Enhancement on Process Stability and Energy Recovery from Two-stage Anaerobic Digestion of Food Waste Using Nanobubble Water

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Hou Tingting

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Hou Tingting

Abstract

With the rapid growth of population and economy, the management of ever-increasing food waste (FW) is becoming a pivotal global issue. “Turning waste into wealth” opens a new path for sustainable utilization and management of wastes. As known, anaerobic digestion (AD) can convert organic wastes into renewable energy like H_2 and CH_4, which may relieve the pressure of energy scarcity and increase the diversity of fuel sources. Compared with the single-stage AD, two-stage AD can overcome the disadvantage of severe acidification in the single-stage system and is also superior in biogas yield, organic matters removal, energy recovery, and process stability. However, the hydrolysis efficiency in the first stage is the key to the improvement of waste energy recovery from complex organic substrates like FW. To enhance hydrolysis efficiency and biogas production, various pretreatment methods have been attempted to treat FW. However, the difficulty in maintaining the operation conditions and the addition of expensive chemicals greatly hinder their practical application. Besides, salinity is another inhibitory factor resulting in lower AD performance of FW and even process failure, especially in methane production. Most previous studies have focused on the effects of salt on AD for hydrogen or methane production. However, the effects of salt on two-stage AD and the related approaches to mitigate the adverse effects of high salinity on hydrogen and/or methane production were seldom addressed. For addressing the inhibition of high salinity on the two-stage AD, improving the microbial activity under the high salinity environment is preferred. Recently, nanobubble water (NBW) technology has been proven as an environmentally friendly method to enhance the AD of organic solid wastes. Moreover, it has been proven that addition of NBW could enhance the activities of microorganisms and/or bacteria under both anaerobic and aerobic conditions.

In this thesis, the enhancement effects on two-stage AD of FW for separate production of hydrogen and methane with N_2-NBW and Air-NBW supplementation were investigated. The underlying mechanisms of NBW addition to each stage were further explored by using the model substrates in the separate hydrolysis/acidogenesis and methanogenesis experiments. In addition, it is the first time to introduce the Air-NBW to mitigate the inhibition caused by high salinity during the two-stage AD of FW. The application of NBW in AD was further optimized and improved the tolerance of methanogens to high salt in combination with the "salt in" strategy. As results:

(1) The highest H_2 and CH_4 yields were obtained from the Air-NBW added reactor, increasing by 38% and 24% in H_2 yield and CH_4 yield, respectively, resulting in a remarkable increase (23%) in energy recovery from the whole AD system. Further investigations indicate that different gas NBW may positively impact the different stages of the AD process. Addition of N_2-NBW only enhanced the hydrolysis/acidification of FW with no significant effect on methanogenesis. By comparison, addition of Air-NBW promoted both stages, most probably due to the collapse of Air-nanobubbles resulting in micro-oxygen environment.

(2) In the Air-NBW added reactors with 0-30 g NaCl/L, hydrogen yield was increased by 21-65% with the subsequent methane yield elevated by 14-43% when compared to the deionized water (DW) group under the same salt concentrations. This study for the first time confirmed that when two-stage AD of FW was exposed to the same level of salt, addition of Air-NBW could achieve the enhancement of enzymatic activity at the individual stage. Results of electron transport system (ETS) activity further demonstrate that addition of Air-NBW may promote the electron transfer associated with the synthesis of hydrogen and methane.

(3) CH_4 yield and inoculum's activity were increased by about 33% and 27% in the NBW pre-domesticated inoculum reactors compared with the corresponding DW group after the second domesticated cycle. The H_2 and CH_4 yields from the two-stage AD of FW were both enhanced in the NBW group. Besides, substituting KOH for NaOH to replenish K^+ in the methanogenic stage effectively alleviated the inhibition of salt on methane production, with a shorter start-up period and improved process stability.

Results from this work suggest the potential application of Air-NBW in the two-stage AD for effectively renewable energy recovery from FW. An efficient approach for H_2 and CH_4 recovery from the two-stage AD of FW under high salinity was also proposed through improving microbial electron transfer and corresponding enzyme activities at each stage.

Key words: Food waste; Nanobubble water; Two-stage anaerobic digestion; KOH; High salinity

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Abbreviations and acronyms

ACP	Acid phosphatase
AD	Anaerobic digestion
ALP	Alkaline phosphatase
BHP	Biochemical hydrogen potential
BMP	Biochemical methane potential
BSA	Bovine serum albumin
COD	Chemical oxygen demand
DW	Deionized water
ETS	Electron transport system
FAN	Free ammonia nitrogen
FAO	Food and Agriculture Organization
FW	Food waste
GB	Glycine betaine
GC	Gas chromatograph
GHGs	Greenhouse gases
HAc	Acetic acid
HBu	Butyric acid
HPr	Propionic acid
HVa	Valeric acid
IA	Intermediate alkalinity
IC	Ion chromatography
MSW	Municipal solid waste
NADH	Nicotinamide adenine dinucleotide
NBs	Nanobubbles
NBW	Nanobubble water
NFOR	NADH ferredoxin oxidoreductase
OLR	Organic loading rate
PA	Partial alkalinity
ROS	Reactive oxygen species
SEM	Scanning Electron Microscopy

S/I	Substrate/Inoculum
SRD	Statista Research Department
sTOC	Soluble total organic carbon
TA	Total alkalinity
TAN	Total ammonia nitrogen
TS	Total solids
UASB	Up-flow Anaerobic Sludge Blanket
VFAs	Volatile fatty acids
VS	Volatile solids
WAS	Waste activated sludge
WBA	World Biogas Association
WHO	World health organization

Chapter 1 Introduction

1.1 Food waste production and related environmental issues

1.1.1 Food waste production

Food and Agriculture Organization (FAO, 2013a) of the United Nations reported that approximately 1.3 billion tons of food used for human consumption was wasted annually, which is a widely cited number because many countries have different opinions on the definition of food waste (FW) leading to the amount of FW in each country cannot be accurately quantified. Additionally, FAO also expressed that the roughly same quantities of food were dissipated in industrialized and less industrialized countries (670 and 630 million tons annually). However, FW is more problematic in industrialized countries, *i.e.*, those countries with per capita FW by consumers between 95-115 kg annually in Europe and North America as against 6-11 kg annually in the low-income Sub-Saharan Africa and South/Southeast Asia regions (FAO, 2014). With the rapid growth of population and economy, it has been estimated that the per capita FW production would be increased by 44% from 2005 to 2025 in the global, especially in the Asiatic countries, *i.e.*, the calculative per capita FW production in China and India were increased from 86.12 to 126.73 kg/year and 55.69 to 86 kg/year, respectively (Adhikari et al., 2006). Statistics and projections of global FW by Huang et al. (2020) show that the production of FW is growing rapidly, with the expectation to more than twice by 2050.

In Japan, the generation of FW is divided into five stages in the supply chain, including agricultural production stage, storage and transportation stage, commodity stage, household consumption stage, and end-of-life stage (Liu et al., 2016). As known, most Japanese companies usually cater to their customers to improve the quality of products by adjusting the size of products or removing a large number of edible parts or expired food, resulting in a large amount of food being wasted. Babalola (2015) reported that ~19 million tons of FW were generated annually, including ~11.3 million tons from wholesale, retail, catering, and restaurant activities food manufacturing and ~7.7 million tons from household preparation and cooking in Japan. These data could raise the concern that a large amount of FW production inevitably causes the negative impacts on the environment.

1.1.2 Environmental issues by FW

Greenhouse gases (GHGs) emission from FW is one of the biggest threats to the environment. FAO (2013b) estimated that ~3.3 billion tons of CO_2 equivalent of GHGs released into the atmosphere annually from FW. World Biogas Association (WBA, 2018) also reported that the GHGs caused by FW accounted for 8% of the world's anthropogenic GHGs emissions per year, which was 4.4 gigatons of CO_2 equivalent. Other environmental issues involved include the appropriation of land resources, formation of photochemical ozone, foul-smelling odor and leachate during transportation and storage, and so on (Chen et al., 2021; Tonini et al., 2018). In brief, FW must be properly managed to reduce the risk of environmental pollution, which will also be a huge challenge facing the world.

1.2 Management of FW

1.2.1 Landfilling, incineration, and composting of FW

Landfilling and incineration are the most common traditional methods for FW disposal, and 70-75% of FW is dumped in landfills in the world (FAO, 2020). Slorach et al. (2019) reported that ≥ 50% of household FW in United Kingdom was landfilled or incinerated. According to Liu et al. (2016), ~ 50% FW produced from food manufacturing industries is used for animal feed, together with 13% recycled as compost, 3% used as energy, 12% reduced through dehydration treatment, and 19% disposed by incineration or landfilling in Japan. On the other hand, 94% of FW from households is disposed by incineration or landfilling with only 4% for recycling in Japan. However, the highest global warming potential (195 kg CO_2 equivalent) is from landfilling because about 32% of the waste gas produced by landfills is not captured and discharged directly into the atmosphere (Slorach et al., 2019). Additionally, large amounts of land resources have been occupied by landfilling, and serious pollution of groundwater and surrounding soil caused by landfill leachate generated is also not to be ignored.

Reportedly, Germany has forbidden the dumping of untreated municipal solid waste (MSW) directly into landfills after May 2005, where pretreatments by thermal, mechanical and/or biological methods are required to reduce the organic components of MSW (Sormunen et al., 2008). China also firstly piloted the mechanically biologically technology at Tianziling landfill in Hangzhou (Zhang et al., 2020b). This strategy to a certain extent enables the separation of biodegradable organic components which could be used as recycled fuel.

As it is known, Japan is short of available land for landfilling and thus historically adopted incineration as the FW disposal method of preference. Statista Research Department (SRD, 2021) reported that Japan had about 1082 incinerators in operation in 2018, and more than 70% of MSW was treated by incineration. However, the proportions of heat and electricity generated by incineration plants are unsubstantial. In 2015, incineration plants capable of recovering heat only accounted for 38%, and this figure may increase as the technology improving (Babalola, 2015). As estimated by Yano and Sakai (2016), one third of the incinerators should be replaced by 2020 and the remaining two thirds should be done by 2030 in Japan. Besides, a large number of harmful substances (dioxins and mercury compounds) and GHGs (carbon dioxide, nitrogen oxides, and sulfur dioxide) are produced during the incineration process (Melikoglu et al., 2013). Therefore, more sustainable and more environmentally friendly management strategies for FW should be addressed and developed.

Composting is a commercialized method for treating biodegradable organic matter in FW to produce the valuable soil modifiers and fertilizers, with a relatively lower environmental impact and higher economic profits in comparison to landfilling and incineration (Uçkun Kiran et al., 2014). However, a large amount of leachate is generally produced during the composting process of FW due to its high moisture content. Cekmecelioglu et al. (2005) claimed that when the moisture content of substrates exceeded 60%, the problems of leachate and odor would occur in the first 15-20 days. In addition, various airborne particles including bioaerosols would be released in the composting facilities, which were associated with a variety of adverse health effects, including allergies, acute toxic effects, and infectious diseases (Gao et al., 2021).

1.2.2 Anaerobic digestion of FW

Fortunately, FW is attracting more and more attention as a potential feedstock to produce chemicals, value-added materials, and fuels due to its abundant nutritional content and biodegradable organic matters.

The continuous growth of population and the scarcity of resources bring about the exhaustion of energy, and the importunity and dependence on energy continue to reach record levels. Nowadays, recycling bioenergy (biogas, bioethanol, butanol, and so on) from FW is increasingly attracting the society's attention. As known, anaerobic digestion (AD) can convert organic wastes into renewable energy like H_2 and CH_4, which may relieve the pressure of energy scarcity and increase the diversity of fuel sources. FW is becoming a preferred feedstock for AD because of its high moisture content and biodegradability (Talan et al., 2021; Boonpiyo et al., 2018). Additionally, from the viewpoint of environmental and economic aspects, bioconversion of organic wastes could reduce the risk of environmental pollution and the disposal cost (Awasthi et al., 2018). Therefore, AD is becoming more widely embraced in the world due to its trinity superiority of wastes management, energy recovery, and friendly ecological circulation. Since 2011, the demonstration projects of FW treatment have been carried out in China, and 95% of them were AD-based waste-to-energy projects. Additionally, China's "13th Five-Year Plan" emphasized a goal of 30% FW disposal rate by 2020, mainly through the AD-based waste-to-energy project (Zhang et al., 2020a). Yano and Sakai (2016) calculated that in Japan about 4 and 9 million tons per year of FW were treated in AD facilities in 2020 and 2030, which accounted for 29.2% and 69.5 % of the total amount of FW generated, respectively. This indicates that an increasing amount of FW is being recycled through AD technology. Bernstad and la Cour Jansen (2012) and Morris et al. (2013) reviewed the 25 and 82 studies of life cycle assessment about FW, indicating that AD of FW is more environmentally friendly.

1.3 Challenging aspects of AD of FW

The simplest and most common AD system is known as single-stage AD, in which the microbial community involved includes hydrolytic-acidifying bacteria and methanogens perform all the steps (hydrolysis/acidification and methanogenesis) in a single digester. However, the optimal and environmental conditions of hydrolytic-acidifying bacteria and methanogens are quite different, and thus the two-stage AD process is introduced, in which hydrolysis/acidification and methanogenesis are carried out separately in two reactors to ensure the optimal growth environment of each microorganism.

Many single-stage anaerobic digesters of FW have been reported to have unstable performance and even process failure due to reactor acidification. Liu and Liao (2019) found that the rapid production of large amounts of volatile fatty acids (VFAs) due to the highly biodegradable components in FW may result in a reduced pH and thus inhibit the activity of methanogens at the early stage of AD. Many previous studies manifested that a preferable pH range is one of the crucial factors for the stability of AD because methanogens are highly sensitive to pH, and the optimal pH range has been reported as 6.5-7.2 or 6.0-80 (Alavi-Borazjani et al., 2020; Ward et al., 2008). In addition, as reported by Moeller and Zehnsdorf (2016), the daily power generation halved from 13.5 to 6.9 MWh/d during just 2 days due to over-acidification in a full-scale AD digester, correspondingly to a decline of biogas production and the instability of the process.

1.3.1 Balance between hydrolysis/acidification and methanogenesis in the two-stage AD

For the organic solid waste easily degradable (*i.e.*, FW in this study), the implementation of the two-stage AD system as a viable alternative can reduce the instability and the risk of acidification of the process (Srisowmeya et al., 2020).

In the first stage, the complex organic compounds can be maximally degraded into short-chain variations of VFAs under slightly acidic condition (pH = 5.0-6.0), from which hydrogen as clean energy with high specific energy content (142 kJ/g) can be produced as another important intermediate in addition to VFAs (Silva et al., 2018; Yuan et al., 2019). During the second stage, the produced VFAs from the first stage can be converted into methane by methanogens under relatively neutral condition (pH = 7.0-8.0) (Voelklein et al., 2016). Thus, two-stage AD is deemed to be promising to reduce organic loads and improve overall energy conversion efficiency besides the generation of the two high-calorific gases, *i.e.*, H_2 and CH_4.

Most previous studies have proven that the two-stage AD has higher energy recovery potential, degradation efficiency of substrates, and organic load handling capacity. For instance, as reported by Baldi et al. (2019), the biogas production and volatile solids (VS) degradation rate from the two-stage anaerobic co-digestion of FW and activated sludge were increased by 26% and 9% in comparison to the single-stage AD of FW, respectively. Similarly, compared to the single-stage AD of extruded lignocellulosic biomass, the energy yield and chemical oxygen demand (COD) removal efficiency of the liquid and solid streams were increased by 33%, 18% and 16%, 14%, respectively in the two-stage AD process (Akobi et al., 2016). According to Micolucci et al. (2018), it was found that a removal efficiency by the two-stage AD of FW was higher 17% than that by the single-stage AD of FW, and the sludge disposal after the two-stage AD process reduced 33% when a digestate dewatering post-treatment was followed. This mirrored that the final disposal cost of the residue would be cut down though a two-stage AD reactor. Feng et al. (2020) reviewed that the maximum organic loading rate (OLR) in the single-stage AD reactor was only within 4.0 g-VS/(L·d), while for the two-stage AD reactor it was 5.7-7.7 g-VS/(L·d). Apart from these, the diversity of archaea in the two-stage AD process was more abundant than that in the single-stage AD process, indicating that the two-stage AD is more conducive to the growth of microorganisms. Kinnunen et al. (2015) observed that in the two-stage AD system, the number of methanogenic archaea and Shannon index were increased about 5.0 and 2.4 times than those in the single-stage system. These data state that two-stage AD is deemed to be promising to improve organic loads and overall energy conversion efficiency besides the generation of the two high-calorific gases, *i.e.*, H_2 and CH_4.

1.3.2 Low hydrogen production from the first stage of two-stage AD

In recent years, hydrogen is recognized as the cleanest energy with high calorific value and zero pollution from its combustion products, catching much global fancy. Among the various hydrogen production technologies, microbial conversion hydrogen from wastes is supposed to be the simplest and most economical approach. Simultaneously, FW has also been proven as an applicable substrate for biological hydrogen production due to its high moisture content and rich carbohydrate content (Talan et al., 2021). Lay et al. (2003) stated that the high solid organic wastes rich in carbohydrates were twentyfold higher hydrogen production potential than those rich in fats and proteins.

However, the composition of FW is complex, rich in carbohydrates (41-62%), proteins (15-25%), and lipids (13-30%) (Boonpiyo et al., 2018). Although the high carbohydrates content in FW can be easily converted, the high contents of proteins, lipids, and some recalcitrant cellulosic materials may greatly reduce the hydrolysis efficiency (Kim et al., 2019). On the other hand, despite the proven advantages of low energy requirements, easily operation, sustainability, and so on, the biological hydrogen fermentation technology couldn’t compete with commercial fossil fuel hydrogen production processes in terms of cost, efficiency, and reliability (Show et al., 2012). The low hydrogen yield in the first stage might therefore be the major limiting factor for the two-stage AD (Yuan et al., 2019). To enhance hydrolysis efficiency and biogas production, various pretreatment methods have been attempted to treat FW. For instance, Ding et al. (2017) examined the effect of hydrothermal pretreatment on AD of FW, attaining the maximum hydrogen and methane yields about 43.0 and 511.6 mL/g-VS under the optimum condition (140℃, 20 min), increasing by 24% and 32% when compared to the untreated FW, respectively. Acidic (pH = 2) and alkaline (pH = 11-12) pretreatments are also frequently adopted to treat FW due to their simple operations (Kim et al., 2014; Jang et al., 2015), from which hydrogen production can be largely improved while adversely impacted by the operation mode. Although these pretreatments improved the hydrolysis of FW and thus enhanced biogas production to some extent, the difficulty in maintaining the operation conditions and the addition of expensive chemicals greatly hinder their practical application.

1.3.3 Salinity inhibition to the two-stage AD of FW process

Generally, the salinity of FW is high (20-50 g NaCl/L) (He et al., 2019) because salt is one of the seasonings widely used in the cooking process. Kuwabara et al. (2020) reported that salt intake is higher in Japan (≥ 11 g per day) than in western societies according to the diverse eating habits, indicating a higher salt concentration in FW in Japan. As known, high salinity (> 10 g NaCl/L) usually increases the osmotic pressure outside the cell and causes the outflow of intracellular water or even the cell rupture, which strongly inhibits the metabolism of non-halophilic bacteria (Li et al., 2018). Therefore, the excessive salt present in organic wastes, *i.e.*, FW, could also inhibit the activities of microorganisms at all stages of AD (hydrolysis/acidification and methanogenesis) to varying degrees, resulting in lower AD performance and even process failure. Results of previous studies on salt inhibition to the two-stage AD are summarized in Table 1-1.

Up to the present, most studies only focused on the effects of salt on AD for hydrogen or methane production, and few studies have been conducted to improve the efficiency of AD under high salt concentrations. Currently few technologies are available to effectively mitigate the inhibition of high salinity on AD of FW, excepting by simple dilution with water or mixing with other low-salt organic wastes. According to the results from previous studies, under high salinity conditions, the high osmotic pressure and inappropriate enzyme linkages lead to plasmolysis and loss of cell activity, which are the main reasons for inhibited AD processes (Kim et al., 2009; Lee et al., 2012). Therefore, enhanced microbial activity may be an appropriate strategy to mitigate salt inhibition on AD of high salinity FW. Now usually increased tolerance of microorganisms to salt by the synthesis of themself or adding externally osmoprotectant, allowing microbes to accumulate osmolytes (compatible solutes) to balance their cytoplasm (Salma et al., 2020). Amino acids and quaternary ammonium compounds, *i.e.*, proline, choline, glycine betaine, carnitine, and trehalose, have been adopted as common osmoprotectant in AD of high salinity organic wastes to recover methanogenic activity and increase methane production (Liu et al., 2019b; Zhang et al., 2016). Liu et al. (2019b) attempted to ameliorate salt inhibition on methane production from kitchen waste AD system by adding osmoprotectant (glycine betaine (GB)), and 2.0 and 2.5 g/L GB were found to be the optimal dosages for the 5 and 10 g Na^+/L reactors resulting in 29.07% and 61.76% improvements in methane yield, respectively. However, the production cost of osmoprotectant is high, and a superfluous addition of GB may adversely affect the methane production. Apart from this, the osmolytes are organic substances that may be consumed by microorganisms as carbon or nitrogen sources, resulting in an instability in continuous AD systems. Therefore, other methods are still necessary to seek for to improve the methanogens activity in a high salt environment.

