corn straw by using NBW or DW pre-augmented sludge are shown in Fig. 4-4. Almost no gas was produced from the blank treatments (only the inoculum augmented by DW, N_2-NBW and O_2-NBW), and there is no obvious difference in methane content in all the reactors. The methane yields from the NBW pre-augmented sludge reactors increased sharply before day 16 and reached a plateau afterwards. The final methane yield from the DW pre-augmented sludge reactors was 115.9±3.5 NmL/g-VS after 24 days' AD of corn straw. As expected, the highest methane yield, averagely 141.7±5.8 NmL/g-VS ($p = 0.03 < 0.05$) was achieved in the O_2-NBW pre-augmented sludge reactors, followed by the N_2-NBW reactors (127.3±2.6 NmL/g-VS) ($p = 0.03 < 0.05$), increasing by 22.3% and 9.8%, respectively when compared with the control reactors. Fu *et al*. (2016) claimed that microaeration could enhance methanogenesis of corn straw. When calcium peroxide was added into AD of lignocellulosic biomass, methane production could be enhanced by 11% (Eom *et al*., 2019). Xu *et al*. (2018) reported that *Bacillus Subtilis* micro-aerobic pretreatment of cellulosic material improved methane production and cellulose removal by 17.4% and 7%, respectively from AD of corn straw. Seen from this comparison analysis, it is certain that the NBW pre-augmented anaerobically digested sludge as inoculum could achieve the comparable enhancement effect on methane production from corn straw.

The mass fractions of organic C, H, O and N in the dry corn straw were determined as 39.5%, 5.7%, 35.9% and 1.9% (TS basis), respectively (Table 4-1). According to Eq. 2-4 in Chapter 2, the TMP of corn straw was calculated as 223.3 NmL/g-VS. The BD value denotes the biodegradability of corn straw during the AD process according to Eq. 2-5 in Chapter 2. The highest BD was achieved in the O_2-NBW pre-augmented sludge reactors, about 63.5%, followed by N_2-NBW (57.0%) and DW (51.9%) reactors (Table 4-2). These results show that the biodegradation of corn straw can be enhanced by the anaerobically digested sludge after NBW

pre-augmentation, which has been further evidenced by the methane production from the BMP tests.

(2) Gompertz model analysis

The methane yields from all the reactors were fitted to the Gompertz model, and the results are also summarized in Table 4-2. The Gompertz model parameters are usually used to better understand the methanogenesis process. The maximum methane yields from the model simulation were similar to the experimental data, indicating that the cumulative methane yield curve fitted well with the Gompertz equation. This is further supported by the high correlation coefficient (R^2=0.993-0.998). As for the O_2-NBW pre-augmented sludge reactors, the lag-phase time (λ) was the shortest among all the test reactors. As reported by Xu *et al*. (2018), a higher bacteria proportion is responsible for the shorter lag-phase time obtained. In this work it is hypothesized that O_2-NBW pre-augmentation may enrich the hydrolytic and methanogenetic bacteria in the anaerobically digested sludge, thus shortening the lag-phase time of AD, which is still under investigation. In addition, a shorter lag phase can improve the economic benefits of the AD system. Results from kinetic modeling further indicate that use of NBW pre-augmented anaerobically digested sludge is promising for the enhancement of methane production from corn straw and its AD efficiency.

4.3.5 Implication of this study to practice

NBW has unique merits for the AD process, such as low cost, simple operation and strong pertinence. Compared with other pretreatment technologies, including acid, alkali and steam explosion, NBW-based AD system has low ecological risk with relatively low energy consumption. In this study, we first attempted NBW pre-augmentation of anaerobically digested sludge and then checked its effects on methane production from corn straw in lab-scale tests, which is also very important to better understand the fundamentals involved. NBW-based AD technology is still at its preliminary stage. Results from this study show that the NBW augmented anaerobically digested sludge could contribute to enhanced methane production from corn straw with no need of increasing reactor volume like previous studies. Future research should be directed to its great potentials for pilot- and/or full-scale AD systems, and their long-term practical application and commercial tests.

4.4 Summary

Anaerobically digestion sludge was subjected to NBW pre-augmentation to improve its bioactivity and methane production from corn straw via AD. The O_2-NBW pre-augmented sludge reflected the highest microbial bioactivity, achieving 22% increase in methane yield (141.7±5.8 NmL/g-VS), and 88.2% and 22.9% increments in cellulose (38.4%) and hemicellulose (35.4%) reductions during AD of corn straw in comparison to the control (DW reactors), respectively. This work demonstrates the concept and possibility of NBW pre-augmentation of anaerobically digested sludge and then the improvement of methane production from corn straw, targeting no increase of AD reactor volume in practice and sustainable management of lignocellulosic biomass.

Table 4-1 Characteristics of corn straw and anaerobically digested sludge used in this study.