1.4 Nanobubble water and its potential application in the two-stage AD of FW

1.4.1 Nanobubble water

Nanobubble water (NBW), containing tiny bubbles with diameter < 1000 nm, has unique characteristics. An interesting feature is that nanobubbles (NBs) can remain stable in solution for a long time due to its lower buoyancy. Oh et al. (2015) reported that the H_2-NBs could exist stably 121 d in gasoline fuel. Azevedo et al. (2016) revealed that the size and bubble number density were no remarkable differences within the 14 days' storage period in the water. Wang et al. (2019) compared the lifetimes of four different NBs in the deionized water (DW) (H_2-NBW, Air-NBW, N_2-NBW, and CO_2-NBW) and obtained the consistent results, the major size of NBs barely changed, and the number of NBs decreased over time and dropped to about 10^8 bubbles/mL during the 14 days. Zeta potential also predicts the stability of the NBs in the system because the suspended particles with a higher zeta potential (positive or negative), the stability of the system could be relatively higher. Uchida et al. (2011) and Ushikubo et al. (2010) reported that the zeta potential of Air-NBs ranged from −17 to −20 mV under pH 5.7-6.2, while the zeta potential of O_2-NBs varied from −34 to −45 mV under pH 6.2-6.4. When the pH value was 7.0-8.0, the zeta potential of different gas type NBW was higher than −20 mV and followed a descending order as H_2-NBW > Air-NBW > N_2-NBW > CO_2-NBW (Wang et al., 2019). Other unique characteristics of NBW include negative charge on their surfaces, high mass transfer efficiency, and large specific surface area of NBs, which may facilitate the chemical reaction, physical adsorption, and mass transport at the gas-liquid interface (Gurung et al., 2016). Most notably, it has been reported that NBs in the NBW will collapse, resulting in the generation of OH· free radicals that is a highly reactive oxygen species (ROS) and is conducive to degrade organic matters (Ahmed et al., 2018; Ghadimkhani et al., 2016). Ahmed et al. (2018) detected the large amounts of OH· free radicals produced in the O_2-NBW and Air-NBW in comparison to the N_2-NBW and CO_2-NBW. Therefore, these unique characteristics may broaden the application of NBW in various fields.

1.4.2 Applications of NBW

In recent decades, more and more researchers have paid attention to the application of NBW in agricultural, industrial, medical, and other fields, especially in the environmental field, because of its zero pollution.

Applications of NBs in agriculture include aquaculture, seed germination, and animals' husbandry, and plantation. For the industrial applications, NBs technology is commonly used for surface cleaning, food processing, energy systems, material modification, cosmetics, etc. In the medical treatment, NBs technology is mainly used in combination with ultrasound for cancer, ultrasound imaging contrast agent, malaria detection, and so on. Up to now, a large proportion of the NBs application in the environmental remediation was concentrated on the water treatment, including aeration, flotation, adsorption and removal of suspended solids, removal of refractory organic pollutants, advanced oxidation, disinfection, bleaching, etc. Khan et al. (2020) and Temesgen et al. (2017) summarized the achievements in the application of NBs as a new vision in water treatment, stating that the advantages of NBs technology lie in reducing the size of the treatment system, running time, disposal cost, and improving the efficiency of removing pollutants. Specific applications of NBs technology and corresponding results are summarized in Table 1-2.

It is worth mentioning that NBW has been also applied in the AD systems, reflecting its beneficial effects. Wang et al. (2019) first discovered that addition of different NBW (H_2-NBW, CO_2-NBW, Air-NBW, and N_2-NBW) to the waste activated sludge (WAS) AD reactors can increase methane production by about 21%, 20%, 18%, and 14%, respectively. Yang et al. (2019) and Wang et al. (2020a) indicated that addition of NBW could enhance the hydrolysis of WAS and cellulose. These previous studies proved that NBW as a simple and environmentally friendly pretreatment strategy may add more benefits for AD of organic wastes. Up to the present, however, no report is available about the effect of NBW on hydrogen production from AD, especially from the two-stage AD process of high organic content wastes (like FW in this study). In addition, the application of NBW in AD also needs to be further optimized.

1.5 Improvement on the microbial activity in the two-stage AD of FW

It's undeniable that microorganism plays a major role in the success of AD system, and its activity seriously affects the performance of AD. Adverse environmental factors (such as high salinity in this study) will threaten the microbial activity and thus reduce the efficiency of AD. Therefore, enhancement of the microbial activity may be as an effective method to solve the low biogas production from the two-stage AD process.

1.5.1 NBW supplementation

As reported, NBW supplementation can enhance the activities of microorganisms or bacteria under both anaerobic and aerobic conditions for wastewater treatment (Agarwal et al., 2011; Ghadimkhani et al., 2016; Temesgen et al., 2017). Liu et al. (2017b) introduced the germination rates of seeds increasing about 6-25% in the different gas NBW, which was attributed to the higher efficiency of nutrient fixation or utilization in the NBW system and exogenous ROS, as an active factor for plant growth and development, was produced by the NBW. Liu et al. (2017b) also found that NBW could induce genes expression related to cell division and expansion by analyzing the corresponding gene expression of barley embryo transcriptome in low concentration H_2O_2 solution or NBW. According to Guo et al., (2019), Sobieszuk et al. (2021), and Weber and Agblevor (2005), the supplementation of NBW showed favorable effects on the growth of lactic acid bacteria (*Lactobacillus acidophilus* 1028), yeast (*Saccharomyces cerevisiae*), and fungus (*Trichoderma reesei*). Xiao and Xu (2020) also found that adopting the NBs technology had 6 times higher dehydrogenase activity compared with the coarse bubble and enhanced the microbial metabolism and the proliferation of microorganisms.

The above reports have proved that addition of various types of NBW can improve microbial metabolism. However, the effect of NBW on microbial activity under high salinity conditions has not been determined. Therefore, it is necessary to investigate whether adopting NBW can overcome the adverse factors (such as low hydrolysis efficiency and the inhibition of salinity on microorganisms) by improving the microbial activity in the AD of FW.

1.5.2 "Salt in" strategy

The "salt in" strategy can be considered another feasible method for enhancing the tolerance of microorganisms under high salt conditions. The mechanism of the "salt in" strategy was explained as the accumulation of inorganic ions in the cytoplasm to regulate the internal osmotic pressure of microorganisms in response to the high salinity environment (Müller et al., 2005).

As known, salinity will induce K^+ efflux from cells, causing osmotic pressure imbalance and ultimately cell lysis and death, and thus K^+ is an essential element for regulating intracellular osmotic pressure (PlemenitaÅ et al., 2014). Definitely, potassium ion as a favorable alternative osmolyte in cells can be transported through the unique potassium pump system on the cell membrane (Martin et al., 1999; Zhang et al., 2016). Therefore, supplementing potassium ion as accelerator exploits a new train of thoughts for improving the biological treatment process of high salinity wastes. Gul et al., (2019) reported that moderately supplemented potassium can reduce the concentration of Na^+ in tissues and cells and alleviate the deleterious effects of Na^+ toxicity on wheat growth and yield. Gan et al. (2021) also found that the proportion of genus *Lactobacillus* was increased from 22.45% to 72.78%, 81.64%, 76.53% and 85.63% at the end of processing when NaCl was partially replaced with 0% (control), 30%, 50%, and 70% of KCl. In an anaerobic membrane bioreactor exposed to high salinity for a long time, the supplementation of potassium also alleviated the inhibition effect of salt on methanogens and improved the methane production and COD removal efficiency (Muñoz Sierra et al., 2018).

In the two-stage AD of FW, due to the low pH at the end of the hydrolysis/acidification stage, the remaining organic slurries from the first stage as the substrate in the methanogenic stage usually needs to adjust the pH value with a base such as NaOH to satisfy the requirements of methanogens. If KOH is used instead of NaOH as the pH regulator, it may not only reduce the additional increase of Na^+ but also supplement K^+ to mitigate the inhibition of high salinity on methane production in the methanogenesis stage AD of high salinity organic wastes.

1.6 Research objectives and thesis structure

Aiming at the problems of low biogas production in the two-stage AD of FW caused by low hydrolysis efficiency and inhibition of high salinity, this study introduced the NBW technology to determine the effects of NBW supplementation on two-stage AD of FW for separate production of hydrogen and methane. It is the first time to investigate the respective behaviors of hydrogen and methane production in two-stage AD of FW under different salt concentrations and introduce the Air-NBW to mitigate the inhibition caused by high salinity during two-stage AD of FW. In addition, in order to improve the practical application potential of NBW in AD system, it was applied in the pre-domestication stage of anaerobically digested sludge and combined with the “salt in” strategy, so as to reduce the construction volume of the reactor and maximize the energy recovery from FW without adding any chemical agents. The thesis structure is revealed in Fig. 1-1. Specifically, the thesis consists of the following 5 chapters.

(1) Chapter 1 Introduction

Firstly, the production of FW in the world and the harm of FW to the environment are described. Secondly, the methods of FW disposal, especially the strategy adopted by Japan, were summarized. Thirdly, AD of FW and the challenging aspects of AD of FW were introduced in detail. Fourthly, the main applications of NBW were described, and the possibility of its application in the two-stage AD system of FW was proposed. Moreover, the “salt in” strategy was also introduced as a common solution to improve the tolerance of microorganisms in high salt environment. Finally, the objectives of this study and the structure of the thesis were obtained.

(2) Chapter 2 Enhanced energy recovery via separate hydrogen and methane production from two-stage anaerobic digestion of food waste with nanobubble water supplementation

According to the previous study (Wang et al., 2019), N_2-NBW and Air-NBW were selected for the tests due to their different initial anaerobic (N_2-NBW) or micro-aerobic environment (Air-NBW) created after addition. Both hydrogen and methane production from the two-stage AD of FW were monitored. The underlying mechanisms of NBW addition to each stage were further explored for the first time by using the model substrates in the separate hydrolysis/acidification and methanogenesis experiments. Finally, the enzyme activities with or without NBW addition were also evaluated to evidence the above mechanisms.

(3) Chapter 3 Addition of air-nanobubble water to mitigate the inhibition of high salinity on co-production of hydrogen and methane from two-stage anaerobic digestion of food waste

This is the first time investigated the response behaviors of hydrogen and methane co-production from the two-stage AD of FW under the different salt concentrations. And then the effects of Air-NBW on mitigating high salinity inhibition on AD of FW were evaluated by analyzing the variations of VFAs, soluble proteins, and soluble carbohydrates. Ultimately, underlying mechanisms were preliminary explored by monitoring the enzyme activities and electron transport system (ETS) activity under Air-NBW addition at different salt concentrations.

(4) Chapter 4 Supplementation of KOH to improve salt tolerance of methanogenesis in two-stage anaerobic digestion of food waste using pre-domesticated anaerobically digested sludge by air-nanobubble water

This experiment was conducted in two steps to eliminate the adverse effects of high salinity (20 g NaCl/L) on the two-stage AD of FW. Firstly, using DW or Air-NBW domesticated the anaerobically digested sludge, which was used as inoculum in the two-stage AD of FW. The microbial activity of inoculum at the end of the pre-domestication process were investigated by measuring the ETS activity, and cumulative methane yield was also recorded during the pre-domestication process. Secondly, the addition of KOH adjusted the pH of the methanogenic stage to 7.5, 8.0, and 9.0, respectively, exploring the effect of different K^+ dosage on methanogenic by using Air-NBW pre-domesticated anaerobically digested sludge under the high salt concentration. For detailed analysis, the variations of organic matters, total alkalinity (TA), total ammonia nitrogen (TAN), free ammonia nitrogen (FAN) and ETS activity were determined.

(5) Chapter 5 Conclusions and future research

In this chapter, the results of each experiment were summarized, and the future research was put forward based on the above studies.

Table 1-1 Summary of previous studies on salt inhibition in anaerobic digestion

Substrate	Inhibition concentration of salt (g/L)	Results	Reference
Kitchen waste	> 8	A sharp decrease of methane yield was observed with addition of > 8 g NaCl /L (causing 17-80% inhibition)	(Anwar et al., 2016)
Food Waste	> 4	Results showed that 2-4 g/L salt addition was the optimal addition dosage for AD systems	(Li et al., 2019)
Food Waste	3.5-5.5 (moderate inhibition) > 8.0 (seriously inhibition)	It was also reported that 3.5-5.5 g Na^+/L caused moderate inhibition on the activities of methanogens, and 8.0 g Na^+/L seriously inhibited the production of methane	(Chen et al., 2008)
Kitchen waste	5-10 (moderate inhibition) ≥ 20 (completely inhibition)	It induced a 20.4% inhibition of methanogenic activity at an Na^+ concentration of 5 g/L and a 57.2% inhibition of methanogenic activity at a Na^+ concentration of 10 g/L.	(Liu et al., 2019b)
Concentrated municipal sewage	≤ 5	The methane production was slightly affected when the NaCl concentration was quite low (≤ 5 g/L); with NaCl concentration increasing continuously (10 and 50 g/L), had obvious influence on inhibiting methane production.	(Gao et al., 2019)
Sludge (heat-treated at 90°Cfor 15 min)	< 6	A high concentration of NaCl (6 g/L) suppressed fermentative hydrogen production.	(Kim et al., 2009)
Food waste	≤ 20	When the sodium concentration reached 20 g/ L, however, the inhibition of hydrogen production began.	(Cao and Zhao, 2009)
Food waste	> 40	40 g NaCl/L produced higher H_2 along with volatile fatty acids. Microbial consortia were well acclimatized up to 40 g NaCl/L but got suppressed beyond (50 g NaCl/L).	(Sarkar et al., 2020)

Table 1-2 Summary of the specific applications of nanobubbles (NBs) technology and corresponding results

Application fields	NBs type	Results	Reference
Agriculture	Air-nanobubble water (NBW)	• The total weights of sweetfish and rainbow trout increased from 3.0 and 50.0 kg to 10.2 and 148.0 kg in the Air-NBW, enhancing about 59% and 14% when compared with the normal water, respectively.	(Ebina et al. 2013)
	Ozone NBs	• Ozone NBs can effectively reduce pathogenic bacteria in the cultivation system of Nile tilapia fish	(Jhunkeaw et al., 2021)
	O_2-NBW	• The irrigation of O_2-NBW effectively improved the nitrification and nitrogen fixation function and diversity of soil microorganisms, enhanced soil fertility, and promoted the high yield of sugarcane.	(Zhou et al., 2020)
Industry	Air-NBW	• OH· radicals produced in the process of Air-NBW cleaning membrane fouling surface will combine with humic acid and other organic pollutants and decompose organic matter.	(Ghadimkhani et al., 2016)
	NBs	• Flotation recoveries were increased about 23% by using the quartz conditioned with NBs	(Rosa and Rubio, 2018)
Medical treatment	Vapor NBs	• Vapor NBs can identify individual malaria parasite–infected *Anopheles* mosquitoes in a few seconds	(Lukianova-Hleb et al., 2015)
	NBs	• NBs effectively target FR-positive tumor cells and selectively kill tumor cells by intracellular explosion in *vitro* and in *vivo*.	(Shen et al., 2018)
	O_2-NBW/Air-NBW	• NBW was significantly decreased the tumor size in mice, especially using the O_2-NBW.	(Mahjour et al., 2019)
Environmental remediation	Air-NBW	• NBs was to accelerate the Pb (II) adsorption process by 366%.	(Kyzas et al., 2019)
	Air-NBW	• Air-NBW could accelerate the metabolism of organisms, facilitating the seed germination and the biofilm growth.	(Xiao and Xu, 2020)
	Ozone NBW	• Ozone NBW can remove 99% of trichloroethylene in groundwater	(Hu and Xia, 2018)

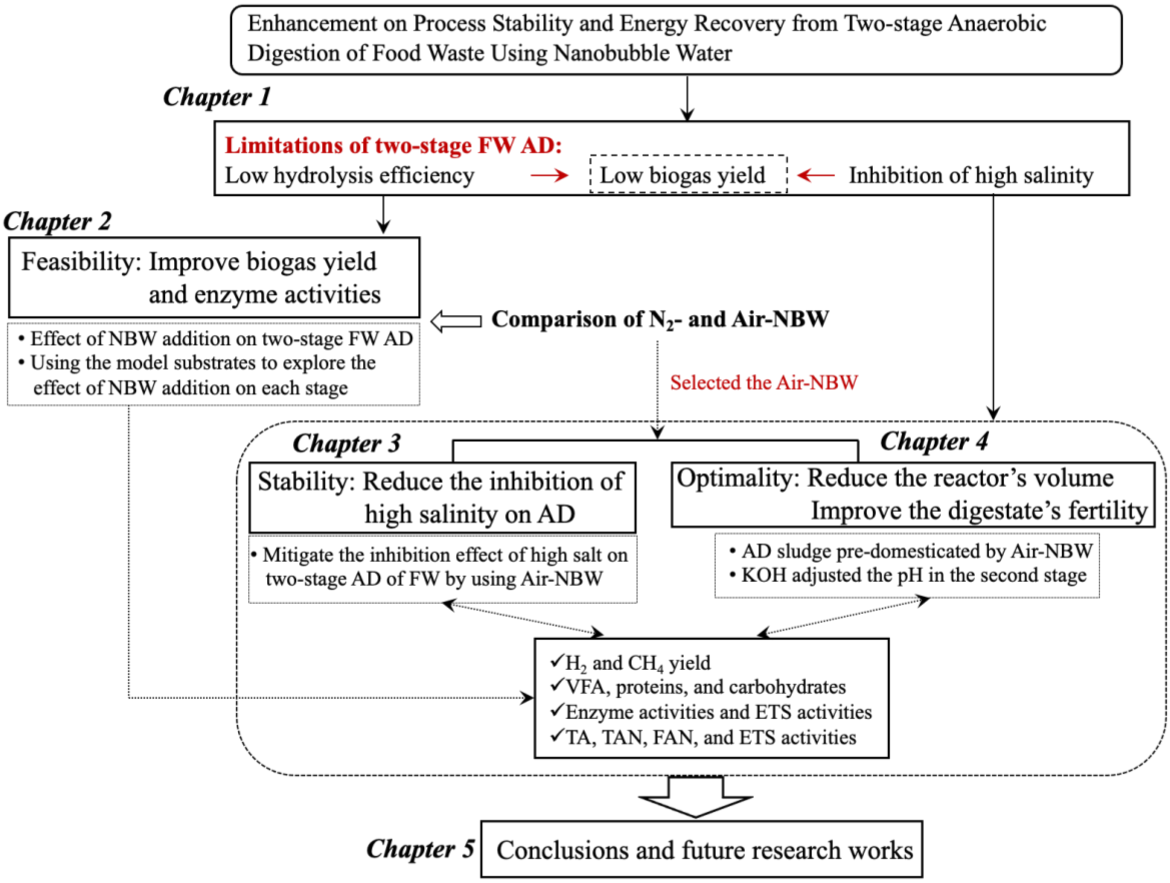

Fig. 1-1 The experimental framework of this thesis. (FW-food waste, NBW-nanobubble water, AD-anaerobic digestion, VFA-variations of volatile fatty acid, ETS- electron transport system, TA-total alkalinity, TAN-total ammonia nitrogen, FAN-free ammonia nitrogen)

Chapter 2 Enhanced energy recovery via separate H_2 and CH_4 production from two-stage anaerobic digestion of food waste with nanobubble water supplementation

2.1 Background

MSW as the main source of organic wastes is estimated to be annually 2.3 billion tonnes by 2025, with FW accounting for the largest proportion (> 50%) (Ahamed et al., 2016; Qin et al., 2019). If not handled properly, one million tonnes of FW would cause 4.5 million tonnes of CO_2 emission from landfills. In contrast, if being treated by AD, one million tonnes of FW could produce enough electricity to power 29,818 households (Kosseva, 2009). As a result, AD is considered as an appropriate option for the disposal of FW. Two-stage AD can separate the complicated AD process into two main stages (hydrolysis/acidification and methanogenesis) to ensure the optimal growth environment for the corresponding microbial groups. However, the low hydrolysis efficiency and low hydrogen yield were the mainly limiting factors in the two-stage AD system. NBW technology as a novel methane has been applied for environmental remediation due to its unique characteristics and zero pollution (Azevedo et al., 2019; Hu and Xia, 2018). It is worth mentioning that NBW addition has also been proven as an environmentally friendly method to enhance AD of organic solid wastes such as WAS and cellulosic materials. Up to the present, however, the effects of NBW on microorganisms during the various stages is limited, and no report is available about the effect of NBW on hydrogen production from AD, especially the two-stage AD process of high organic content wastes (like FW in this study).

2.2 Materials and methods

2.2.1 FW, inoculum and NBW

FW was directly collected from the dormitory kitchen at the University of Tsukuba, Japan, which was mainly composed of peels of vegetables and fruits with smaller amounts of meat and leftovers. The indigestible components such as bones, chopsticks and plastics were manually removed before the FW was smashed and homogenized into slurry using a food grinder. The processed FW was stored at 4°C before use. Anaerobically digested sludge was used as inoculum, which was sampled from a wastewater treatment plant in Tsuchiura, Ibaraki Prefecture, Japan and kept at 4°C in the refrigerator prior to use. The physicochemical characteristics of FW and inoculum are showed in Table 2-1.

The NBW, *i.e.*, N_2-NBW or Air-NBW was prepared using the NBW generator (HACK FB11, Japan), and the pressure and flow rate of gas and water were controlled at 0.22-0.25 MPa, 0.05 and 3 L/min for 20 min under the optimal generation conditions with NBs size of ~140 nm and the bubble particle concentration > 100 million particles/mL, following the specific manufacturing process according to previous works (Wang et al., 2019; Yang et al., 2019). Air used for the Air-NBW generation was taken directly from the natural environment of the laboratory, and the purity of N_2 used for the N_2-NBW generation was ≥ 99.9995%.

2.2.2 Experimental set-up of the two-stage AD of FW

The Schott Duran serum bottles with identical specifications (250 mL in total volume with 150 mL of working volume) were used in both stages of experiments. In the batch biochemical hydrogen potential (BHP) tests, each bottle was loaded with the same amount (20.0 g wet weight) of FW and 60 mL of inoculum. After that, 70 mL of DW, N_2-NBW, or Air-NBW was added to make the total working volume of each bottle to be 150 mL, in which the DW group was used as a blank control. In addition, the initial total VS in each bottle was 13.96 ± 0.49 g/L and the substrate to inoculum (S/I) ratio was about 6.5 (VS basis). As reported by Yuan et al. (2019), a high S/I ratio could inhibit the activity of methanogens in the untreated inoculum and achieve a higher hydrogen yield. More specifically, all the initial pH values were adjusted to 5.50 ± 0.10 using a 2 M HCl solution in the BHP tests, which is regarded as the optimal pH for the enzyme activity of hydrogenase and could inhibit the activity of methanogens to avoid methane production (Liu et al., 2019a).

In the batch biochemical methane potential (BMP) tests, the remaining acidified slurries or mixtures from the BHP tests were used as substrates for the second stage of AD to produce methane. 80 mL of acidified slurry and 50 mL of inoculum (at an S/I ratio (VS basis) of 4.5 and initial total VS of 7.18 ± 0.40 g/L) were added into each bottle and then 20 mL of DW, N_2-NBW, or Air-NBW was added to make the total working volume of each bottle to be 150 mL.

In this stage, all the initial pH values were adjusted to 7.50 ± 0.10 using a 2 M NaOH or HCl solution, which has been reported as the optimal pH for methanogens (Liu et al., 2019a). Additionally, the inoculum was activated for about two weeks under mesophilic condition (38 ± 1°C) until no gas production, and the total solids (TS) and VS of inoculum were about 0.67 ± 0.01% and 0.42 ± 0.01% after activation, respectively.

Before starting the experiments, all the bottles were flushed with N_2 gas (purity ≥ 99.9995%) for 2 min for 3 times to ensure anaerobic conditions and then incubated in a temperature-controlled incubator (38 ± 1°C) for 68 h during the first stage of AD for hydrogen production and 24 d during the second stage of AD for methane production. In the BHP tests, the first gas sample was taken 8 h later, and then regularly collected over time every 12 h to detect the contents of H_2 and CO_2. In the BMP tests, the gas sample was regularly collected over time every 2 d to detect the contents of CH_4 and CO_2.

2.2.3 Effect of NBW addition on each stage in the two-stage AD by using the model substrates

The two-stage AD consists of two steps: hydrolysis/acidification and methanogenesis. The hydrolysis stage that can break down carbohydrates, proteins, and lipids into monosaccharides, amino acids, and long-chain fatty acids is crucial for complex organic compounds like FW (Dong et al., 2019). In this experiment, the effect of NBW addition on each stage in the two-stage AD system was investigated by using the model substrates (protein (bovine serum albumin, BSA), glucose, and acetic acid (HAc)).

In this part, all the experiments were carried out in 100 mL glass serum bottles, and divided into three groups (Group-1, Group-2, and Group-3). Group-1 was carried out to illustrate the effect of NBW addition on the hydrolysis process. In this group, 1500 mg/L of protein as the model substrate together with 25 mL of inoculum was loaded. Moreover, the total working volume was made up to 50 mL with 25 mL of DW, N_2-NBW, or Air-NBW, respectively. The concentration of protein in the filtrated supernatant from each bottle was daily monitored during the 8 days' hydrolysis.

Group-2 was conducted to evaluate the effect of NBW addition on the acidogenesis step and glucose (1500 mg/L) was the model substrate. All the other operation conditions were the same as the hydrolysis step. During the examination, the concentration of glucose in the filtrated supernatant from each bottle was analyzed by sampling every 3 h for 12 h.

Group-3 was used to assess the effect of NBW addition on methanogenesis. HAc was used as the model substrate in this group because HAc was the main contributor to methane production according to the VFAs measurement in this study. A mixture with 90 mg of HAc (1500 mg/L) and 30 mL of inoculum was added into the designated bottle, then 30 mL of DW, N_2-NBW, or Air-NBW was added to make the total volume up to 60 mL. The biogas composition was analyzed every 2 days to calculate the cumulative methane yield during the 10 days' methanogenesis stage.

Before starting all the above experiments, each bottle was firstly sealed with a rubber stopper and resin crimp cap, and then flushed with high-purity N_2 gas (≥ 99.9995%) for 1 min for 3 times to remove oxygen and finally placed in a temperature-controlled incubator (38 ± 1°C).

2.2.4 Microbial enzyme activities at each stage

To a great extent, the stability and efficiency of AD of organic wastes depend on the activity of microorganisms, and the main functional microorganisms in each stage are different in the two-stage AD process (Ma et al., 2019). Thus, the major microbial enzyme activities were evaluated in this study, aiming to provide insights into the effect of NBW addition on the two-stage AD of FW.

Four extracellular hydrolases, *i.e.*, alkaline phosphatase (ALP), acid phosphatase (ACP), α-glucosidase, protease (at the end of the first stage), and coenzyme F_{420} (at the end of the second stage) were measured. For their analysis, the activities of ALP, ACP, and α-glucosidase were measured according to Yang et al. (2020). 0.1% p-nitrophenyl phosphate disodium salt (pnpP), 0.1% pnpP, and 0.1% p-nitrophenyl-α-D-glucopyranoside were used as substrates for the determination of ALP, ACP, and α-glucosidase, respectively. Besides, the activity of protease was analyzed according to the Folin-Phenol reagent method with 10 mg/mL of casein as the substrate (Dai et al., 2017). Finally, the activity of coenzyme F_{420} was measured according to the method described by Dong et al. (2019).

In this study, the relative enzyme activity was calculated to evaluate the effect of NBW addition on microorganisms according to Eq. 2-1.

$$\text{Relative enzyme activity (\%)} = \frac{A_{NBW}}{A_{DW}} \times 100 \qquad 2\text{-}1$$

where A_{NBW} and A_{DW} represent the absorbance at the same wavelength of the NBW added sample and DW added sample, respectively.

2.2.5 Analysis methods and calculations

The TS and VS of FW and inoculum were based on the standard methods (APHA, 2012). All liquid samples were filtered through 0.45 μm membrane filters before being analyzed and all the measurements were performed in triplicate. The concentrations of protein and glucose were determined by Lowry's method and Phenol-sulfuric acid method, respectively (Dubois et al., 1956; Lowry et al., 1951). Soluble total organic carbon (sTOC) was determined by a total carbon analyzer (TOC-VCSN with ASI-V autosampler, Shimadzu, Japan). pH value was determined with a semi-solid pH meter (Testo 206, Germany). According to the detailed description by Huang et al. (2016), the concentration of individual VFA, namely HAc, propionic acid (HPr), *iso*-butyric acid (*iso*-HBu), *n*-butyric acid (*n*-HBu), *iso*-valeric acid (*iso*-HVa), or *n*-valeric acid (*n*-HVa), was determined by a gas chromatograph (GC-8A, Shimadzu, Japan) equipped with Unisole F-200 30/60 column and flame ionization detector.

For the gas samples, the volume of biogas production was measured using 50 and 20 mL of gas-tight syringes and normalized to standard temperature and pressure (25°C, 1 atm). The contents of H_2, CH_4, and CO_2 in biogas were analyzed using a gas chromatograph (GC-8A, Shimadzu, Japan) after sampling 1 mL biogas produced, and the volume of hydrogen or methane was calculated according to Eq. 2-2 (Fu et al., 2020a).

$$V_t = C_t \cdot V_{biogas,t} + V_{head} \cdot (C_t - C_{t-1}) \qquad 2\text{-}2$$

Additionally, the production of hydrogen or methane during the interval between *t* and *t-1* was calculated based on VS_{added} in this study, according to Eq. 2-3.

$$Y_t = V_t / \text{Total } VS_{added} \qquad 2\text{-}3$$

where V_t (H_2 or CH_4) (mL) is the volume of hydrogen or methane produced during the interval between times *t* and *t-1*; C_t (%) and C_{t-1} (%) are the contents of hydrogen or methane at times *t* and *t-1*; $V_{biogas,t}$ (mL) is the volume of biogas produced at time *t*; V_{head} (mL) is the headspace volume of the bottle; Y_t (H_2 or CH_4, mL/g-VS_{added}) is the yield of hydrogen or methane during the interval between times *t* and *t-1* based on the total amount of VS_{added}.

In the BHP and BMP tests, the experimental data of cumulative hydrogen or methane yield in each stage was fitted to the modified Gompertz model as in Eq. 2-4 (Xing et al., 2017).

$$H_t \text{ or } M_t = P\, exp\{-exp[\frac{R_{max}}{P}(\lambda\text{-}t) + 1]\} \quad 2\text{-}4$$

where H_t or M_t (mL/g-VS_{added}) is the specific hydrogen or methane yield at a given time t; P (mL/g-VS_{added}) is the maximum hydrogen or methane production potential; R_{max} (mL/(g-VS_{added}·h) or mL/(g-VS_{added}·d)) is the maximum hydrogen or methane production rate; λ (h or d) is the lag time; t (h or d) is the experimental duration; and e (Euler's number) = 2.7183.

In this study, the energy recovery from the two-stage AD of FW with or without the NBW addition was calculated according to Eq. 2-5.

$$E_r = 12.78\times Y_{H_2,\, yield} + 40.03\times Y_{CH_4,\, yield} \quad 2\text{-}5$$

where E_r (kJ/g-VS_{added}) is the energy recovery from the two-stage AD of FW; (L/g-VS_{added}) is the hydrogen yield in the first stage; $Y_{H_2,\, yield}$ and $Y_{CH_4,\, yield}$ (L/g-VS_{added}) is the methane yield in the second stage. The heating value of H_2 (12.78 kJ/L) or CH_4 (40.03 kJ/L) under 1 atm and 25°C was estimated according to the density and calorific value of H_2 or CH_4 gas, *i.e.*, 0.09 kg/m^3 or 0.72 kg/m^3 and 142 kJ/g or 55.6 kJ/g, respectively (Silva et al., 2018).

2.2.6 Statistical analysis

All the reactors operation were conducted in duplicate with the measurements being in triplicate. The data were processed by Microsoft Excel 2010 and expressed as the mean value ± standard deviation. One-way analysis of variance was also applied for statistical analysis and statistical significance was assumed at $p < 0.05$. Origin 9.0 (Origin Lab, trial version) was used to draw and analyze the fitting curves.

2.3 Results and discussion

2.3.1 Hydrogen production from the first stage of two-stage AD of FW

(1) *Hydrogen yield and kinetics analysis*

The results of hourly H_2 production and cumulative H_2 yield in the BHP tests with addition of DW, N_2-NBW, or Air-NBW are shown in Fig. 2-1. It's important to mention that no methane production was detected during the whole H_2 production process. The hourly H_2 productions from all the bottles were almost identical during the first 20 h (p = 0.98 > 0.05) and then increased to the peak values of 1.09 ± 0.00, 1.26 ± 0.04, and 1.34 ± 0.00 mL/(g-VS_{added}·h) at the 32nd h in FW+DW, FW+N_2-NBW, and FW+Air-NBW, respectively (Fig. 2-1a). In addition, hourly H_2 productions from the NBW added reactors were also higher than that from the control with DW addition after reaching the peak values. These results indicate that addition of NBW can enhance H_2 production. Similarly, the cumulative H_2 yields from the NBW added reactors were greater than that from the DW added reactor (Fig. 2-1b). It is interesting to notice that the maximum cumulative H_2 yield (27.31 ± 1.21 mL/g-VS_{added}) was obtained in the Air-NBW added reactor, about 38% higher than that from the control (19.79 ± 0.07 mL/g-VS_{added}). Meanwhile, the cumulative H_2 yield (25.42 ± 0.37 mL/g-VS_{added}) from the N_2-NBW added reactor was 28% higher than that from the control. Some researchers reported that OH· radicals were produced via collapse of cavity bubbles, which can be explained by the Young-Laplace equation (Ghadimkhani et al., 2016). Therefore, the enhancement of cumulative H_2 yield by NBW addition may be due to the fact that NBs in the NBW will break up when contacted with organic matters in FW, resulting in the generation of OH· free radicals that can help oxidize and decompose the organic matters thus leading to accelerated hydrolysis of FW (Ghadimkhani et al., 2016; Takahashi et al., 2007). On the other hand, the uneven mass transfer of organic matters in FW is regarded as one of the main reasons for its low biogas production efficiency. A previous work by Hu and Xia (2018) indicated that micro-nanobubbles might promote mass transfer efficiency, achieving enhanced utilization and conversion efficiency of organic substrates. In comparison, although N_2-NBW can ensure a strictly anaerobic environment, the cumulative H_2 yield was 10% lower than that from the Air-NBW added reactor. This implies that the introduction of trace amounts of air or O_2 would increase rather than inhibit hydrogen production. This observation is consistent with Fu et al. (2020a) who obtained the maximum hydrogen yield of 24.3 mL/g VS at an oxygen loading of 5 mL/g VS, about 70% higher than that without micro-aeration during the two-stage AD of corn straw. As reported, improved H_2 production under the micro-oxygen condition can be attributable to the rapid hydrolysis of carbohydrates and proteins (Sarkar and Venkata Mohan, 2017). Besides, Johansen and Bakke (2006) and Rafieenia et al. (2017) claimed that proper aeration can enhance the activities of hydrolytic enzymes like cellulase and protease. Restated, the micro-aerobic environment was realized by adding Air-NBW in these tests, which needs further in-depth investigations before being applied in practice.

The relevant parameters of the first stage were obtained by fitting the BHP experimental data to the Modified Gompertz model (Table 2-2). The results show that the highest P_{H2} (27.66 mL/g-VS$_{added}$) and $R_{H2, max}$ value (4.37 mL/(g-VS$_{added}$·h)) were obtained in the Air-NBW added reactor, which was consistent with the actual experimental results in the BHP tests. In addition, the lag time (λ) was reduced slightly in the NBW added reactors (16.88-17.10 h) when compared to the control (17.56 h). It indicates that although the addition of NBW resulted in some change of the original environment for the microorganisms, it didn't affect the start-up of the reactor.

(2) *VFAs evolution in the first-stage AD*

The individual VFA and total VFAs concentration at the end of BHP tests are shown in Fig. 2-2a. In comparison to the control, a higher total VFAs concentration was detected under NBW addition, increasing by about 9% and 10% in the FW+N_2-NBW and FW+Air-NBW reactors, respectively. It is worth noting that HAc and HBu were the major VFA during the hydrogen production stage, amounting to > 90% of the total VFAs concentration, in agreement with previous observation (Yuan et al., 2019).

In this study, the highest HAc production (2,954.09 ± 48.34 mg/L) was detected in the FW+Air-NBW reactor, which is corresponding to its highest H_2 production. However, the HBu concentration followed a descending order of FW+N_2-NBW (1,889.62 ± 0.45 mg/L) > FW+Air-NBW (1,775.84 ± 61.51 mg/L) > FW+DW (1,583.59 ± 2.12 mg/L). This means that addition of Air-NBW is more conducive to HAc production, while addition of N_2-NBW is beneficial for HBu production. The molar ratio of HBu to HAc (B/A ratio), as a proportional indicator of H_2 yield from per mole hexose (Ghimire et al., 2015), is plotted in Fig. 2-2b. Only a slight increase in B/A ratio was noticed in the NBW added reactors, which was 0.40, 0.45, and 0.42 in the FW+DW, FW+N_2-NBW, and FW+Air-NBW reactors, respectively. This result indicates that addition of NBW may not alter the metabolic pathway to produce hydrogen, and the increase of hydrogen yield might be contributed by the improved hydrolysis as discussed later.

2.3.2 Methane production from the second stage of two-stage AD of FW

The remaining hydrolyzed and acidified organic slurries or mixtures at the end of BHP tests were further used as the substrates for methane production in the subsequent BMP tests.

The results of daily CH_4 production and cumulative CH_4 yield are presented in Fig. 2-3. Regarding the daily CH_4 production (Fig. 2-3a), all the test reactors behaved almost same during the first 6 days and reached their peak values on day 8. However, the FW+Air-NBW reactor achieved the highest peak value of 133.90 ± 0.56 mL/(g-VS_{added}·d), in comparison to 87.22 ± 6.28 and 115.17 ± 0.20 mL/(g-VS_{added}·d) from the FW+DW and FW+N_2-NBW reactors, respectively. This result is in agreement with the VFAs concentrations in the three reactors (Fig. 2-2a). It has been claimed that a high VFAs concentration is beneficial for methane production (De Gioannis et al., 2017). The BMP tests lasted 24 d and the cumulative CH_4 yields from the NBW added reactors were significantly higher than the control (with DW added, $p = 0.01 < 0.05$, Fig. 2-3b). Similarly, the cumulative CH_4 yield followed a descending order of FW+Air-NBW (373.63 ± 3.58 mL/g-VS_{added}) > FW+N_2-NBW (347.63 ± 7.05 mL/g-VS_{added}) > FW+DW (300.93 ± 3.24 mL/g-VS_{added}), increasing by 24% and 16% when compared with the FW+DW reactor, respectively. This observation clearly shows that addition of NBW can positively affect both hydrogen and methane productions in the two-stage AD of FW, especially under Air-NBW addition. This result also proves once again that the micro-aerobic environment created by Air-NBW addition can promote rather than inhibit AD process. Similar phenomenon could be found in a previous study (Wang et al., 2020b), in which the enhancement of methane production from AD of cellulose was achieved with the supplementation of O_2-containing gas NBW. At the end of methanogenesis, the final VS degradation rates in the FW+DW, FW+N_2-NBW, and FW+Air-NBW reactors were 65.97 ± 0.92%, 71.18 ± 0.04%, and 74.33 ± 0.21%, respectively, indicating that addition of NBW can improve VS decomposition thus enhance hydrogen and methane productions from the two-stage AD of FW. The above results also suggest that NBW-based two-stage AD process can successfully improve energy recovery from the AD of FW with no chemicals addition and no secondary pollution.