Characteristics	Corn straw	Anaerobically digested sludge
pH	ND	7.27±0.01
Total solid (TS, %)	91.10±0.02	2.100±0.007
Volatile solid (VS, % of TS)	83.40±0.02	74.760±0.001
Organic C (% of TS)	39.5	ND
Organic H (% of TS)	5.7	ND
Organic O (% of TS)	35.9	ND
Organic N (% of TS)	1.9	ND
Crystallinity (%)	31.0±0.01	14.0±0.01
Cellulose (%)	32.5±0.8	14.3±1.7
Hemicellulose (%)	28.4±0.4	12.3±1.5
Lignin (%)	8.6±1.2	5.6±2.3

ND-not determined.

Table 4-2 Methane production, crystallinity index, and parameters estimated from the modified Gompertz model by fitting to the experimental data.

Pre-augmented sludge reactors	TMP (NmL/g-VS)	BMP (NmL/g-VS)	BD (%)	Modified Gompertz model				Crystallinity reduction (%)
				P	R_{max}	λ	R^2	
DW		115.9±3.5 c	51.9	122.5	8.4	1.0	0.998	17.4±0.08 [b]
N_2-NBW	223.3	127.3±2.6 b	57.0	136.2	9.9	1.4	0.993	20.2±0.54 [ab]
O_2-NBW		141.7±5.8 a	63.5	145.9	13.4	0.9	0.996	21.1±0.01 [a]

BD-biodegradability, DW-deionized water, BMP-biochemical methane production, P-simulated maximum methane yield (NmL/g-VS), NBW-nanobubble water, R^2-correlation coefficient, R_{max}-maximum methane production rate (NmL/g-VS/day), TMP-theoretical methane production, λ-lag phase time (day). Data are expressed as mean ± SD, and data with different superscript letters denote significant difference at $p < 0.05$.

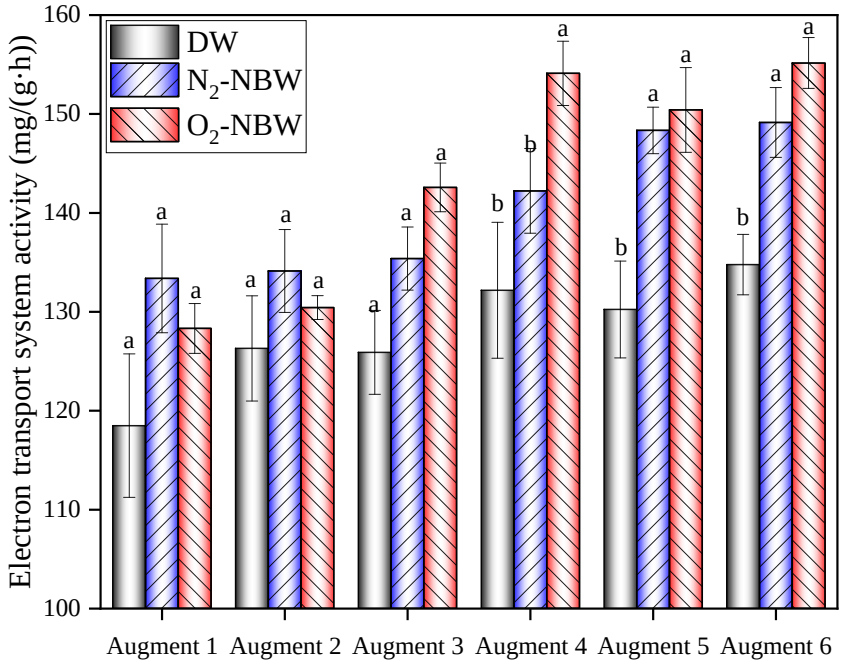

Fig. 4-1 Changes in electron transport system activity during six times of pre-augmentation by DW, N_2-NBW and O_2-NBW with corn straw as sole substrate. DW-deionized water, NBW-nanobubble water. Data are expressed as mean ± SD, and the data with different superscript letters denote significant difference at $p < 0.05$.