The methanogenesis stage was also described with the parameters of P, R_{max}, and λ by fitting the experimental data to the Modified Gompertz model as shown in Table 2-2. The highest P_{CH4} (375.25 mL/g-VS_{added}) and $R_{CH4, max}$ (174.84 mL/(g-VS_{added}·d)) were also obtained in the Air-NBW added reactor, followed by the N_2-NBW added reactor (P_{CH4} = 346.58 mL/g-VS_{added}, $R_{CH4, max}$ = 133.90 mL/(g-VS_{added}·d)) and the control (P_{CH4} = 300.93 mL/g-VS_{added}, $R_{CH4, max}$ = 100.29 mL/(g-VS_{added}·d)). When compared to the control, a remarkable increase in $R_{CH4, max}$ by about 34% was obtained in the N_2-NBW added reactor, and an increase by about 74% was achieved in the Air-NBW added reactor. This observation further confirms that addition of NBW is beneficial for methane production. In addition, the lag phase of methanogenesis was not significantly different among the three test conditions, about 4.02-4.44 d.

2.3.3 Effect of NBW addition on the specific individual step involved in the two-stage AD by using the model substrates

The effects of NBW addition on the hydrolysis/acidogenesis and methanogenesis were explored by using the model substrates and the results are shown in Fig. 2-4. As seen, the hydrolysis and acidogenesis steps were significantly enhanced with addition of NBW, especially under Air-NBW addition (p = 0.02 and 0.01 < 0.05). The concentration of protein was detected to decrease by 1,190.18 ± 36.07, 1,397.56 ± 45.08, and 1,433.62 ± 9.02 mg/L, achieving protein degradation rates of 67%, 79%, and 83% in the FW+DW, FW+N_2-NBW, and FW+Air-NBW reactors, respectively (Fig. 2-4a). Similarly, the concentration of glucose decreased by 1,098.54 ± 76.5, 1,208.7 ± 39.78, and 1,315.8 ± 0.00 mg/L, resulting in glucose consumption rates of 69%, 76%, and 83% in the FW+DW, FW+N_2-NBW, and FW+Air-NBW reactors, respectively (Fig. 2-4b). The increase in degradation and consumption rates of these two model substrates further illustrates that addition of NBW enhanced the hydrolysis and acidogenesis during the two-stage AD process. For the methanogenesis (Fig. 2-4c), the cumulative methane yields were 94.89 ± 4.51, 100.93 ± 4.92, and 126.79 ± 5.12 mL/g HAc in the FW+DW, FW+N_2-NBW, and FW+Air-NBW reactors, respectively. Most recently, Yang et al. (2019) found that addition of N_2-NBW did not significantly enhance the methanogenesis of acetate, which agreed with the result from this study. The cumulative methane yield was only increased by 6% in the N_2-NBW added reactor when compared to the control ($p = 0.85 > 0.05$) in this study. However, the methanogenesis was significantly enhanced under Air-NBW addition as its cumulative methane yield increasing by 33% ($p = 0.04 < 0.05$). Therefore, the positive effect of N_2-NBW addition on the two-stage AD of FW could attribute to the enhancement of hydrolysis/acidogenesis, while addition of Air-NBW can not only promote the hydrolysis/acidogenesis of FW but also enhance the methanogenesis.

2.3.4 Effects of NBW addition on the microbial enzyme activities at each stage

The relative activities of ALP, ACP, α-glucosidase, and protease at the end of the first stage (hydrolysis/acidification) and coenzyme F_{420} at the end of the second stage (methanogenesis) were determined and calculated to provide insights into the effects of NBW on microorganisms in the two-stage AD of FW. Clearly, the activities of four extracellular hydrolases under NBW addition were higher than those in the control (Fig. 2-5a). In particular, addition of Air-NBW achieved the highest activities of ALP, ACP, α-glucosidase, and protease, increasing by 29%, 47%, 14%, and 8% in comparison to the control, respectively. It has been reported that phosphatases including ALP and ACP can catalyze the hydrolysis of organic phosphate esters, and protease is capable of hydrolyzing soluble proteins into amino acids (Mu and Chen, 2011; Wan et al., 2020). In addition, the activity of α-glucosidase in the FW+N_2-NBW reactor increased by 23% compared with the control. α-glucosidase is considered as a crucial enzyme for the conversion of disaccharides and oligosaccharides into monosaccharides (Ni et al., 2020). The increase of bioactivity in these hydrolases verifies that addition of NBW could enhance the hydrolysis of FW under the test first-stage conditions.

As it is known, coenzyme F_{420} plays a critical role in the process of methanogenesis, which represents the conversion activity of the electron donor-driven by redox proton translocation in the methanogenic archaea (Mu and Chen, 2011). Methanogens have been generally considered as strictly anaerobic microbes and trace amounts of oxygen may be toxic to them (Botheju, 2011). However, Fig. 2-5b shows that the relative activity of coenzyme F_{420} increased by 4% and 34% in the FW+N_2-NBW and FW+Air-NBW reactors in comparison to the control, respectively. As reported, *Methanobacterium* and *Methanosarcina* can tolerate limited oxygen environment (Botheju, 2011; Fu et al., 2016b). Due to addition of Air-NBW in the first stage, the microorganisms may have chance to expose to the micro-oxygen environment; thus, aerobic or facultative anaerobic bacteria present in the first stage can grow fast and consume the limited oxygen quickly, resulting in quick transformation of the micro-oxygen environment into anaerobic condition in the second stage. In this study, addition of Air-NBW had a significantly positive effect on the activity of coenzyme F_{420} with improved methane production. This observation further confirms that the improvement of hydrogen and methane productions from the two-stage AD of FW through Air-NBW supplementation may involve in the enhancement of microbial activities in both stages, namely hydrolysis/acidification and methanogenesis.

2.3.5 Energy recovery from the two-stage AD of FW with or without NBW addition

The energy recovery from H_2 and CH_4 in the two-stage AD of FW with or without NBW addition was analyzed as shown in Table 2-3. It is worth noting that the two-stage AD of FW achieved much higher energy recovery (12.30 kJ/g-VS_{added} in FW+DW) than one-stage AD using the same FW (9.68 kJ/g-VS_{added}) (Hou et al., 2020). This observation to some extent is in agreement with a previous work by Yuan et al. (2019) who reported 10% higher energy yield obtained in the two-stage AD of FW than the one-stage AD of FW. Similarly, De Gioannis et al. (2017) adopted two-stage AD of FW and obtained 20% increase in energy recovery when compared with the one-stage AD of FW. As pointed out by Fu et al. (2020a), the first stage of two-stage AD can be regarded as biological pretreatment of high organic content wastes (such as FW in this study), in which hydrogen energy recovery and substrates pretreatment are realized simultaneously. In this study, owing to addition of Air-NBW, the increment in energy recovery from the two-stage AD of FW is about 58% than the one-stage AD of FW. This indicates that addition of Air-NBW can greatly improve the performance of two-stage AD of FW, achieving more energy recovery from FW when compared with one-stage AD. In this study, the highest total energy recovery was obtained from the FW+Air-NBW reactor (15.31 ± 0.16 kJ/g-VS_{added}), increasing by 24% when compared to the FW+DW reactor. Previous studies have reported that technologies such as hydrothermal, acid/base pretreatment, and aeration can improve the energy yields from anaerobic digestion of FW. Rafieenia et al. (2017) claimed that the pre-aerated (at an air flow rate of 5 L/h for 24h) protein rich FW was the best in terms of total energy generation, amounting to 9.64 kJ/g VS. Ding et al. (2017) applied hydrothermal pretreatment (140°C for 20 min) on FW, and gained 18.8 kJ/g VS energy yield. In fact, supplementation of Air-NBW can also be considered as an environmentally friendly technology due to no chemicals added and no secondary pollution with more renewable energy recovery from the two-stage AD of FW. Provided that the increased energy output outweighs the additional energy demand used for manufacturing NBW, addition of NBW in industrial application has practical significance. Therefore, the economic assessment of NBW addition in the two-stage AD of FW for co-production of hydrogen and methane was conducted in this study, and Air-NBW addition was used as an example.

The production capacity of the NBW generator is 12-22 L/min (the median generation capacity (17 L/min) was used for calculation) at a power consumption of 0.75 kW. When 1 tonne of FW is treated, the required 4,500 L of Air-NBW will consume 3.31 kW·h of electricity. The comparative economic assessment results between the FW+DW reactor and FW+Air-NBW reactor are shown in Table 2-4. The generated electricity from the FW+Air-NBW reactor increased about 23% when compared to the FW+DW reactor.

2.4 Summary

Addition of NBW can achieve higher H_2 and CH_4 yields from the two-stage AD of FW than the control, especially under Air-NBW addition which increased H_2 yield by 38% and CH_4 yield by 24%. Addition of N_2-NBW only enhanced the hydrolysis/acidification of FW with no significant effect on methanogenesis. By comparison, addition of Air-NBW promoted both hydrolysis/acidification stage and methanogenesis stage most probably due to the collapse of Air-nanobubbles resulting in micro-oxygen environment, reflecting by the enhanced activities of four extracellular hydrolases at the end of hydrolysis/acidification and coenzyme F_{420} at the end of methanogenesis, respectively. The relative activities of hydrolase increased by 8-47% in the first stage, and the activity of coenzyme F_{420} was significantly enhanced by 34% in the second stage with addition of Air-NBW. Therefore, the developed two-stage AD of FW with Air-NBW supplementation can enhance hydrogen and methane production simultaneously, greatly increasing energy recovery (by 23%) from the whole AD system.

Table 2-1 Physicochemical characteristics of the food waste and inoculum used in this study.

Parameter	Food waste	Inoculum
Total solids (TS, %)[a]	13.08 ± 0.12	0.70 ± 0.01
Volatile solid (VS, %)[a]	8.93 ± 0.18	0.45 ± 0.03
Total volatile fatty acids (VFAs, mg/L)	5,287.74 ± 67.57	9.77 ± 1.70
Soluble total organic carbon (sTOC, mg/L)	10,191.74 ± 21.63	171.61 ± 0.22

[a] Wet weight basis.

Table 2-2 Parameters estimated by fitting the experimental data from the first stage and second stage AD tests to the Modified Gompertz model.

Stage/Reactors	Parameters in the modified Gompertz model			
The first stage AD of FW				
	P_{H2} (mL/g-VS_{added})	$R_{H2, max}$ (mL/(g-VS_{added}·h))	λ (h)	R^2
FW+DW	19.84 ± 0.05	3.87 ± 0.08	17.56 ± 0.08	0.9999
FW+N_2-NBW	24.52 ± 0.15	4.32 ± 0.14	16.88 ± 0.22	0.9996
FW+Air-NBW	27.66 ± 0.29	4.37 ± 0.22	17.10 ± 0.41	0.9990
The second stage AD of FW				
	P_{CH4} (mL/g-VS_{added})	$R_{CH4, max}$ (mL/(g-VS_{added}·d))	λ (d)	R^2
FW+DW	300.93 ± 2.78	100.29 ± 4.10	4.02 ± 0.17	0.9981
FW+N_2-NBW	346.58 ± 3.49	133.90 ± 6.77	4.05 ± 0.19	0.9974
FW+Air-NBW	375.25 ± 3.01	174.84 ± 8.33	4.44 ± 0.15	0.9981

Notes: AD-anaerobic digestion; VS-volatile solids; FW-food waste; DW-deionized water; NBW-nanobubble water; P_{H2} or P_{CH4}-maximum hydrogen or methane production potential; $R_{H2, max}$ or $R_{CH4, max}$ -maximum hydrogen or methane production rate; λ-lag time.

Table 2-3 Energy recovery from the two-stage AD of FW with or without NBW addition.

Reactors	Energy recovery (kJ/g-VS_{added})		
	H_2 production in the first stage	CH_4 production in the second stage	Two-stage AD
FW+DW	0.25 ± 0.00	12.05 ± 0.13	12.30 ± 0.13
FW+N_2-NBW	0.32 ± 0.00	13.92 ± 0.28	14.24 ± 0.28
FW+Air-NBW	0.35 ± 0.02	14.96 ± 0.14	15.31 ± 0.16

Notes: AD-anaerobic digestion; VS-volatile solids; FW-food waste; DW-deionized water; NBW-nanobubble water.

Table 2-4 Reults of economic assessment between the FW+DW reactor and FW+Air-NBW reactor when treating 1 ton of FW.

Reactors	H_2 yield (L)	Heating value of H_2 (kJ)	CH_4 yield (L)	Heating value of CH_4 (kJ)	Total heating value (kJ)	Electricity generation (kWh)
FW+DW	1,767	22,582	26,873	1,075,726	1,098,308	305.09
FW+Air-NBW	2,439	31,170	33,365	1,335,601	1,366,771	376.35

Notes: The unit conversion from kJ to kWh is 1/3600. AD-anaerobic digestion; FW-food waste; DW-deionized water; NBW-nanobubble water.

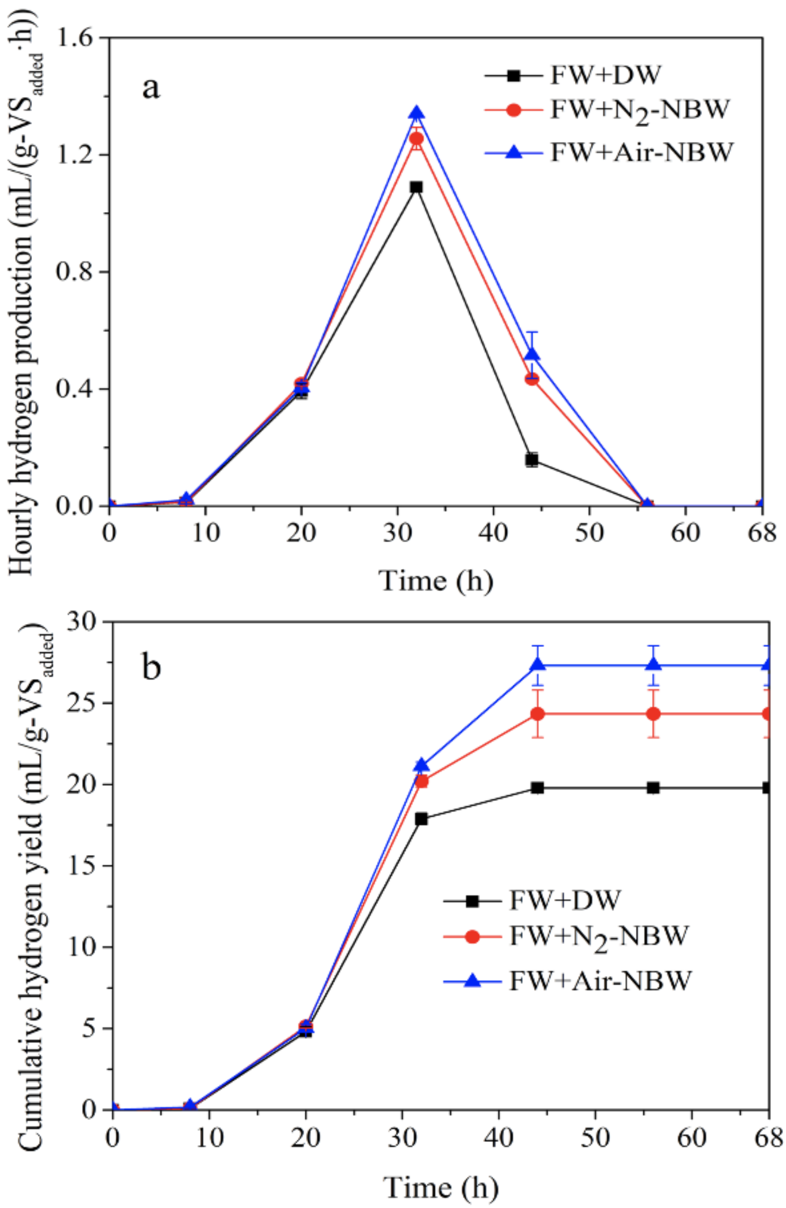

Fig. 2-1 Time courses of hourly H_2 production (a) and cumulative H_2 yield (b) under DW and NBW (N_2-NBW and Air-NBW) addition. (VS-volatile solids; FW-food waste; DW-deionized water; NBW-nanobubble water)

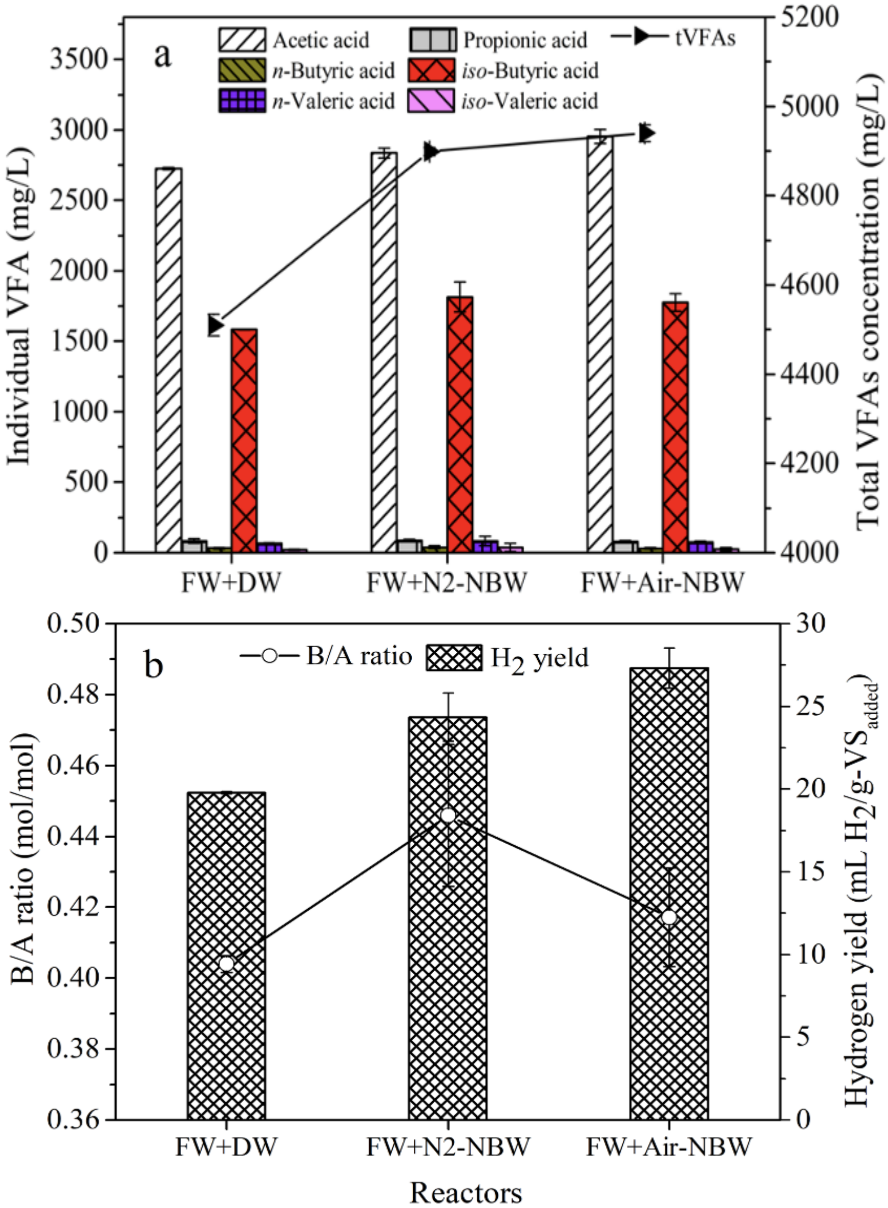

Fig. 2-2 The individual VFA and total VFAs concentrations (a) and the molar HBu/HAc (B/A) ratio and H_2 yield (b) at the end of the first stage under DW and NBW (N_2-NBW and Air-NBW) addition. (VFA-volatile fatty acid; DW-deionized water; NBW-nanobubble water; HBu-butyric acid; HAc-acetic acid)