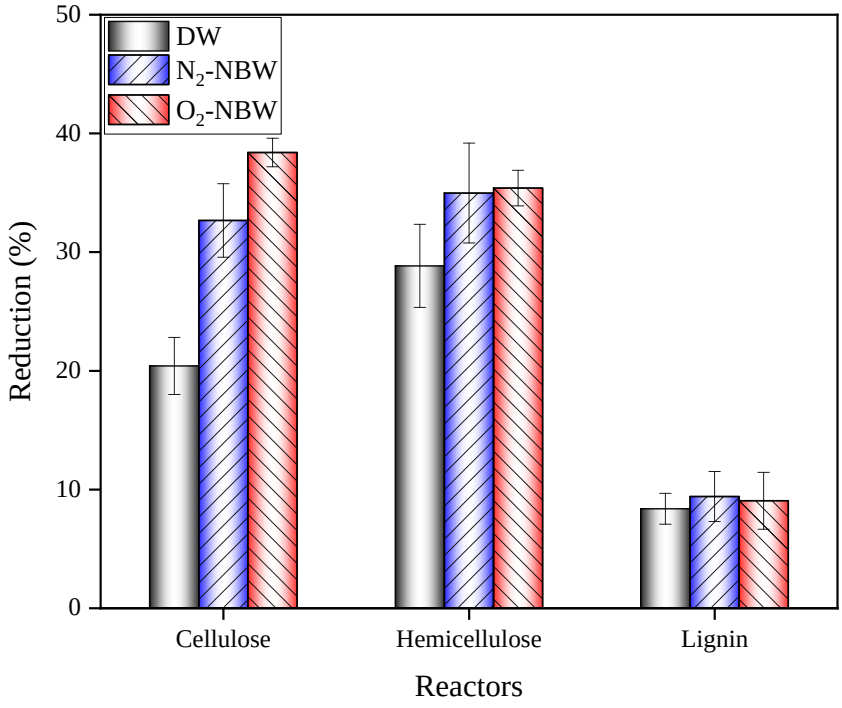

Fig. 4-2 Reductions of cellulose, hemicellulose and lignin by the DW, N_2-NBW and O_2-NBW pre-augmented anaerobically digested sludge with corn straw as sole substrate. DW-deionized water, NBW-nanobubble water.

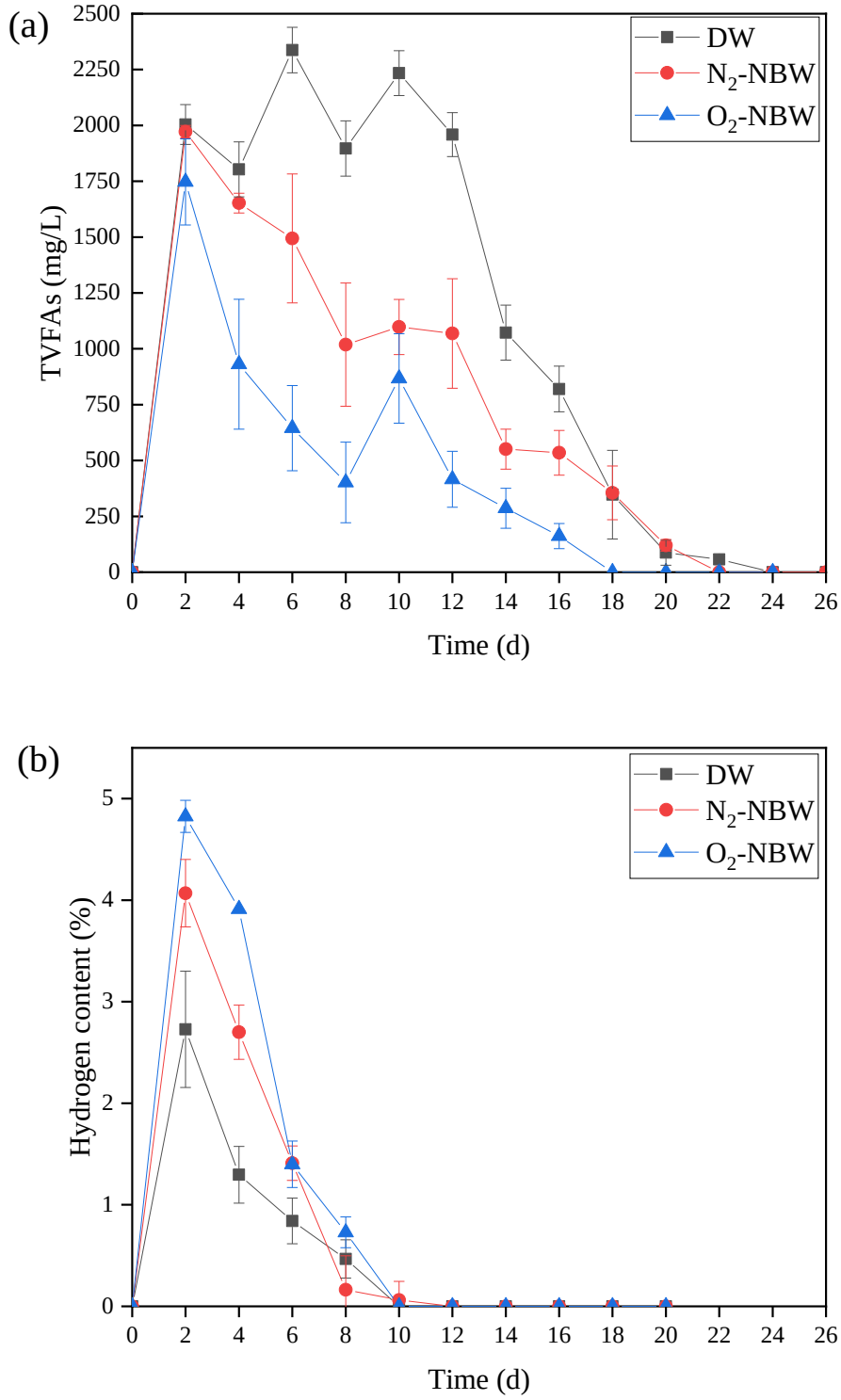

Fig. 4-3 Variations of total volatile fatty acids (TVFAs), a) and hydrogen content (b) in the DW, N_2-NBW and O_2-NBW pre-augmented anaerobically digested sludge reactors during anaerobic digestion of corn straw. DW-deionized water, NBW-nanobubble water.

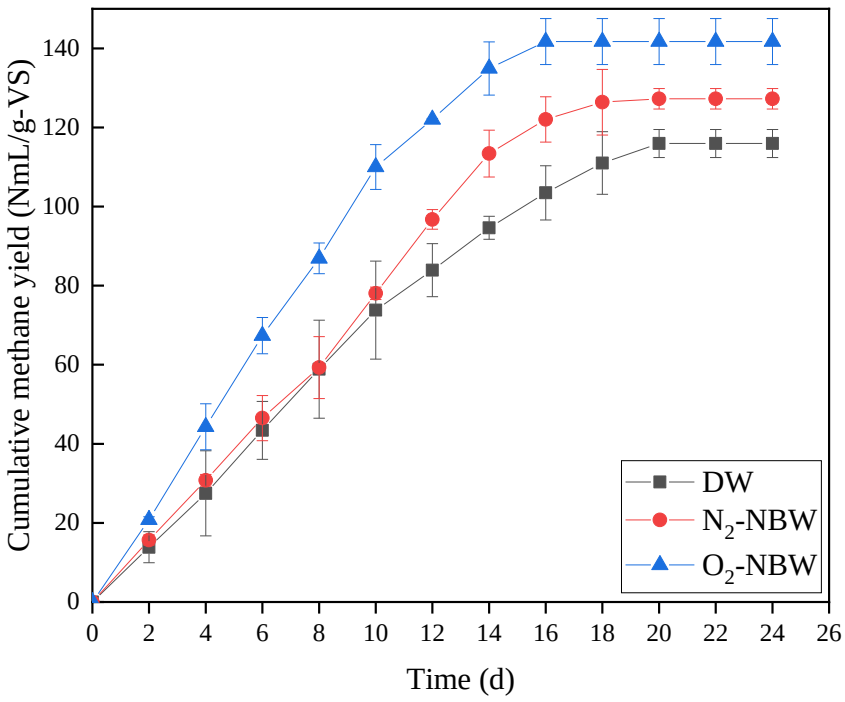

Fig. 4-4 Cumulative methane yields from the DW, N_2-NBW and O_2-NBW pre-augmented anaerobically digested sludge reactors during the 24 days' anaerobic digestion of corn straw. DW-deionized water, NBW-nanobubble water.

Chapter 5 Mechanisms analysis

The novel NBW-based AD system developed in this research can realize enhanced lignocellulosic biomass degradation and methane production. This chapter summarized the possible mechanisms involved in this novel NBW-based AD system according to the results from chapters 2-4.

5.1 Enhanced hydrolysis in the NBW-based AD system

Hydrolysis of cellulose particles can generate an oligosaccharide layer under high cellulose loading condition, which could create a mass transfer resistance (Vaquerizo *et al.*, 2018), thus increasing the resistance to the surrounding environment (Bouchard *et al.*, 2016). The high-mobility water molecules can diffuse from the bulk phase to the surface of the cellulose particles where the oligosaccharide layer is formed (Vaquerizo *et al.*, 2018), which is then attacked by the high-mobility water molecules in NBW. NBW has a positive effect on overcoming the obstacles of cellulose hydrolysis to produce the oligosaccharide layer (Fig. 5-1). On the other hand, the hydrophobic and negatively charged surface of NBs is capable to adsorb trace metals that are essential for anaerobic microorganisms (Yang *et al*., 2007). Small molecules of organic matters and trace elements can be adsorbed on the surface of NBs and act on the oligosaccharide layer together with the microorganisms. Results from the current research provide a new alternative to acquire more methane as renewable energy from high cellulose loading AD process with NBW supplementation, which can help to establish and maintain the sustainable society by utilizing the huge amount of lignocellulosic biowaste in the world.

5.2 Enriched microbial communities in the NBW-based AD system

The hydrolysis-acidification of cellulose can be enhanced with the increased production of hydrolysate (VFAs) for more CH_4 production by the methanogens under NBW condition. The enhanced hydrolysis of high loading cellulose can also provide abundant carbon and nutrient sources for the growth of microorganisms, further enriching microbial communities. To better understand the correlation between CH_4 production and microbial community structure in the

high loading cellulose AD process with NBW addition, bacterial and archaeal communities were analyzed through Illumina sequencing. NBW addition can also enhance community of hydrolytic bacteria and methanogenic archaea (Fig. 5-2). *Firmicutes* can produce extracellular enzymes (cellulase), and then involve in the degradation of organic compounds and the formation of VFAs (Zou *et al*., 2018). *Aminicenantes*, *Thermotoga*, *Chloroflexi*, *Bacteroidetes*, *Spirochaetae* and *Synergistetes* can enhance the hydrolysis lignocellulosic biomass, which has been proven in this study that the reactors with NBW addition yielded higher concentration of hydrolyzate (VFAs) during the hydrolysis-acidification stage of cellulose. *Methanobacterium, Methanomassiliicoccus, Methanospirillum, Methanolinea* and *Methanosaeta* can also be enhanced with NBW addition, which could use the above hydrolysate for methane production. Taking all the results together, the enhanced methane production from the NBW-based AD of lignocellulose is also associated with the enriched bacterial and archaeal communities under NBW addition condition.