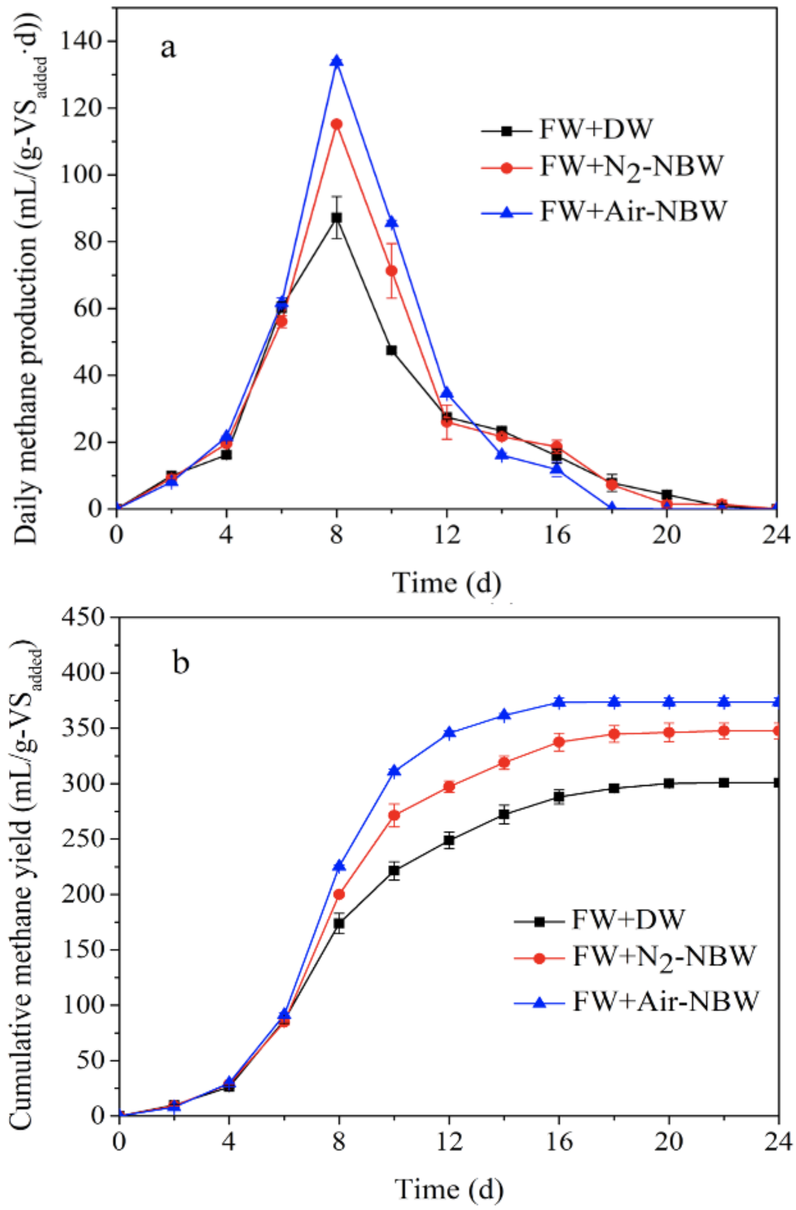

Fig. 2-3 Time courses of daily CH_4 production (a) and cumulative CH_4 yield (b) under DW and NBW (N_2-NBW and Air-NBW) addition. (FW-food waste; DW-deionized water; NBW-nanobubble water)

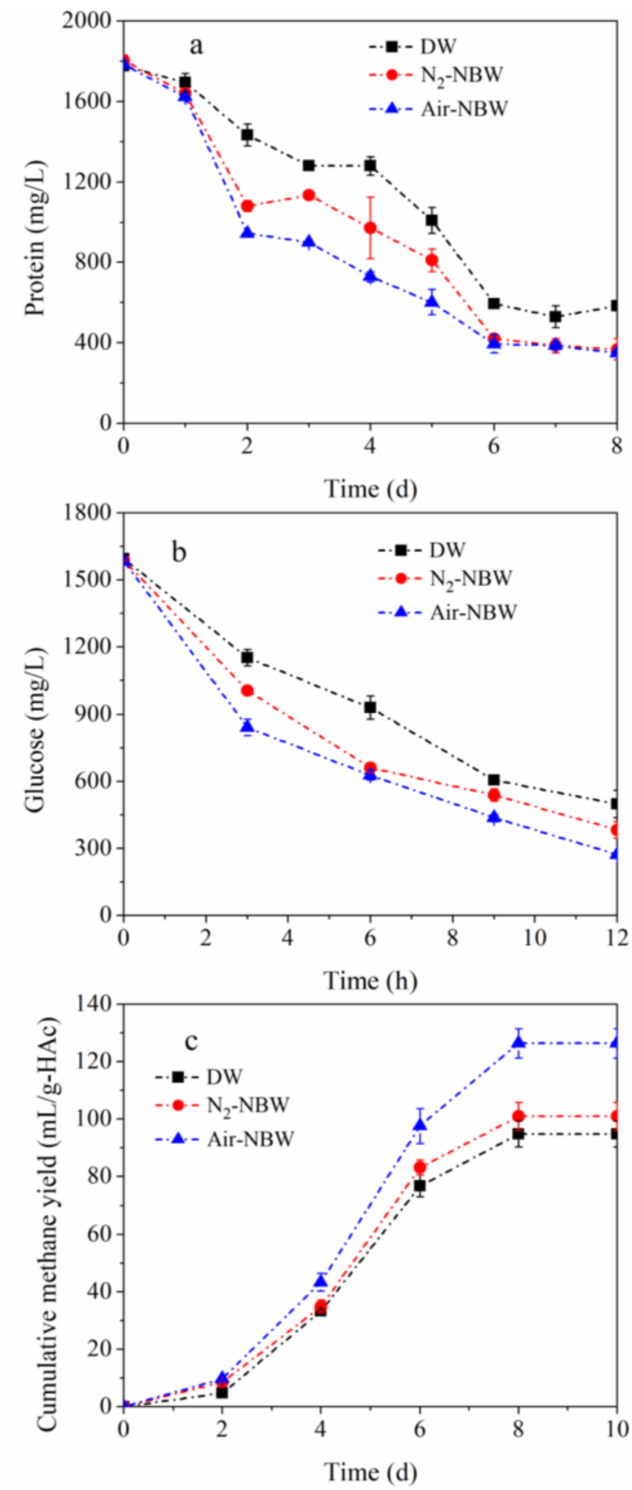

Fig. 2-4 Effects of NBW (N_2-NBW and Air-NBW) addition on the specific individual step involved in two-stage anaerobic digestion by using the model substrates. (DW-deionized water; NBW-nanobubble water; HAc-acetic acid)

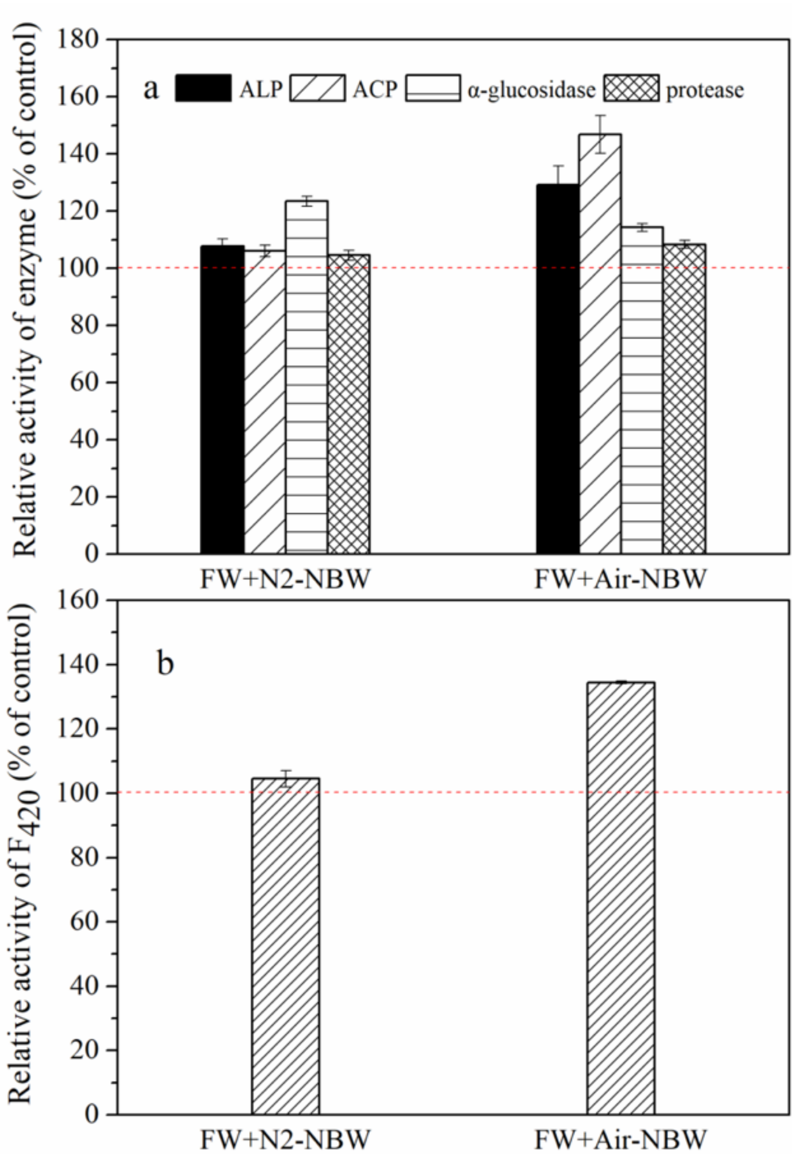

Fig. 2-5 Effects of NBW (N_2-NBW and Air-NBW) addition on the relative activity of ALP, ACP, protease, α-glucosidase, and protease at the end of the first stage (a), and the relative activity of coenzyme F_{420} at the end of the second stage (b). (ALP-alkaline phosphatase; ACP-acid phosphatase; FW-food waste; DW-deionized water; NBW-nanobubble water)

Chapter 3 Addition of air-nanobubble water to mitigate the inhibition of high salinity on co-production of H_2 and CH_4 from two-stage anaerobic digestion of food waste

3.1 Background

Salinity as one of the most stressful abiotic factors that limits the hydrogen or methane production from AD has attracted increasing attention rapidly. FW is becoming an increasingly preferred feedstock for AD but normally contains a high salinity because salt is extensively used as a food flavoring agent. In particularly in Japan, a much higher level of Na^+ consumption by most Japanese was found than the world health organization (WHO) recommendation, which is related to the Japanese diet (Asakura et al., 2014). At present, one of the effective methods to solve the inhibition of salt on AD is to improve the tolerance and activity of microorganisms in high salt environment. In general, amino acids and quaternary ammonium compounds have been adopted as common osmoprotectant in AD of high salinity organic wastes to recover methanogenic activity and increase methane production (Liu et al., 2019b; Zhang et al., 2016). However, the high production cost of osmoprotectant could greatly hinder their application, and a superfluous addition of osmoprotectant may adversely affect the methane production. Recently, NBW has been specifically applied to enhance the bioactivities of enzymes involved in AD systems of various organic solid wastes. For instance, Yang et al. (2019) and Wang et al. (2020b) found that addition of NBW enhanced hydrolase activities in the AD of waste activated sludge and ETS activity in the AD of cellulose. Wang et al. (2020a) reported that addition of Air-NBW enhanced the cellulose crystallinity reduction by 20% and the hydrolytic and methanogenic bacteria were enriched in the high cellulose loading AD system. According to the results from the chapter 2, it indicated that the addition of Air-NBW was observed to not only enhance the activities of hydrolase at the end of hydrolysis/acidification stage but also improve the activity of coenzyme F_{420} at the end of methanogenesis stage in the two-stage AD of FW. Besides, the negative charge on the surface of the NBs may adsorb cations (Meegoda et al., 2019), possibly to adsorb Na^+ in a high-salt environment to prevent them from entering the cell and cell rupture. Thus, this chapter is the first time investigated the response behaviors of hydrogen and methane co-production from the two-stage AD of FW to different salt concentrations. And then the effects of Air-NBW introduction on mitigating high salinity inhibition on AD of FW were evaluated.

3.2 Materials and methods

3.2.1 Raw materials and Air-NBW

According to Liu et al. (2017a) and He et al. (2019), the simulated FW comprised rice (25%), noodle (22%), pork (16%), cabbage (29%), and tofu (8%). Before starting the experiments, the FW was smashed and homogenized into slurries using a food grinder and stored at -20°C in the refrigerator. Inoculum was same with the chapter 2 (2.2.1). The physicochemical characteristics of the FW and inoculum are summarized in Table 3-1. Air-NBW was selected in this study according to the chapter 2.

3.2.2 Effects of Air-NBW addition on two-stage AD of FW at different salt concentrations

All experiments of both stages were performed in the closed Schott Duran serum bottles of 250 mL with the effective volume of 150 mL. In the first stage, according to the VS content of FW and inoculum in Table 1, 6.5 g raw FW (wet weight) and 70 g inoculum were added to each reactor at a substrate to S/I of 4:1 (VS basis). Then each bottle was diluted to the effective volume with DW or Air-NBW. As a result, the initial total VS was 12.76 ± 0.17 g/L. Afterwards, different amounts of salt (NaCl) were added to these reactors to obtain the pre-designed NaCl concentrations of 0, 5, 10, 20, and 30 g/L, respectively. At this stage, the initial pH was adjusted to the optimal pH (5.50 ± 0.20) considering the activity of hydrogenase. The reactors were denoted as FW+DW-0, FW+DW-5, FW+DW-10, FW+DW-20, and FW+DW-30 when DW was applied at salt concentrations of 0, 5, 10, 20, and 30 g/L, respectively. Similarly, the reactors were labelled as FW+Air-NBW-0, FW+Air-NBW-5, FW+Air-NBW-10, FW+Air-NBW-20, and FW+Air-NBW-30, respectively when Air-NBW was supplemented.

In the second stage, anaerobically digested sludge was firstly activated for about two weeks under 38 ± 1°C until no gas production, with TS and VS of 0.80 ± 0.01% and 0.55 ± 0.01%. 50 g remaining acidified slurry from the first stage was added as substrate to each bottle together with 50 g activated inoculum (S/I = 2:1 (VS basis)) to produce methane. Then each bottle was diluted to the effective volume with the DW or Air-NBW, resulting in an initial total VS of 6.01 ± 0.70 g/L with initial pH being adjusted to 7.50 ± 0.10. Similarly, different amounts of NaCl were supplemented to each reactor to make the final NaCl concentrations to be 0, 5, 10, 20, or 30 g/L in the reactor with the same designations as in the first stage.

Before starting the experiments, all the reactors were flushed with high purity nitrogen (purity ≥ 99.9995%) around 2 min for 3 times to remove oxygen and then placed in a thermostatic incubator at 38 ± 1℃ for 92 h in the first stage and 36 d in the second stage, respectively.

3.2.3 Analytical methods

The analytical methods of general parameters and calculations were as the same as in Chapter 2 (2.2.5). The inhibition degree was calculated by Eq. 3-1.

$$\text{Inhibition degree (\%)} = \frac{(Y_{t,\ FW+DW,\ 0} - Y_{t,\ FW+DW/Air\text{-}NBW,i})}{Y_{t,\ FW+DW,\ 0}} \times 100\% \qquad 3\text{-}1$$

where $Y_{t,\ FW+DW,\ 0}$ is the cumulative H_2 or CH_4 yield in FW+DW-0; $Y_{t,\ FW+DW/Air\text{-}NBW,\ i}$ is the cumulative H_2 or CH_4 yield in FW+DW or FW+Air-NBW at NaCl concentration of i (i = 5, 10, 20, or 30 g/L).

The undissociated HAc and HBu concentrations in all the reactors were calculated according to Eq. 3-2 (Cao and Zhao, 2009).

$$pH = pKa + lg\frac{[A^-]}{[HA]} \qquad 3\text{-}2$$

where pKa is dissociation constant (4.76 for HAc and 4.81 for HBu); $[A^-]$ and $[HA]$ are the dissociated acid and undissociated acid concentrations, respectively.

The inhibition on hydrogen or methane production from AD of FW under high salinity to a great extent depends on its impacts on microbial activities (Chen et al., 2018). More specifically, the main functional microorganisms in each stage are different in the two-stage AD process. Besides, the mechanisms involved in the influence of salinity on hydrolase activity in the first stage and coenzyme F_{420} in the second stage remain unclear. Therefore, it is necessary to evaluate the effect of high salinity on the major microbial enzyme activities and whether addition of Air-NBW could alleviate the inhibition of salt on microorganisms. In this study, the activities of α-glucosidase and protease, and coenzyme F_{420} were measured after the first stage and at the end of the second stage, respectively according to the method as chapter 2 (2.2.4).

In addition, the microbial activities during the hydrogen and methane production stages were assessed by monitoring the electron transport rate. The ETS activity was tested using the 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride-electron transport system (INT-ETS) method which has been described in detail previously (Wang et al., 2020).

3.3 Results and discussion

3.3.1 Effect of Air-NBW addition on hydrogen production from the first stage AD of FW at different salt concentrations

(1) *Hydrogen production rate and cumulative hydrogen yield*

As seen in Fig. 3-1a, the peak values of average hourly hydrogen production occurred firstly in the salt-free digesters (FW+DW-0 and FW+Air-NBW-0). When salinity was ≥ 20 g NaCl/L, the peak values of average hourly hydrogen production decreased significantly with the increase of salinity under no and addition of Air-NBW ($p = 2.27 \times 10^{-5}$ for the DW group and 1.63×10^{-6} for the Air-NBW group, both $p << 0.05$). This indicates that high salinity may inhibit hydrogen production, which is consistent with the negative effects of salt on hydrogen production in previous studies. For instance, Kim et al. (2009) obtained 30%, 60%, and 90% inhibition on hydrogen production at 2.41, 5.36, and 10.14 g Na^+/L, respectively. Similarly, Zhang et al. (2017) observed that hydrogen production could be gradually decreased with the increase of salinity and the inhibitory effect was more obvious at salinity ≥ 15 g NaCl/L. It is noteworthy that the peak values of average hourly hydrogen production in the Air-NBW group (6.38, 4.89, 3.79, 1.22 and 1.15 mL/(g-VS_{added}·h) were higher than those in the DW group (5.79, 4.39, 3.17, 0.85, and 1.01 mL/(g-VS_{added}·h) under the test salinity conditions (0-30 g NaCl/L).

The cumulative hydrogen yield showed similar trends (Fig. 3-1b) and the maximum hydrogen yield of 197.55 ± 0.63 mL/g-VS_{added} was obtained from FW+Air-NBW-0, about 28% higher than that from FW+DW-0 (154.63 ± 0.70 mL/g-VS_{added}). It is worth mentioning that FW+Air-NBW-5 (185.64 ± 2.53 mL/g-VS_{added}) also obtained approximately 20% higher than FW+DW-0. Moreover, the cumulative hydrogen yields from the Air-NBW added reactors were increased by 30%, 21%, 65%, and 65% in comparison to the DW added reactors at 5, 10, 20, and 30 g NaCl/L, respectively. As seen, salt inhibition on hydrogen production was more obvious in the reactors with DW addition, *i.e.*, 14%, 25%, 77%, and 82% inhibition degrees occurred in FW+DW-5, FW+DW-10, FW+DW-20, and FW+DW-30, respectively (Fig. 3-2a). When Air-NBW was supplemented, however, the inhibition degree was significantly reduced, which were 10%, 63%, and 70% in FW+Air-NBW-10, FW+Air-NBW-20, and FW+Air-NBW-30, respectively. This observation indicates that addition of Air-NBW can effectively alleviate the inhibition of salt on hydrogen production from the two-stage AD of FW. As reported previously, the inhibition of high concentration salt on AD was mainly caused by the loss of microbial activity (Anwar et al., 2016; Chen et al., 2018). However, Air-NBW supplementation can significantly increase the activity of hydrolase in the two-stage AD of FW, which may be the reason for the reduced inhibition of salt on hydrogen production under addition of Air-NBW.

The kinetic parameters were estimated as shown in Table 3-2. The maximum hydrogen production potential (P_{H2}) and the maximum hydrogen production rate ($R_{H2,\ max}$) were decreased with the increase of salt concentration, regardless of whether Air-NBW was added or not. However, the values for the reactors with Air-NBW addition were higher than those for the corresponding reactors with DW addition. Upon the increase in salt concentration, the lag time (λ) seemed to increase correspondingly. In contrast, Air-NBW group had about 23-42% shorter λ (λ = 6.00-14.64 h) in comparison to the DW group (λ = 10.33-19.17 h). This observation implies that addition of Air-NBW can accelerate the start-up of AD process even under salt inhibition conditions.

(2) *Potential impact of salt on the hydrolysis/acidification upon Air-NBW addition*

The variations of individual VFA, soluble proteins, and soluble carbohydrates may indirectly reflect the degree of hydrolysis/acidification. Therefore, VFAs were monitored in the first stage as shown in Fig. 3-2b. A remarkably negative effect on VFAs production was noticed with the increase of salt concentrations ($p = 1.19 \times 10^{-6}$ for the DW group and $p = 1.74 \times 10^{-8}$ for the Air-NBW group, both $p \ll 0.05$). It has been found that VFAs yield was inhibited at > 6 g NaCl/L, with the production being reduced at least by 44.8% at > 9 g NaCl/L (Liu et al., 2017a). Zhao et al. (2016) claimed that VFAs production was enhanced when salt concentration was < 8 g/L, while severely inhibited when the salt concentration was further increased. In this study, higher total VFAs productions were achieved from the Air-NBW added reactors, increasing by 6-18% when compared to DW added ones. This observation is in agreement with that of hydrogen yield, probably because acid production is usually accompanied by hydrogen production.