5.3 Micro-oxygen environment provided by O_2-NBW may promote the cellulose hydrolysis during AD process

The hydrophilic and negatively charged surfaces of NBs can attract H^+ and NAD^+, which then can help to attack the oligosaccharide layer by cellulolytic and microorganisms together with cellulase due to the largely increased surface and contact area. The destruction of the oligosaccharide layer may enhance the hydrolysis of cellulose through cellulase activity. The highest ORP value was detected in the O_2-NBW reactor (-412±14 mV). It also gained the highest ETS activity owing to the collapse of O_2-NBs and release of O_2, which can provide a micro-oxygen environment that drives the synthesis of ATP and increases the biological activity. Results from the microbial community structure analysis suggest that the micro-oxygen environment might promote the hydrolysis stage of AD. O_2-NBW addition was found to enrich facultative bacteria and hydrogenotrophic methanogens. Besides, O_2-NBW addition can also enhance metabolisms of microbes, further improving methane production (30%) from cellulose. Thus, we first confirmed that O_2-NBW could provide micro-oxygen environment with excellent performance on AD of cellulosic biomass, promoting its application in the future.

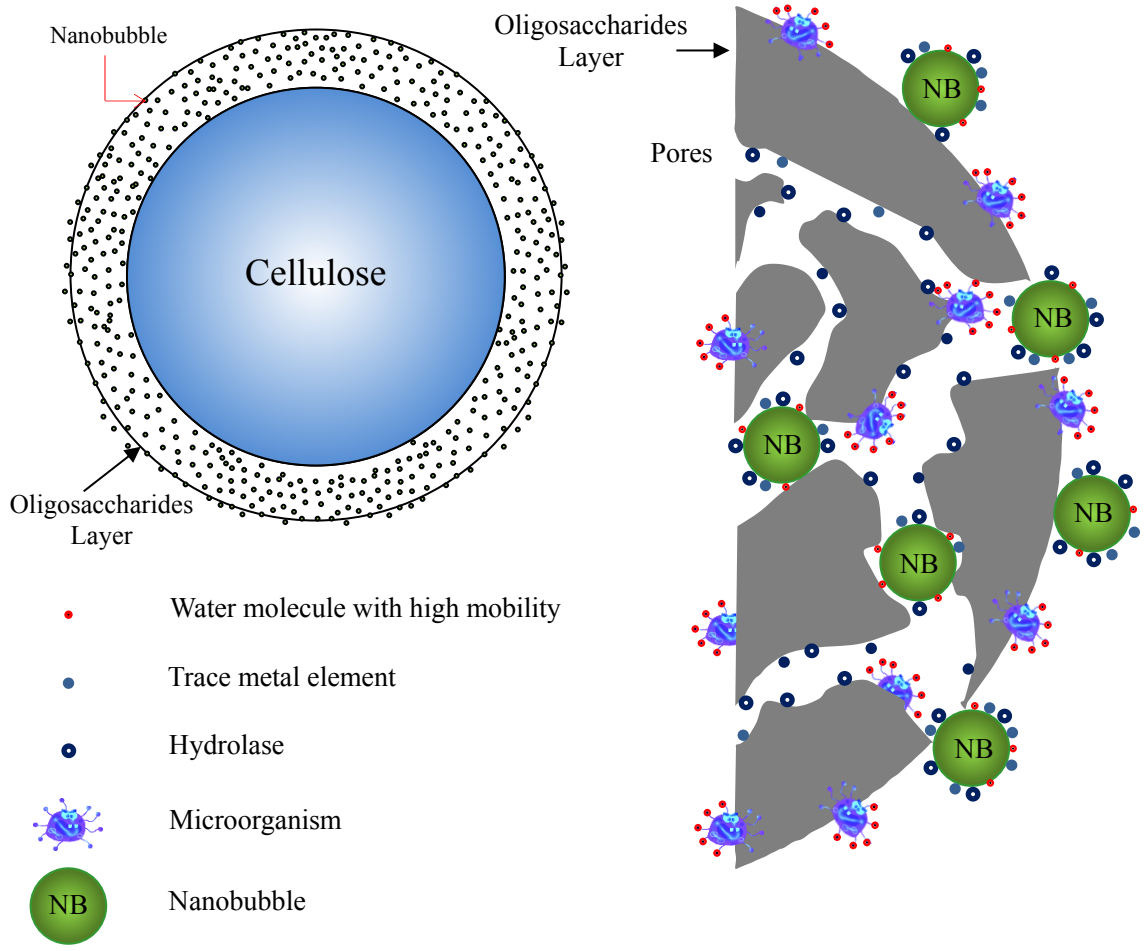

Fig. 5-1 Enhanced hydrolysis involved in the anaerobic digestion of cellulose under NBW addition.

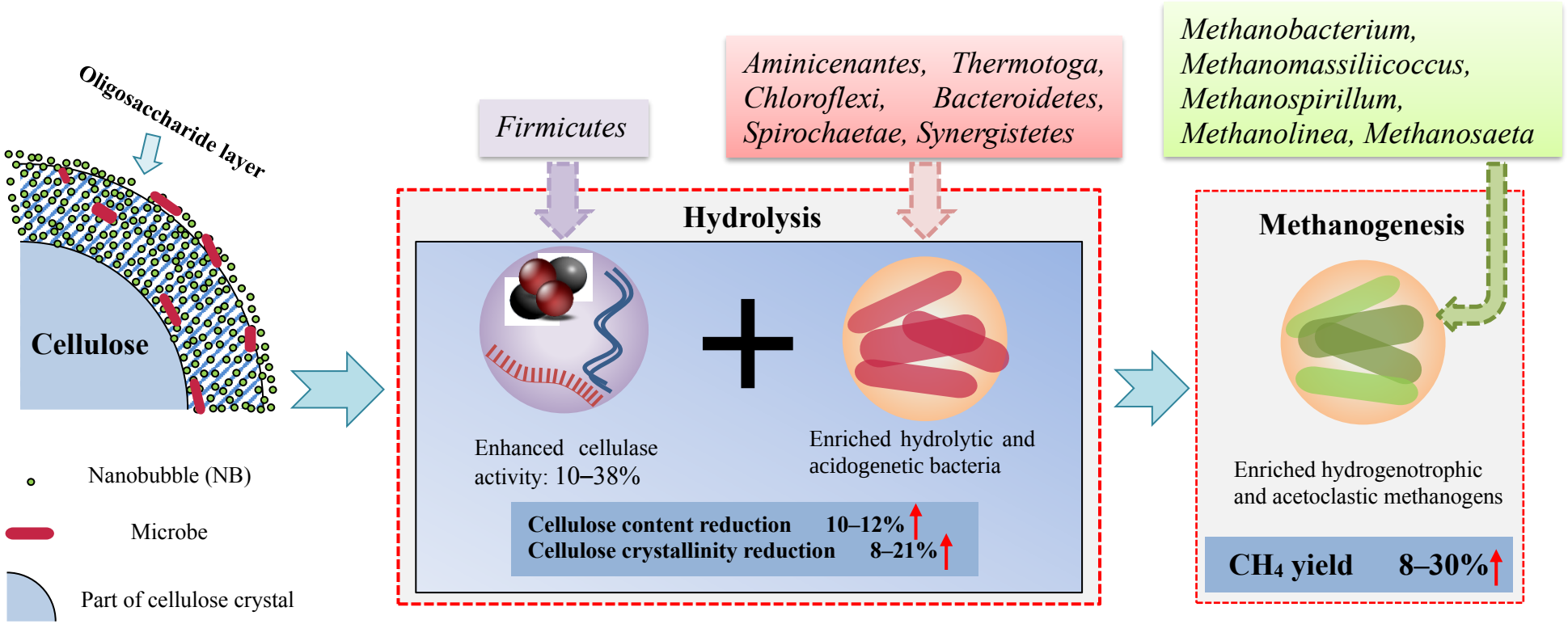

Fig. 5-2 Enriched microorganisms involved in the anaerobic digestion of cellulose under NBW addition.