Regarding the changes of individual VFA concentration, a clear H_2 production through the HAc and HBu pathway was noticed in all the reactors. This indicates that the HBu-type fermentation was the main fermentation type in this study according to Zhang et al. (2017). However, at high salinity levels, HAc and HBu productions were reduced, which may be the main reason for the lower H_2 yields in the high salinity reactors.

Ginkel and Logan (2005) detected that a larger amount the undissociated HAc and HBu was present under lower pH conditions, that led to the inhibited H_2 production. As it is known, the undissociated HBu may exert more negative effect on H_2 yield than the undissociated HAc. Table 3-3 summarizes the changes of final pH, undissociated HAc and HBu concentrations in all the reactors. As shown, the concentrations of undissociated HAc and HBu were increased with the increase of salinity, while a lower increase was detected in the Air-NBW added reactors (Table 3-3). Heuvel et al. (1992) claimed that the undissociated acid at the critical inhibitory concentration (50 mM) may greatly increase the cell maintenance energy requirements and deplete the ATP reserves necessary for the metabolic switch to solventogenesis. In this study, addition of Air-NBW reduced the accumulation of undissociated acid, which might be resulted from its enhancement effect on microbial activity as discussed later.

Soluble proteins and carbohydrates play a crucial role in microbial growth and hydrogen production by anaerobic fermentation (Laspidou and Rittmann, 2002). As shown in Figs. 3-3a and b, the initial concentrations of soluble proteins and carbohydrates were 1,014.15 ± 78.82 and 2,278.26 ± 128.12 mg/L in all the reactors. After the hydrogen production stage, the concentrations of soluble proteins and carbohydrates declined remarkably in the reactors with lower salinity (0-10 g NaCl/L). However, higher concentrations of soluble proteins and carbohydrates were always detected in the reactors with higher salinity (≥ 20 g NaCl/L), especially in the DW added reactors. Zhao et al., (2017) revealed that the concentrations of soluble carbohydrates and proteins were increased from 8,596 and 2,156 mg COD/L to 12,054 and 3,124 mg COD/L when NaCl concentration was increased from 2 to 15 g/L in the AD system of FW. This observation suggests that high salinity may accelerate the release of proteins and carbohydrates due to the bacterial protective responses to high salinity stress (Wang et al., 2013). It was noted that the declines in soluble proteins and carbohydrates were more remarkable in the Air-NBW group: (1) they had about 14%, 21%, 48%, 68%, and 113% higher declines in soluble proteins concentrations, and (2) about 11%, 16%, 7%, 13% and 14% higher declines in soluble carbohydrates concentrations than the reactors with DW addition when NaCl concentration varied from 0 to 30 g/L. On the one hand, this phenomenon indicates that a prolonged exposure of microorganisms to high salinity may lead to cell lysis or dissolution, reducing the hydrolysis of proteins and carbohydrates. On the other hand, it also suggests that addition of Air-NBW rather than DW may not only ameliorate the above negative effect but also promote the hydrolysis of organic matters under high salinity conditions. Wang et al. (2017) applied Scanning Electron Microscopy (SEM) and observed more pores and channels on the surface of sludge after being treated with air micro-bubbles, which is conducive to the formation of zoogloea.

(3) *Activities of ETS and extracellular hydrolases during the first stage AD of FW*

In this study, the microbial activity indicated by the electron transport rate and extracellular hydrolases was monitored during the first stage. As illustrated in Fig. 3-4a, the ETS activity was detected to increase and then decrease along with the lower salt concentrations (≤ 10 g NaCl/L); however, in the DW added reactors with 20 and 30 g NaCl/L, a continuous decline of ETS activity was noticed. The highest ETS activity, 71.78 mg/(g·h), was obtained at 44 h in FW+Air-NBW-0. At the end of the first stage, the ETS activities in the Air-NBW added reactors were 5-92% higher than the DW added reactors at the test salt concentrations. This observation implies the enhanced electron transfer effect under Air-NBW addition condition, and thus the increased bioactivity of microbes, which could be ascribed to the involvement of Air-NBs in electron transfer with resultant enhanced synthesis of hydrogen from their negatively charged surfaces. A detailed explanation is given in Section 3.3.3.

Fig. 3-4b displays the relative activities of α-glucosidase and protease in all the reactors at the end of the first stage with the extracellular hydrolases of FW+DW-0 being as the reference (100%). Clearly, high salinity may inhibit the activity of hydrolase to some extent when compared to the salt-free reactors. For instance, the relative activity of α-glucosidase was decreased by 13%, 15%, 24%, and 38% in FW+DW-5, FW+DW-10, FW+DW-20, and FW+DW-30, respectively. However, less inhibition was observed on the activity of protease when compared to that of α-glucosidase. For example, the relative activity of protease did not decrease but increased slightly by 2% at salt concentration of 5 g/L. And the relative activity of protease was decreased by 8%, 17%, and 29% in FW+DW-10, FW+DW-20, and FW+DW-

30, respectively. Besides, it is worth noting that 9-20% higher α-glucosidase and 0.4-22% increase in protease activities were detected in the Air-NBW added reactors than those in the DW added reactors at salt concentrations of 0-30 g/L, reflecting the higher bioactivities of anaerobes in the former reactors. Therefore, supplementation of Air-NBW is regarded as an effective strategy to enhance microbial activities under high salinity, even for hydrogen production from AD of FW.

3.3.2 Effect of Air-NBW addition on methane production from the second stage AD of FW at different salt concentrations

(1) *Methane production rate and cumulative methane yield*

In the second stage, the remaining hydrolyzed and acidified organic slurries at the end of the first stage were used as the substrates for methane production. Much attention was paid to the effect of salt on methane production from the second stage AD of FW with or without Air-NBW addition. The daily methane production as shown in Fig. 3-5a clearly indicates some prolongation effect of salt on the reactor start-up and progress. More specifically, when the salt concentration was 20 g/L (FW+DW-20), the peak daily methane production was reached on day 30, with 18 days later than FW+DW-0; furthermore, when the salt concentration was 30 g/L (FW+DW-30), the reactor failed to reach the peak daily methane production till the end of the experiment (day 36). There are some halophilic anaerobic microbial communities (Lefebvre et al., 2006), while these halophilic microorganisms are limited, and non-halophilic microorganisms require a long time to accommodate the high salinity condition. Therefore, when coping with high salinity FW, anaerobic microbes should be given enough time to adapt to the high salinity environment, thus a longer start-up time was required at high salinity (like 20 and 30 g NaCl/L in this study). The lag time (λ) estimated by the Modified Gompertz model shows the similar result (Table 3-2).

Regarding the cumulative methane yield (Fig. 3-5b), the methane yield was significantly reduced at high salinity, especially in the DW added reactors. In the Air-NBW added reactors, the cumulative methane yields were 322.23 ± 0.76, 288.39 ± 2.63, 277.04 ± 3.03, 202.27 ± 6.23, and 65.24 ± 0.57 mL/g-VS_{added} at 0, 5, 10, 20 and 30 g NaCl/L, about 19%, 16%, 20%, 14%, and 43% higher than those of DW added reactors (269.96 ± 7.35, 247.55 ± 12.96, 231.19 ± 19.45, 178.06 ± 8.39, and 45.60 ± 3.51 mL/g-VS_{added}), respectively. As seen from Fig. 3-5c, the inhibition degrees of methane production were 8%, 14%, 34%, and 83% in FW+DW-5, FW+DW-10, FW+DW-20, and FW+DW-30, respectively. Compared with previous studies, the two-stage AD of high-salt FW in this study was more resistant to salt inhibition on methane production. For instance, Anwar et al. (2016) claimed that the inhibition degrees of one-stage kitchen waste AD system were 24%, 33%, 56%, and 81% in the reactors at 10, 12, 14, and 16 g NaCl/L, respectively. And 80% and 91% of inhibition degrees were obtained on methane production from one-stage AD of FW at 10 and 15 g NaCl/L, respectively (Zhao et al., 2017). Two-stage AD of FW used in this study may add some mitigation effect of salt inhibition on methane production, as the microorganisms in the first stage have been exposed and got accommodated to the high salinity conditions. Additionally, it is interesting to notice that the methane yields from the Air-NBW added reactors at 0, 5, and 10 g NaCl/L were increased by about 19%, 7%, and 3% in comparison to that from FW+DW-0, and the inhibition degrees of methane production were only 26% and 76% in FW+Air-NBW-20 and FW+Air-NBW-30, respectively. These results imply that addition of Air-NBW could also effectively reduce the salt inhibition on methane production in the second stage AD of FW.

(2) *Variations of organic matters concentration during the second stage AD*

The initial and final concentrations of soluble proteins and carbohydrates in the second stage AD are displayed in Fig. 3-6a. The initial concentrations of soluble proteins in all the reactors were higher than those of soluble carbohydrates. It is probably because that the hydrolysis of proteins in the first stage was not as sufficient as that of carbohydrates, resulting in more residual proteins. The hydrolysis of proteins was reported later than that of carbohydrates and thus required a longer hydrolysis time (Kim et al., 2019), which was also verified in this study. Especially under high salt concentrations, the initial concentrations of soluble proteins and carbohydrates in the second stage were higher, which need to be further hydrolyzed in the second stage with a prolonged the lag time. This could also be one of the reasons for the prolonged start-up of methane production with the increase of salt concentration.

The VFAs produced in the first stage were the primary substrates that could be directly converted into methane by methanogens during the second stage. Therefore, a high initial VFAs concentration in the second stage is conducive to methane production (De Gioannis et al., 2017). As shown in Fig. 3-6b, the higher initial VFAs concentrations were detected in the reactors with low salt concentrations (0-10 g/L), and addition of Air-NBW could produce more VFAs in the first stage, which can be utilized by methanogens in the second stage. However, when the salt concentration was 30 g/L, the total VFAs concentration was not decreased but increased at the end of methane production stage, particularly in FW+DW-30. Due to the hydrogen production metabolic pathway, the first stage was dominated by HBu, which was followed by HAc with little HPr production (the initial VFAs for the second stage). Nevertheless, HPr was accumulated, around 180 and 285 mg/L in the FW+DW-20 and FW+DW-30 at the end of the second stage, respectively. HAc is regarded as the preferable substrate for methane production, while HPr is not conducive to the utilization by methanogens (Capson-Tojo et al., 2018). These data suggest that the inhibition of high salinity (≥ 20 g NaCl/L) on methanogenesis may be attributable to low efficiency of hydrolysis/acidification in the first stage and unfavorable methanogenesis due to the produced HPr.

(3) *Activities of ETS and coenzyme F_{420} in the second stage AD of FW at different salt concentrations*

The ETS activities in the reactors with or without Air-NBW addition were assessed during the 36 days' methanogenesis as shown in Fig. 3-7a. In the reactors with low salinity (0-10 g NaCl/L), significant increase in ETS activity was detected, especially in the Air-NBW added reactors. However, the ETS activities in FW+DW-20 and FW+DW-30 were decreased slightly during the first 12 days, reflecting the negative effect of high salinity on ETS of microorganisms and thus reduced microbial activity. On the contrary, the ETS activities in the FW+Air-NBW-20 and FW+Air-NBW-30 were increased continually during the whole process, indicating that addition of Air-NBW was beneficial for mitigating the adverse effect of high salinity on ETS activity in the methanogenesis. At the end of the second stage, the ETS activity was increased by 9-30% in the Air-NBW added reactors when compared to the DW added reactors. In addition, after the 36 days' methane production, the relative activities of coenzyme F_{420} in all the reactors exhibited the same trend (Fig. 3-7b), *i.e.*, when FW+ DW-0 was taken as the reference (100%), the activity of coenzyme F_{420} was detected to remarkably decrease at high salinity levels, which were 73%, 62%, 54%, and 40% in the DW added reactors, and 130%, 110%, 99%, 72%, and 49% in the Air-NBW added ones at 5, 10, 20 and 30 g NaCl/L, respectively. These results indicate that addition of Air-NBW could enhance methane production by increasing microbial activity even under high salinity conditions.

3.3.3 Preliminary analysis of mechanisms involved in this study

Salt toxicity to microorganisms is predominantly determined by cations (Anwar et al., 2016; Chen et al., 2018) and the main cation in FW is Na^+ because FW salinity mainly derives from food flavoring agents (NaCl). Osmotic pressure would increase sharply at high salt concentrations leading to plasmolysis and ultimately cell lysis and death (Fig. 3-8). This is why high salinity has a negative effect on AD. Meegoda et al. (2019) found that negatively charged NBs in an electrolyte solution (0.001-0.1 M NaCl) could combine with Na^+ to form the electric double layer, which may prevent Na^+ from entering the cell, thus reducing adverse effects of Na^+ on microorganisms. However, the effect of high salinity on the morphology of NBs cannot be ignored. As reported by Meegoda et al. (2019) and Bunkin and Shkirin (2012), low salinity exerts imperceptible effect on physicochemical characteristics of NBs, but when the NBs are exposed to a high salinity solution for a while, the bubble size, surface charge density, and number of negative charges would increase. Nevertheless, the possible effect of these observations on AD is unclear.

As shown in Fig. 3-8, during the H_2 production stage, addition of Air-NBW enhanced the activities of ETS and extracellular hydrolases at different salt concentrations, which are likely attributable to the involvement of Air-NBs in the electron transfer associated with the synthesis of hydrogen and enhanced biosynthesis of hydrolases. To be specifically, the negative charges on the surfaces of NBs could attract H^+, which could be more easily reduced by NADH to H_2 following $NADH + H^+ \rightarrow NAD^+ + H_2$ (Cai et al., 2011). Tanisho et al. (1989) also found that the conversion of NADH to hydrogen could occur on the cell membranes, implying that the hydrogenase involved in the NADH pathway is a membrane-bound enzyme. Therefore, Air-NBs may promote the electron exchange between NADH ferredoxin oxidoreductase (NFOR) and hydrogenase to produce H_2 due to it is negatively charged. For the methanogenesis stage, addition of Air-NBW also improves the activities of ETS and coenzyme F_{420}, most probably attributable to its stimulation effect on electron transfer between bacteria and methanogens. It also indirectly indicated that addition of Air-NBW enhanced microbial activity, resulting in increased CH_4 production. Liu et al. (2015) and Xiao and Xu (2020) have also verified that NBW could accelerate the metabolism of organisms, facilitating the seed germination and the biofilm growth. Still, more direct evidence is demanding for the above viewpoints in the followed-up works.

3.4 Summary

This study investigated the effect of salt (0-30 g NaCl/L) on co-production of hydrogen and methane from two-stage AD of FW, showing that NBW addition may provide a promising approach to mitigate the adverse impacts of high salinity on hydrogen and methane production. The hydrolysis/acidogenesis and methanogenesis stages were seriously inhibited at high salt levels ($\geq$ 20 g NaCl/L). However, addition of Air-NBW ameliorated the inhibition of high salinity on the efficiency of two-stage AD of FW, with hydrogen and methane yields increased by 21-65% and 14-43% in the Air-NBW added reactors in comparison to the DW added ones at 0-30 g NaCl/L. This study for the first time confirmed that when two-stage AD of FW was exposed to the same level of salt, addition of Air-NBW could achieve enhanced enzymatic activity and ETS activity at the individual stage.

Table 3-1 Physicochemical characteristics of the food waste and inoculum.

Parameter	Food waste	Inoculum
Total solids (TS, %)[a]	23.95 ± 0.37	0.80 ± 0.02
Volatile solid (VS, %)[a]	23.55 ± 0.32	0.55 ± 0.01
Total volatile fatty acids (VFAs, mg/L)	925.51 ± 21.72	9.77 ± 1.70
Soluble proteins (mg/L)	11,226.51 ± 85.24	208.86 ± 17.78
Soluble carbohydrates (mg/L)	21,836.00 ± 28.85	18.17 ± 0.26

[a] Wet weight basis.

Table 3-2 Parameters estimated by fitting the experimental data from the two-stage food wastes (FW) anaerobic digestion (AD) tests with deionized water (DW) or Air-nanobubble water (NBW) addition at different salt concentration using the Modified Gompertz model.

Stage/Parameters	Reactors									
The first-stage AD of FW with or without Air-NBW addition at different salt concentration										
	FW+DW-0	FW+DW-5	FW+DW-10	FW+DW-20	FW+DW-30	FW+Air-NBW-0	FW+Air-NBW-5	FW+Air-NBW-10	FW+Air-NBW-20	FW+Air-NBW-30
P_{H2} (mL/g-VS_{added})	156.49 ± 1.02	146.91 ± 2.86	129.03 ± 6.69	33.75 ± 1.52	28.43 ± 0.90	200.79 ± 3.19	195.46 ± 8.10	156.74 ± 6.61	53.86 ± 2.58	44.61 ± 1.42
$R_{H2, max}$ (mL/(g-VS_{added}·h))	17.10 ± 0.15	12.47 ± 0.14	6.13 ± 0.22	4.05 ± 0.51	3.67 ± 0.24	17.85 ± 1.27	13.51 ± 1.68	6.48 ± 0.20	4.86 ± 0.51	3.87 ± 0.17
λ (h)	10.33 ± 0.24	17.16 ± 0.64	17.82 ± 1.25	17.87 ± 1.30	19.17 ± 1.04	6.00 ± 1.18	12.65 ± 2.00	12.88 ± 0.13	13.74 ± 0.54	14.64 ± 1.08
R^2	0.9993	0.9962	0.9901	0.9728	0.9872	0.9965	0.9903	0.9955	0.9737	0.9880
The second-stage AD of FW with or without Air-NBW addition at different salt concentration										
	FW+DW-0	FW+DW-5	FW+DW-10	FW+DW-20	FW+DW-30	FW+Air-NBW-0	FW+Air-NBW-5	FW+Air-NBW-10	FW+Air-NBW-20	FW+Air-NBW-30
P_{CH4} (mL/g-VS_{added})	271.90 ± 2.97	249.21 ± 2.20	231.97 ± 2.25	184.44 ± 0.23	59.87 ± 1.23	323.55 ± 3.38	289.85 ± 2.34	279.17 ± 3.48	208.29 ± 6.23	80.47 ± 0.67
$R_{CH4, max}$ (mL/(g-VS_{added}·d))	122.69 ± 1.20	119.36 ± 0.77	90.77 ± 5.65	52.65 ± 5.80	6.76 ± 0.54	138.14 ± 1.67	132.41 ± 2.77	126.28 ± 1.39	55.50 ± 5.73	9.62 ± 0.41
λ (d)	7.52 ± 0.27	8.56 ± 0.11	10.06 ± 0.23	20.71 ± 0.58	22.66 ± 0.25	7.22 ± 0.26	8.52 ± 0.11	9.88 ± 0.29	20.64 ± 0.57	21.23 ± 0.45
R^2	0.9935	0.9959	0.9965	0.9790	0.9870	0.9941	0.9967	0.9935	0.9796	0.9913

Table 3-3 The final pH value, undissociated HAc, and undissociated HBu at the end of the first stage in each reactor.

Reactors	Final pH	Undissociated HAc (mM)	Undissociated HBu (mM)
FW+DW-0	4.12 ± 0.04	9.94 ± 0.80	17.75 ± 1.22
FW+DW-5	3.84 ± 0.02	14.71 ± 1.46	33.84 ± 0.27
FW+DW-10	3.71 ± 0.06	16.96 ± 1.53	39.78 ± 4.13
FW+DW-20	3.37 ± 0.01	24.76 ± 0.36	30.31 ± 1.58
FW + DW-30	3.14 ± 0.04	32.00 ± 1.62	46.15 ± 3.74
FW+Air-NBW-0	4.36 ± 0.04	6.64 ± 0.49	12.39 ± 1.08
FW+Air-NBW-5	3.96 ± 0.00	12.95 ± 0.15	26.50 ± 0.09
FW+Air-NBW-10	3.85 ± 0.04	12.99 ± 1.35	32.97 ± 2.81
FW+Air-NBW-20	3.58 ± 0.03	19.27 ± 1.06	20.20 ± 1.63
FW+Air-NBW-30	3.39 ± 0.07	25.77 ± 1.23	28.51 ± 0.56

Notes: HAc-acetic acid; HBc-butyric acid; FW-food waste; DW-deionized water; NBW-nanobubble water.