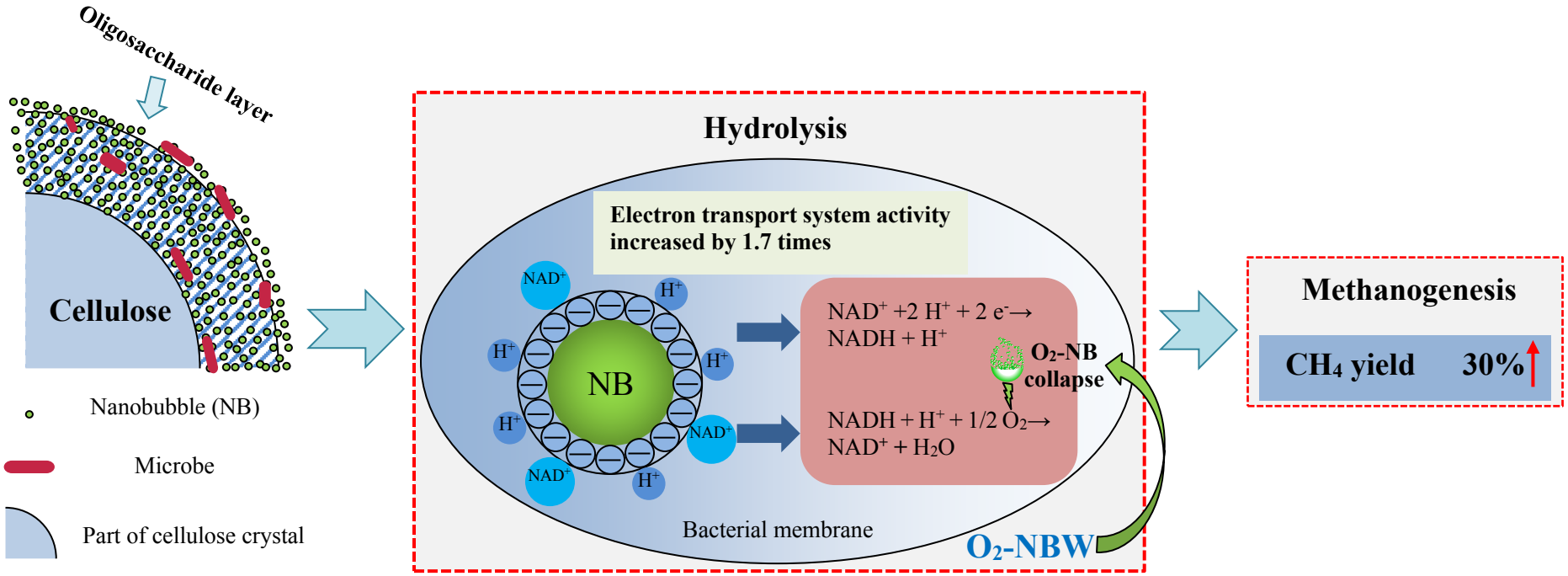

Fig. 5-3 Metabolisms involved in the O_2-NBW supplemented anaerobic digestion of cellulose.

Chapter 6 Conclusions and future research

6.1 Conclusions

In this study, NBW-based AD system was developed to realize simultaneous enhancement on the rate-limiting step of AD process, hydrolysis, and microbial metabolisms, resulting in enhanced resource conservation and management of agricultural resides. The main results regarding methane production, resources conservation and mechanisms analysis during biochemical potential tests on the NBW-based AD system can be summarized as follows.

6.1.1 Cellulose at high loading for methane production via AD supplemented with NBW

The positive effects of high-mobility NBW on the AD of refractory cellulose. VFAs yields were increased by 11–30% during the hydrolysis-acidification stage of cellulose under NBW addition. When Air-NBW was supplemented, 18% increase in CH_4 yield (263.6 NmL/g-VS) and 20% enhancement in cellulose crystallinity reduction (81%) were achieved in comparison to the control. These enhancements are attributable to the greatly enhanced hydrolysis-acidification stage of high cellulose loading AD, most probably due to the microaerobic environment created by Air-NBW addition. These findings can provide valuable insights into the mechanisms involved in the NBW-supplemented AD process. A new alternative to acquire more methane as renewable energy from high cellulose loading AD process with NBW supplementation, which might reduce the consumption of fossil fuel.

6.1.2 O_2-NBW created micro-oxygen environment to enhance methane production from AD of cellulose.

This study provided a promising approach to increase methane yield from AD of cellulose through micro-oxygen environment provided by O_2-NBW. In agreement with the hypothesis, O_2-containing gas NBW supplementation improved methane production from cellulose during AD. 8%–30% increase of methane yield and 8%–14% increase of cellulose reduction were achieved

from AD of cellulose with NBW addition compared to the control (DW addition). Cellulase activity, cellulose crystallinity reduction and ETS activity were also enhanced under NBW addition. O_2-containing gas NBW addition was found to enrich facultative bacteria and hydrogenotrophic methanogens. In this study, we first confirmed that O_2-NBW could provide micro-oxygen environment which has positive effects on methane production from cellulose in the lab-scale tests. O_2-containing gas NBW created micro-oxygen environment could contribute to the enzyme activity and microbial metabolism. Results from this work can also help us better comprehend the fundamentals involved. As seen, O_2-containing gas NBW can create micro-oxygen environment that is conductive to enhanced methane production with no chemical addition and possibly no additional energy consumption during AD.

6.1.3 Anaerobically digested sludge pre-augmented by NBW enhanced methane production from corn straw.

Anaerobically digested sludge was subjected to NBW pre-augmentation to improve its bioactivity and methane production from corn straw via AD. The O_2-NBW pre-augmented sludge reflected the highest microbial bioactivity, achieving 22% increase in methane yield (141.7 ± 5.8 NmL/g-VS), and 88% and 23% increments in cellulose (38.4%) and hemicellulose (35.4%) reductions during AD of corn straw in comparison to the control (DW reactors), respectively. Use of NBW pre-augmented anaerobically digested sludge as inoculum can remarkably enhance methane yield from corn straw, targeting no increase of AD reactor volume in practice and sustainable management of lignocellulosic biomass (Fig. 6-1). This study provided a novel concept for NBW-based AD system of crop residue.

6.2 Future research

The developed NBW-based AD systems in this study can realize simultaneous methane production, lignocellulosic biomass reduction and process stability control. Further studies are still needed to perfect the novel AD system so as to promote its practical applications:

(1) Microbial kinetic, metabolic pathways and genes expression under NBW environment should be explored by using advanced technique such as stable isotope labeling and metabolomics

analysis, which would be helpful to gain a deeper insight into the activity and biochemical pathways of substrate conversion in the NBW-based AD system.

(2) The production procedure for NBW should be developed and optimized, including reduction of preparation cost and increase of NBs concentration.

(3) NBs transfer rate and utilization rate under various operating conditions as well as homogeneity of the liquid should be quantified in the NBW-based AD system.

(4) The NBW-based AD system should also be evaluated using a life cycle analysis of the whole system.

(5) All the major and important parameters such as characteristics of the substrate, quantity, enzyme activity, temperature, enzyme stability and pH should be optimized through cost-effectiveness analysis.

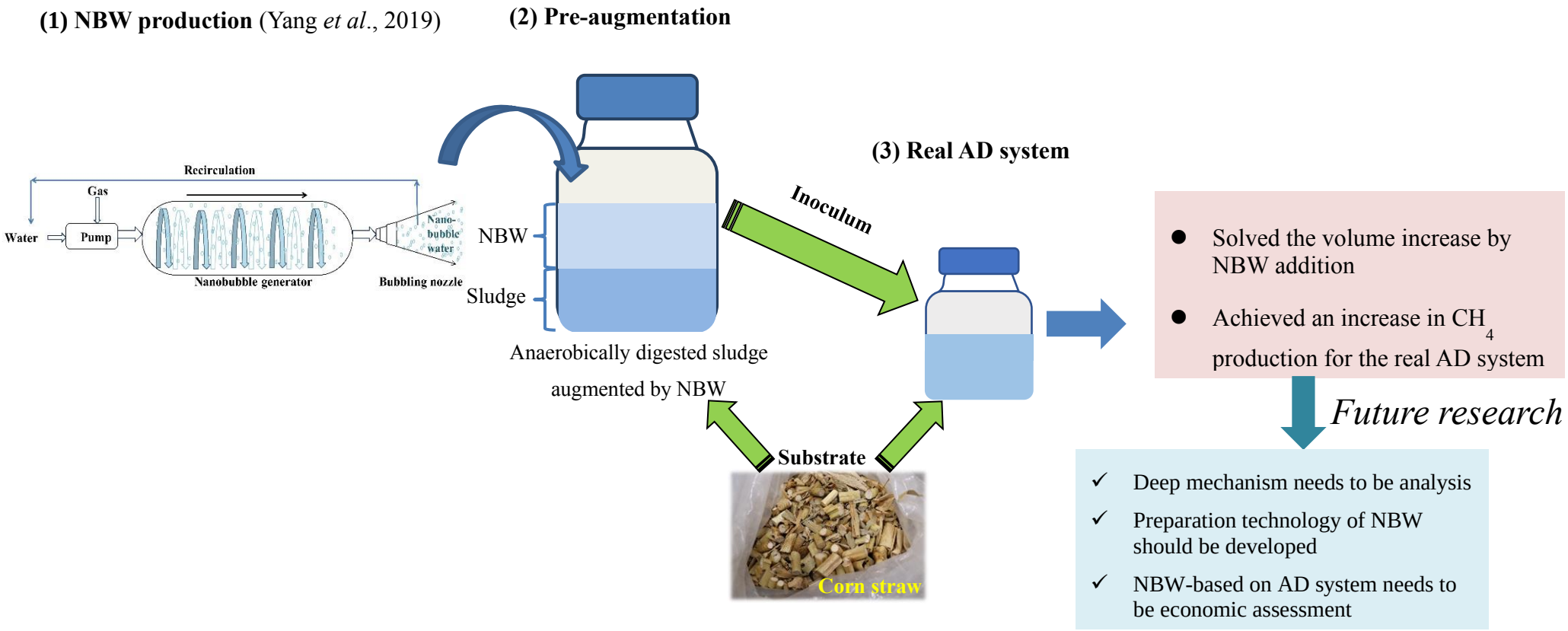

Fig. 6-1 A novel concept of NBW-based AD system. NBW-nanobubble water, AD-anaerobic digestion.

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Publications

1. **Wang, X.**, Yuan, T., Lei, Z., Kobayashi, M., Adachi, Y., Shimizu, K., Lee, D.-J., Zhang, Z., 2020. Supplementation of O_2-containing gas nanobubble water to enhance methane production from anaerobic digestion of cellulose. Chem. Eng. J. 398, 125652.
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3. **Wang, X.**, Yuan, T., Guo, Z., Han, H., Lei, Z., Shimizu, K., Zhang, Z., Lee, D.-J., 2020. Enhanced hydrolysis and acidification of cellulose at high loading for methane production via anaerobic digestion supplemented with high mobility nanobubble water. Bioresour. Technol. 297, 122499.
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