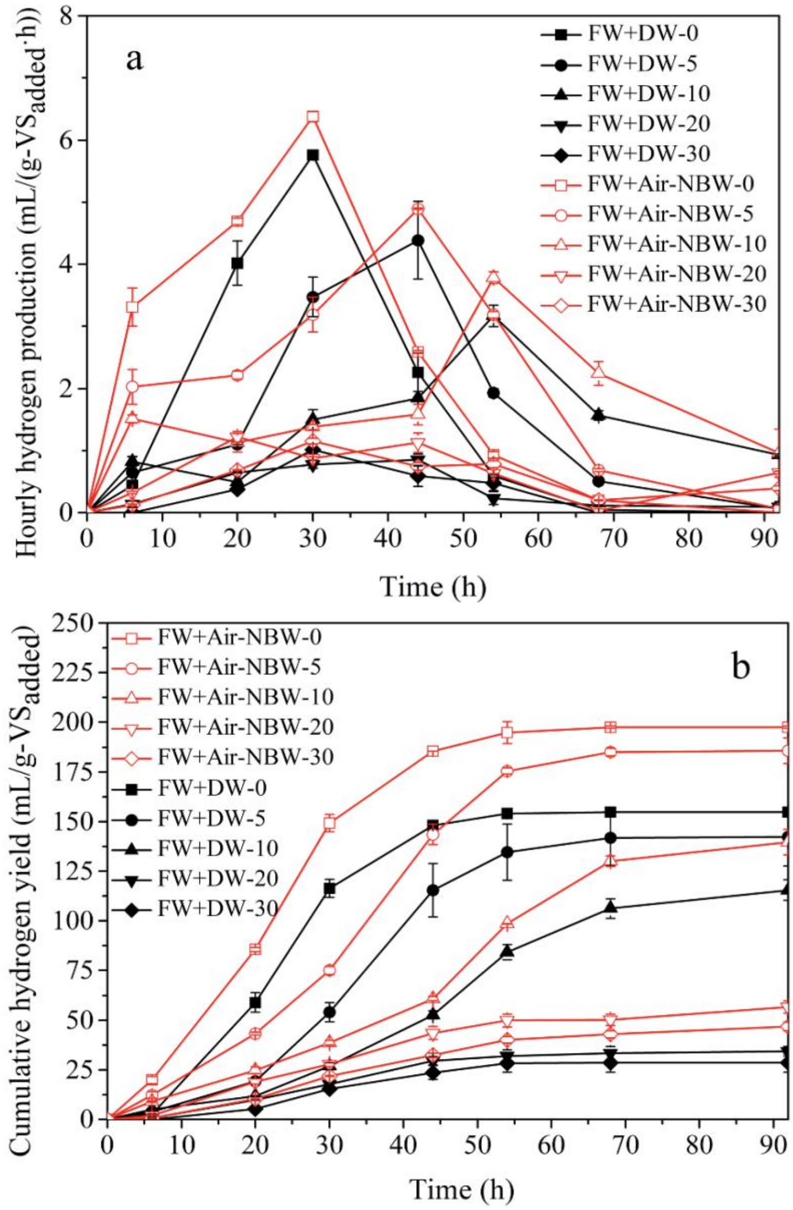

Fig. 3-1 H_2 production rate (a) and cumulative H_2 yield (b) from FW+DW and FW+Air-NBW at 0-30 g NaCl/L. (FW-food waste; VS-volatile solids; DW-deionized water; NBW-nanobubble water)

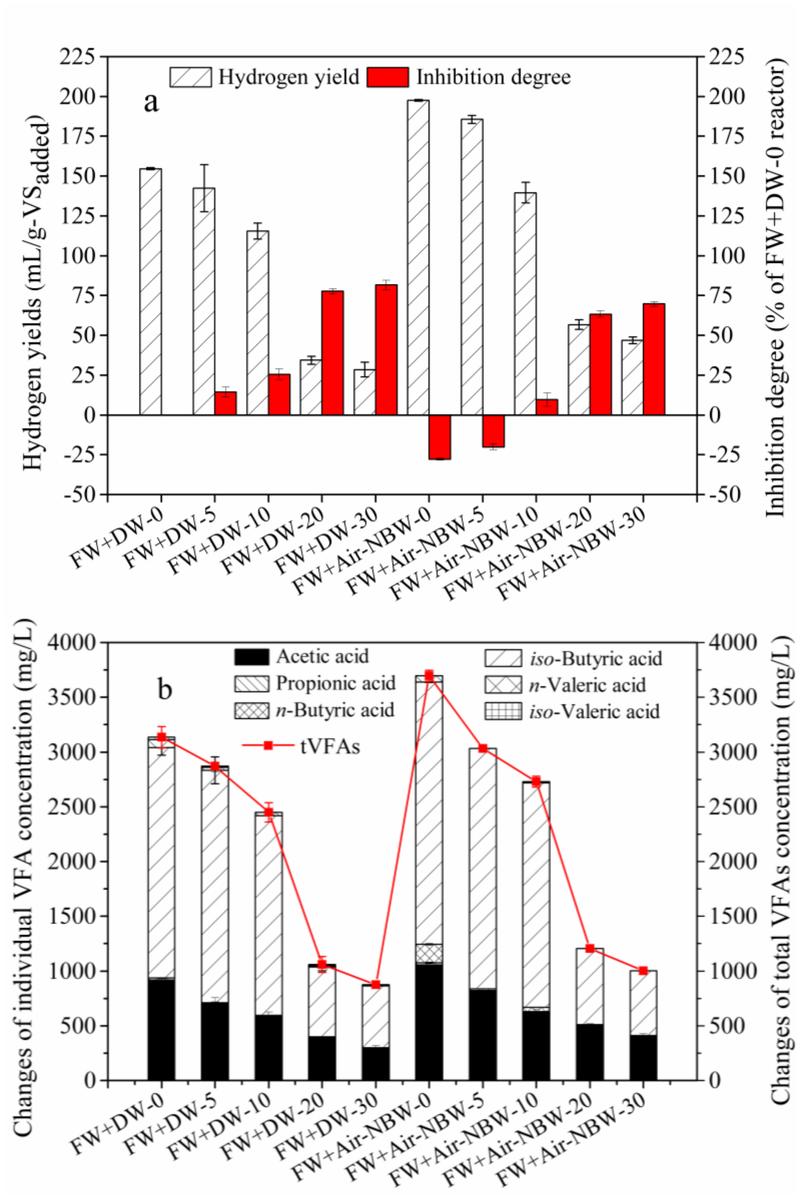

Fig. 3-2 H_2 yield and inhibition degree (a), and the changes of individual VFA and total VFAs concentration (b) at the end of the first stage AD of FW+DW and FW+Air-NBW at 0-30 g NaCl/L. (VS-volatile solids; VFA-volatile fatty acids; AD-anaerobic digestion; FW-food waste; DW-deionized waster; NBW-nanobubble water)

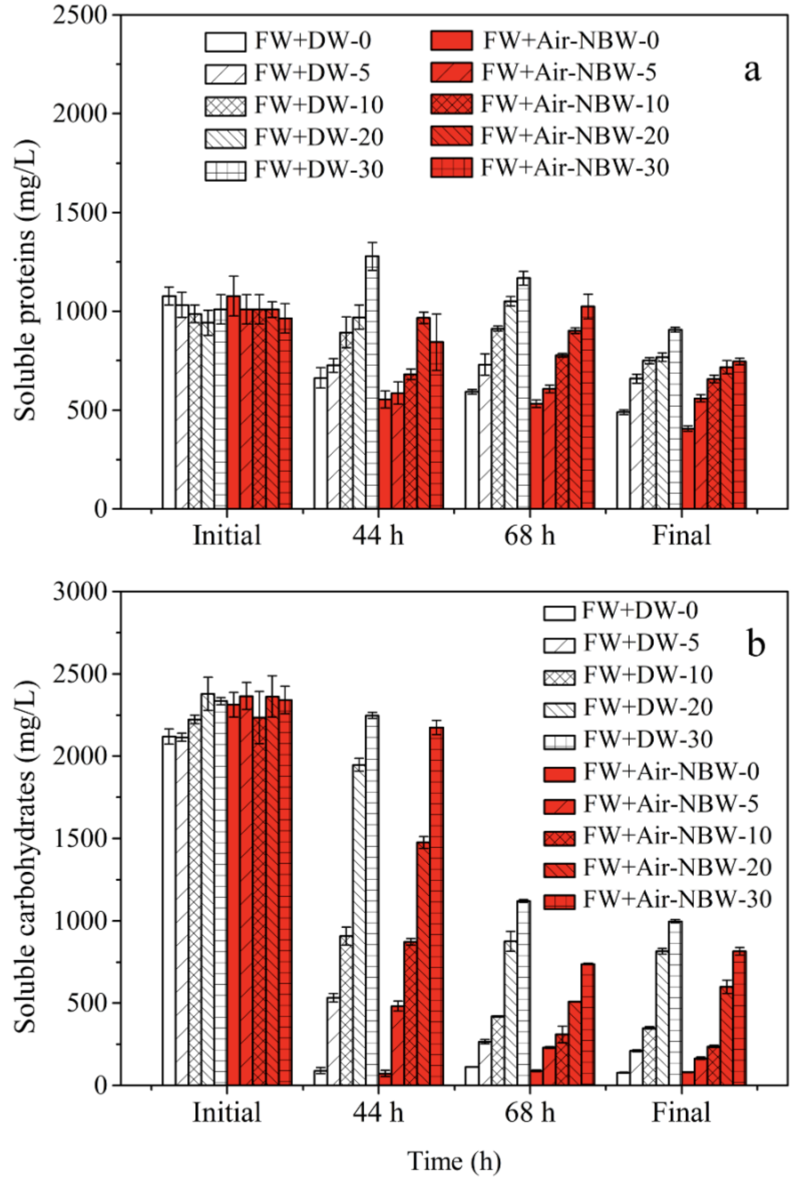

Fig. 3-3 The concentrations of soluble proteins (a) and carbohydrates (b) during the first stage AD of FW+DW and FW+Air-NBW. (VS-volatile solids; VFA-volatile fatty acids; AD-anaerobic digestion; FW-food waste; DW-deionized waster)

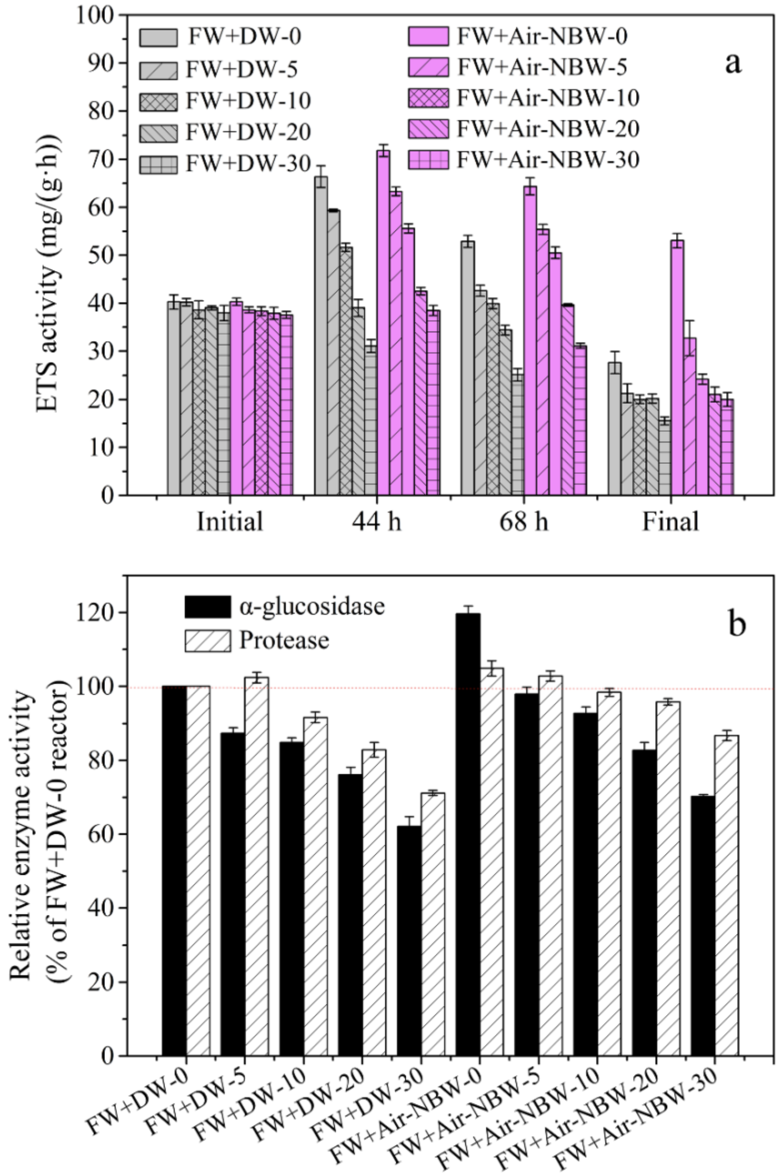

Fig. 3-4 The effects of salt on ETS activity (a) and relative activities of α-glucosidase and protease (b) at the end of the first stage AD of high salinity FW with or without Air-NBW addition. (ETS-electron transport system; FW-food waste; AD-anaerobic digestion; DW-deionized water; NBW-nanobubble water)

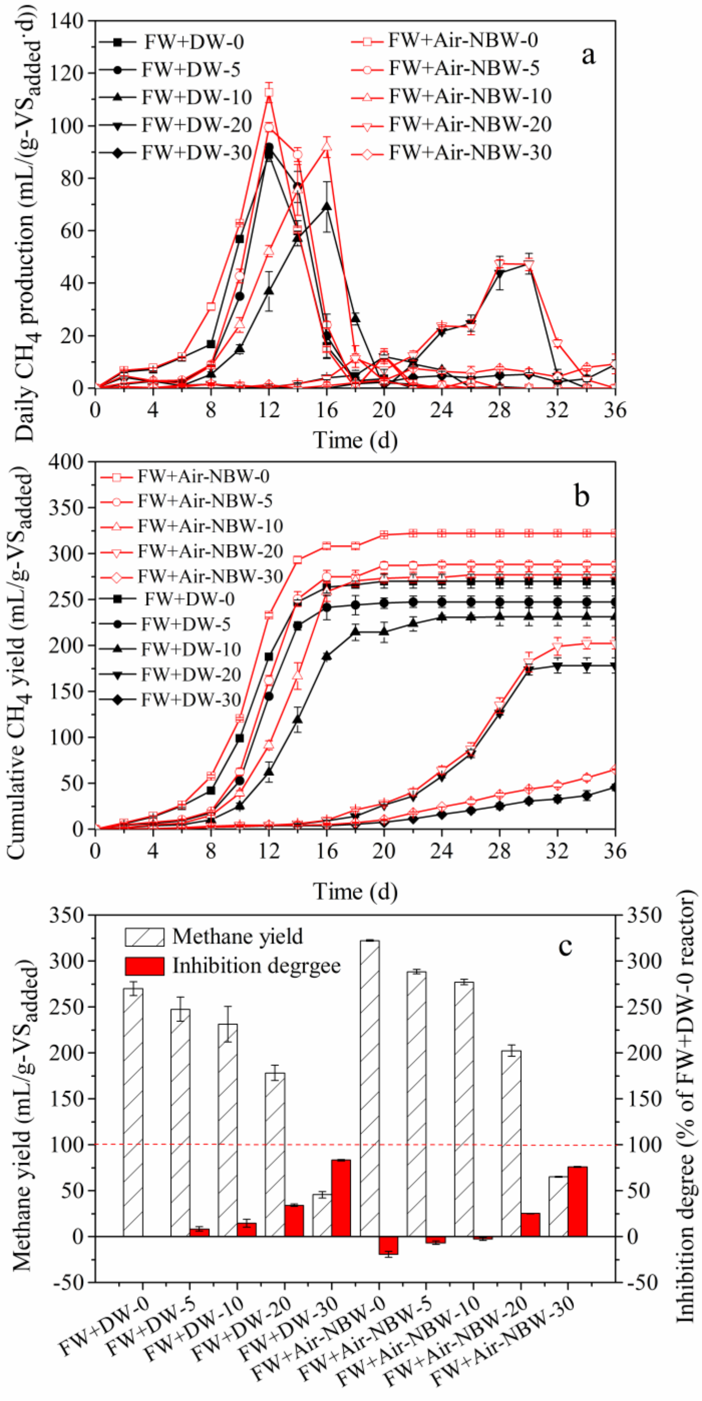

Fig. 3-5 CH_4 production rate (a), cumulative CH_4 yield (b), and CH_4 yield and inhibition degree (c) of FW+DW and FW+Air-NBW during the second stage AD at 0-30 g NaCl/L. (VS-volatile solids; FW-food waste; DW-deionized water; NBW-nanobubble water)

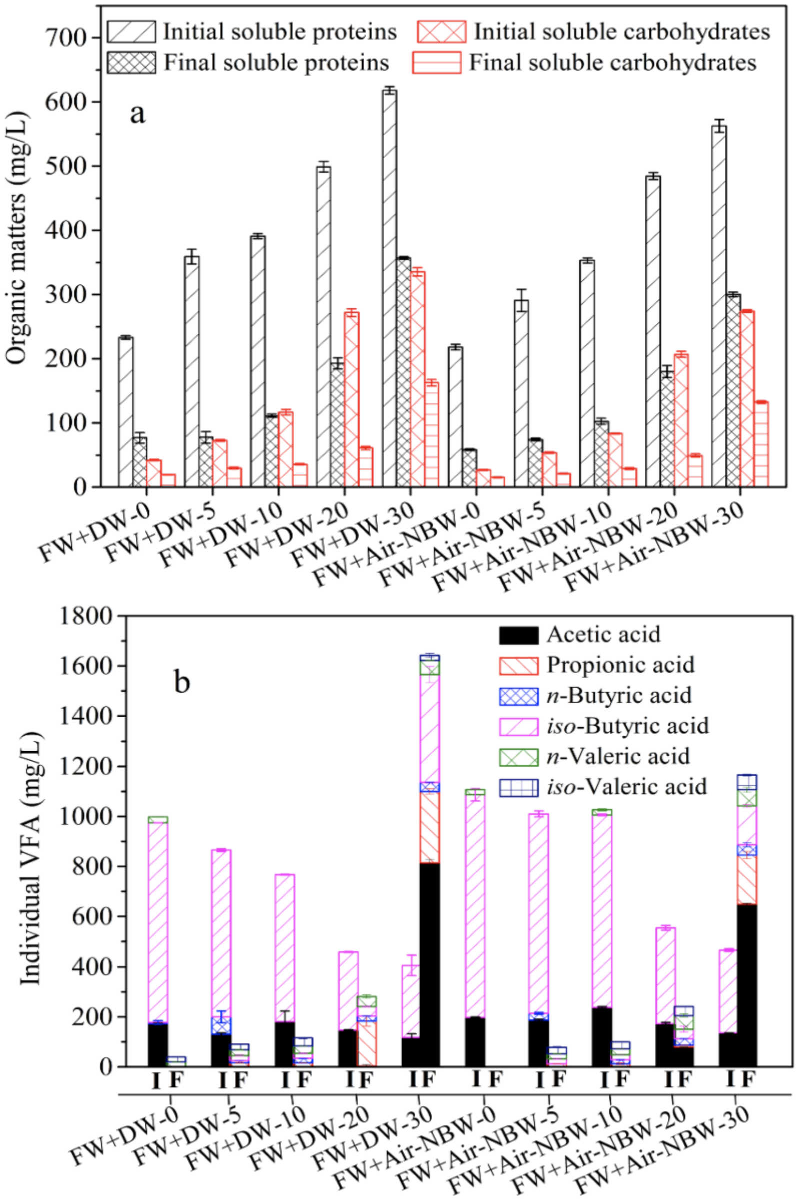

Fig. 3-6 The concentrations of initial and final organic matters (a) and individual VFA (b) during the second stage AD of FW+DW and FW+Air-NBW at 0-30 g NaCl/L. The letters 'I' and 'F' at X-axis denote the initial and final concentrations, respectively. (VFA-volatile fatty acid; VS-volatile solids; FW-food waste; DW-deionized water; NBW-nanobubble water)

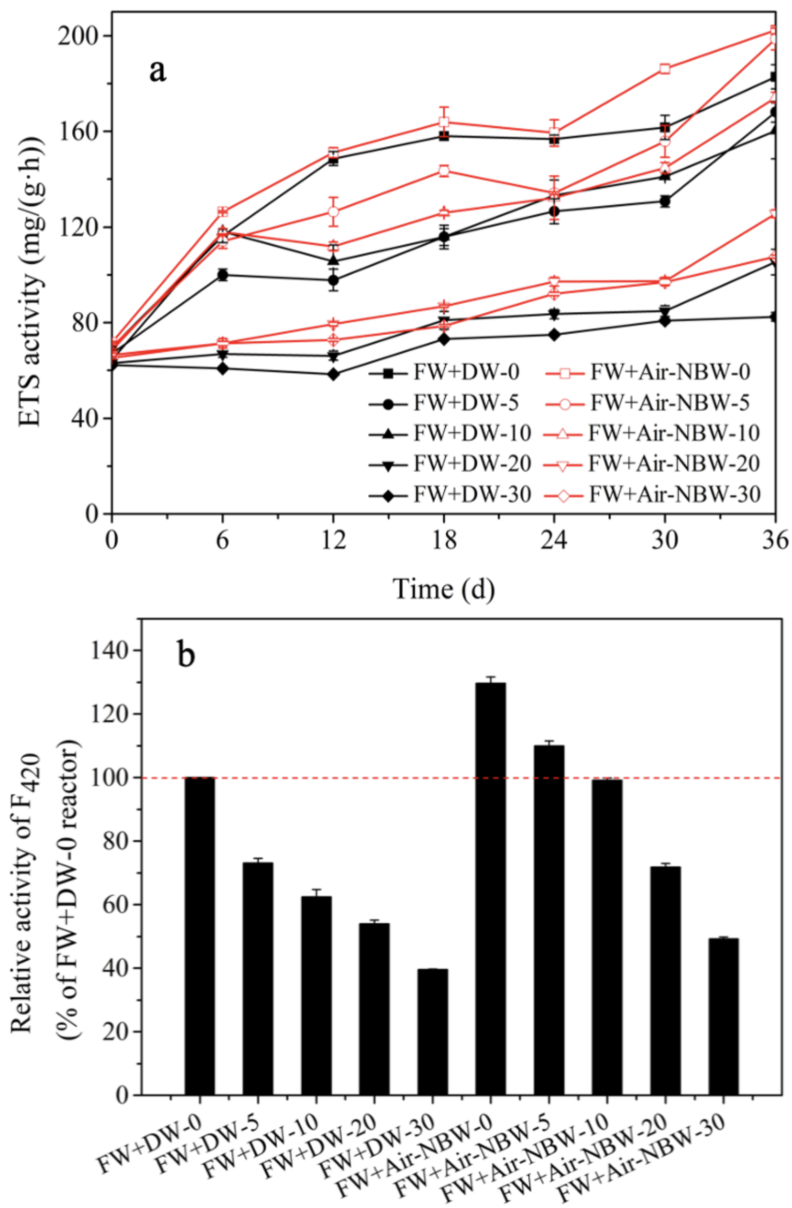

Fig. 3-7 Effects of salt on ETS activity (a) during the methanogenesis and relative activities of coenzyme F_{420} (b) at the end of the second stage AD of FW with or without Air-NBW addition. (ETS-electron transport system; FW-food waste; AD-anaerobic digestion; DW-deionized water; NBW- nanobubble water)

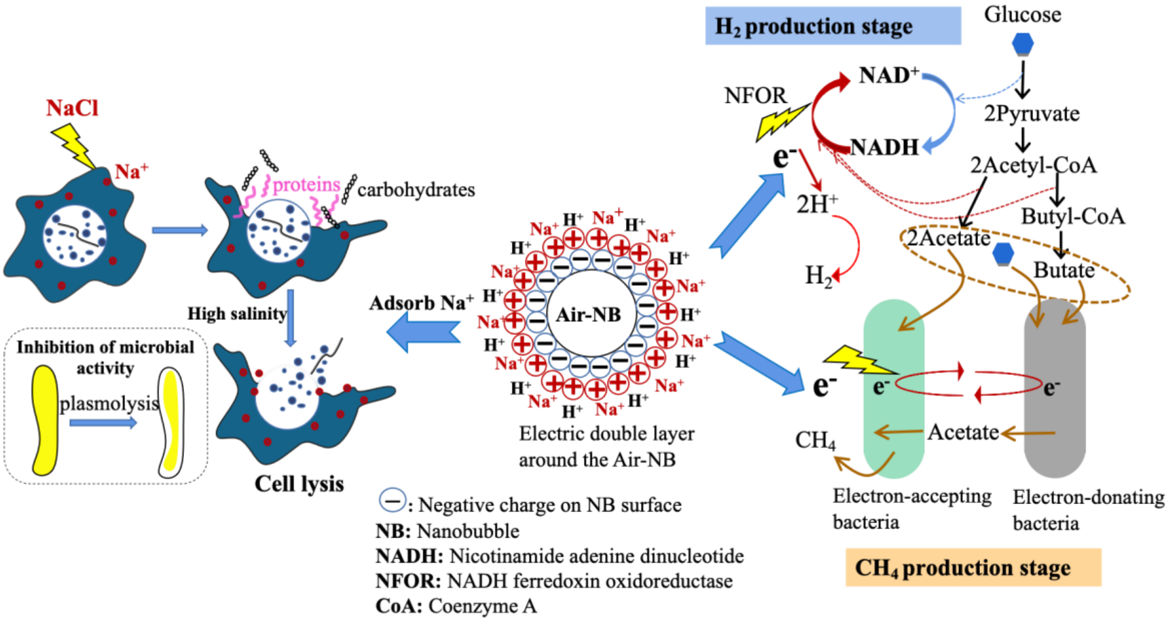

Fig. 3-8 Impact of NaCl on microorganisms and preliminary analysis of mechanisms involved in the enhanced H_2 and CH_4 production from two-stage AD of high salinity FW with Air-NBW addition and resultant promoted electron transfer. (AD-anaerobic digestion; FW-food waste; NBW-nanobubble water)

Chapter 4 Supplementation of KOH to improve salt tolerance of methanogenesis in two-stage anaerobic digestion of food waste using pre-domesticated anaerobically digested sludge by air-nanobubble water

4.1 Background

Many previous studies have investigated the inhibition effects of salt on AD processes, including hydrolysis, acidogenesis, or methanogenesis. Among them, high salt inhibited the methane production more remarkable than the hydrogen and VFAs production because the methanogens are more sensitive to environmental factors like high salt concentration than hydrolytic bacteria (Wang et al., 2018). According to the results from the chapter 3, high salt ($\geq$ 20 g NaCl/L) inhibited the methane production more remarkable than the hydrogen and VFAs production. It was also found that anaerobic microbes should be given enough time to adapt to the high salinity environment when coping with high salinity FW, thus a longer start-up time (about 20 days) is required at 20 g NaCl/L. Additionally, Hierholtzer and Akunna (2014) also found a similar phenomenon, delay of about 15 days in methane production in AD of saline-rich macro-algae. These suggested that the microbial activity was weakened by high salt concentration during the early stage of methanogenesis. Strategies for addressing this problem in improving the microbial activity is preferred. NBW had been put forward and demonstrated to improve the bioactivities of enzymes involved in AD systems, including hydrolases and cellulase (Wang et al., 2020a; Yang et al., 2019). According to previous studies, however, the volume of NBW-based AD reactors is relatively large, which could increase the construction costs. On condition that the anaerobically digested sludge is firstly domesticated by NBW and then as an inoculum added to the AD system may not only increase the activity of microorganisms but also no increase the volume of reactors. On the other hand, “salt in” strategy is another method to maintain the microbial activity under the high salt concentration, and the K^+ is a favorable alternative osmolyte in cells (Martin et al., 1999; Zhang et al., 2016). It is noteworthy that the effects of addition KCl on the AD of saline organic wastewater has been investigated, and the results showed that the methane production was increased by 49.1% with the addition of 1.74 g/L KCl (optimal dosage) when the NaCl content was 20 g/L (Li et al., 2018). In the two-stage AD of FW, due to the low pH at the end of the hydrolysis/acidification stage, the remaining organic slurries from this stage as the substrate in the methanogenic stage usually needs to adjust the pH value with a base such as NaOH to satisfy the requirements of methanogens. Therefore, substitution of NaOH with KOH as a pH regulator may not only reduce the additional increase of Na^+ but also supplement K^+ to mitigate the inhibition of high salinity on methane production in the methanogenic-stage AD of high-salt organic wastes.

4.2 Materials and methods

4.2.1 Anaerobically digested sludge, feedstock and Air-NBW

Anaerobically digested sludge, FW, and Air-NBW were as same as the chapter 3 (3.2.1). The physicochemical characteristics of the FW and inoculum are summarized in Table 4-1. The initial TS and VS of FW and raw sludge were 18.77 ± 0.40% and 18.01 ± 0.03%, 0.88 ± 0.04% and 0.58 ± 0.05%, respectively. Then, the sludge was dewatered through a 1 mm pore size sieve and centrifuging at 4000 rpm for 15 min. The TS and VS of the concentrated sludge were 2.45 ± 0.02% and 1.92 ± 0.02%. The specific manufacturing process of Air-NBW was based on the chapter 2.2.1.

4.2.2 Acclimation of anaerobically digested sludge by DW or Air-NBW

Inoculum plays a pivotal role in anaerobic biological processes because the stability and efficiency of AD have a strong correlation with the number, species, and metabolic activities of microorganisms (Franchi et al., 2018). In this study, the anaerobically digested sludge was pre-domesticated by Air-NBW, improving microbial activity to resist the toxicity of high salt to microorganism.

For pre-domesticating, 200 mL raw concentrated sludge and 10 g FW (wet weight) at the S/I of 0.5 were added in the 500 mL of digesters, and then the DW or Air-NBW were used to adjust the working volume of each digester to 350 mL. Each bottle was purged by pure N_2 for 2 min, 3 times and the initial pH was 6.79 ± 0.42 in the first cycle and 7.51 ± 0.12 in the second cycle with no additional adjustments. All digesters were domesticated at a constant temperature of 38 ± 1°C. After completion, the reaction slurries were dewatered again through a 1 mm pore size sieve and centrifuging at 4000 rpm for 15 min, and then the concentrated sludge was used as the inoculum in the second cycle to repeat the above domestication procedure. During the two cycles of the domestication process, methane production was measured every two days and ETS activity at the end of each cycle (around one month) were detected.

4.2.3 Operation conditions for two-stage AD of high salinity FW

The hydrogen potential tests were carried out in the digesters (100 mL of total volume) with 15 g (wet weight) of FW with the 20.0 g/L of salt (NaCl) concentration and 40 mL of above pre-domesticated anaerobically digested sludge as inoculum were added to each bottle to make the total working volume of each bottle to be 55 mL. A higher initial total VS was achieved in each bottle about 6%, and the S/I ratio was about 6.0 (VS basis). Note that no additional chemical reagents were added to adjust the pH during the first stage. Due to the low initial pH of the FW (4.30), the initial pH of each digester was 6.50 ± 0.20 after completion of the digesters constructed, within the appropriate pH range for hydrogen production. Depending on the different of the pre-domesticated inoculum, the reactors were labelled as DW and Air-NBW.

For methane potential tests, the reactors of identical specifications with the first stage were used and the working volume of reactors were also 55 mL. The residual acidified slurries from the first stage as substrates and pre-domesticated anaerobically digested sludge as inoculum were added in each reactor at the S/I ratio of 2. The initial total VS of each reactor was 1.9% ± 0.02%. Afterwards, to evaluate the effect of KOH as an accelerator on methane production from the AD of high salinity FW, the initial pH of each reactor was adjusted to 7.50, 8.00, and 9.00 using 6 M KOH, respectively. The control group was adjusted the initial pH to 7.50 by using 6 M NaOH. Therefore, the reactors were designated $DW_{control}$-7.50, DW-7.50, DW-8.00, DW-9.00, $NBW_{control}$-7.50, NBW-7.50, NBW-8.00, and NBW-9.00, respectively.

Before starting the all experiments including the both stages, each reactor was sealed with a rubber stopper and purged with pure nitrogen for 2 min, 3 times to remove oxygen and then digested in a temperature-controlled incubator (38 ± 1°C).

4.2.4 Analytical methods and calculations

The basic analysis methods and ETS activity were as same as the chapter 2 (2.2.5) and chapter 3 (3.2.3). The kinetic analysis used the modified Gompertz model to fit the experimental data of each stage as described previously (chapter 2.2.5). Additionally, the alkalinity including TA, partial alkalinity (PA, endpoint pH to 5.75), intermediate alkalinity (IA, pH from 5.75 to 4.30) were based on the standard methods (APHA, 2012). For the determine of the concentrations of NH_4^+ and K^+, the samples were filtered through 0.22 μm filters and then were measured by using the Ion Chromatography (IC, Shimadzu, Japan). The lactic acid was quantified by using a spectrometer (Shimadzu, Japan) and the specific methods was published in Borshchevskaya et al. (2016). FAN was calculated according to the following Eq. 4-1. All measurements were performed in triplicate Zhao et al. (2020a).

$$FAN = \frac{TAN \times 10^{pH}}{e^{6344/(273+T)} + 10^{pH}} \quad \text{4-1}$$

where T is the temperature in Celsius (38°C in this study).

4.3 Results and discussion

4.3.1 Methane yield and ETS activity during the acclimation process

Microorganisms with robust physiology are essential for the stability performance of AD. Faced with a new substrate, the established consortium of anaerobically digested sludge normally needs time to adapt to this new substrate (Vásquez and Nakasaki, 2016). When FW is directly used as a substrate in an ordinary AD system, it could cause an imbalance in the microorganism system, resulting in a slow start-up and low efficiency (Zeng et al., 2019). Therefore, the pre-domestication of anaerobically digested sludge is necessary. In this study, the anaerobically digested sludge was pre-domesticated under a low S/I ratio by adding Air-NBW, expecting to enhance the microbial activity of the inoculum and improve the performance in the subsequent two-stage AD of high salinity FW.

During the two cycles of pre-domestication, the cumulative methane yield was examined, and the results are displayed in Fig. 4-1. As seen, the methane production rate was lower during the first 8 d in the first cycle, and however, the reactors were quickly start-up in the second cycle. In the Air-NBW pre-domesticated sludge reactor, the highest peak value of methane production in the second cycle (42.23 ± 1.47 mL/g-VS_{added}·d) was obtained, increasing about 37% when compared to the DW group (31.59 ± 0.45 mL/g-VS_{added}·d). Additionally, the cumulative methane yield in the Air-NBW reactor (169.88 ± 1.83 mL/g-VS_{added} in the first cycle and 217.46 ± 0.50 mL/g-VS_{added} in the second cycle) was higher around 14% and 33% than that in the DW reactor (149.48 ± 0.20 mL/g-VS_{added} in the first cycle and 163.24 ± 2.96 mL/g-VS_{added} in the second cycle) during both cycles, respectively.

ETS activity also showed the similar results, *i.e.*, it was higher 1.9 and 2.2 times after the first cycle and 3.5 and 4.4 times after the second cycle in the DW and Air-NBW pre-domesticated sludge reactor, respectively (shown in Fig. 4-2). This indicates that the pre-domestication of digested sludge is an effective approach for enhancement of microbial activity. After the second cycle, the highest ETS activity was achieved in the Air-NBW reactor (87.03 mg/(g·h)), increasing by 27% in comparison to the DW reactor (68.69 mg/(g·h)). Xiao and Xu (2020) also found that adopting the NB technology had 6 times higher dehydrogenase activity compared with the coarse bubble and enhanced the microbial metabolism and the proliferation of microorganisms. Similarly, in introducing the current application of NBs technology, Gurung et al. (2016) mentioned that it exhibited greater application prospect in the field of environmental engineering because it immensely promoted the biological activity and mass transfer efficiency. The results of this study also confirmed these views, *i.e.*, digested sludge pre-domesticated by Air-NBW presented a higher biological activity and methane potential.

4.3.2 Performance of hydrolysis/acidification stage

(1) *Hydrogen yield and ETS activity*

In the first stage, the specific hydrogen production, cumulative hydrogen yield, and ETS activity were displayed in Fig. 4-3. The hydrogen production was completed within 48 h and each reactor was quickly start-up (shown in Fig. 4-3a). In particular, the hourly hydrogen production in the Air-NBW group was higher than that in the DW group during the whole process. According to the results from the chapter 3, when the salt concentration was 20 g NaCl/L, the lag time (λ) was 17.87 ± 1.30 h. Other previous research also found that when the salt concentration was 19.19 and 24.49 g/L, the lag time (λ) was 14.6 and 29.2 h, respectively

(Cao and Zhao, 2009). However, the result of this study indicates that the high salinity (20 g NaCl/L) didn't inhibit the start-up of reactors when the pre-domesticated sludge was used as inoculum. The λ was only 3.72 ± 0.06 and 2.90 ± 0.02 h in the DW pre-domesticated inoculum reactor and Air-NBW pre-domesticated inoculum reactor, respectively (Table 4-2). As shown in Fig. 4-3b, the cumulative hydrogen yield was also observed the similar conclusion, namely the hydrogen yield in the Air-NBW group was higher about 46% than the DW group. These data confirms that the performance of hydrogen production could be enhanced under the high salinity condition by inoculating the Air-NBW pre-domesticated anaerobically digested sludge.

For the ETS activity in the first stage (Fig. 4-3b), it was consistent with the hydrogen production results and indirectly reflected the metabolic activity related to electron transfer in microbial cells. High hydrogen production was accompanied by an increase in ETS activity, and the ETS activity in the Air-NBW group was always higher than the DW group. In particular, the ETS activity of the Air-NBW reactors were higher around 1.7 and 2.2 times than the DW reactors at the 6 and 12 h, respectively, suggesting that the Air-NBW pre-domesticated inoculum exhibited the more excellent microbial metabolic activity. This may be attributed that the microbial biomass may be increased after the Air-NBW pro-domestication of inoculum. Zhao et al. (2020b) expressed that the diversity and abundance of archaea and bacterial communities would increase after domesticating. More importantly, Xiao and Xu (2020) reported that the NBs technology could significantly accelerate the formation of biofilm and increase the biofilm thickness. Therefore, using NBW to pre-domesticate inoculum could provide a strategy to strengthen the microbial activity in AD system to resist the adverse factors that affect the AD performance.

(2) *Variation in VFAs and lactic acid concentration during the hydrogen fermentation*

The variations of pH, VFAs, and lactic acid were monitored in the first stage and the results are shown in Fig. 4-4. Multitudinous research has proved that the different hydrogen-producing fermentation pathways (including HBu-type fermentation and mixed-acid fermentation) were formed in the anaerobic fermentation system, which will lead to the accumulation of various end products (Lee et al., 2012). VFAs as the main by-product of hydrogen production accumulated rapidly in the first 12 h and then slowly increased, shown in Fig. 4-4a. At the end of 48 h, the total VFAs concentration was approximately 4,189.30 ± 37.94 and 4,948.41 ± 177.26 mg/L in the DW reactor and Air-NBW reactor, respectively. The concentration of HBu increased by 33% in the Air-NBW reactor compared with the DW reactor, suggesting that the HBu-type fermentation was more remarkable in the Air-NBW reactor. In addition, high carbohydrate content (41-62%) in FW often leads to the accumulation of lactic acid, which is the main product of carbohydrate fermentation (Anna, 2013). However, the lactic acid concentration was not significantly different in each reactor during the first 6 h and then was always higher in the DW reactor than that in the Air-NBW reactor from 12 h (Fig. 4-4b). At the end of the first stage, the lactic acid concentration was 9.50 ± 0.20 and 7.64 ± 0.16 g/L in the DW reactor and Air-NBW reactor, respectively. This indicates that the microbial community may begin to shift toward lactic acid metabolism in the DW reactor, thus explaining the decrease in hydrogen production. Ghimire et al. (2018) investigated the accumulation of lactic acid during hydrogen production and found that the decrease in hydrogen production was related to the metabolic transfer caused by lactic acid accumulation. Anna (2013) has also summarized the reasons for the inhibition of hydrogen production by lactic acid, including the competition of lactic acid bacteria for substrates and the excretion of bacteriocins inhibiting the growth of other microorganisms. In addition, the accumulation of lactic acid also contributed to the lower pH in the DW group (Fig. 4-4c). At the end of the first stage, pH value was 3.83 ± 0.01 and 3.94 ± 0.03 in the DW group and the Air-NBW group, respectively.

4.3.3 Effect of KOH addition on methane production from second stage AD

(1) *Methane yield and ETS activity*

In the second stage, the CH_4 production rate and cumulative CH_4 yield are displayed in Fig. 4-5. As shown in Fig. 4-5a, there is no significant difference of methane production among all the reactors during the first 6 days ($p = 0.38 > 0.05$). Notably, the reactors with the addition of KOH were started up faster than the control group, and the λ in the Air-NBW pre-domesticated inoculum reactors were shorter than that in the DW pre-domesticated inoculum reactors (in Table 4-2). The CH_4 production potential (P_{CH4}) and CH_4 production rate ($R_{CH4, max}$) also reflected the consistent results, and the highest P_{CH4} and highest $R_{CH4, max}$ were obtained in the NBW-8.0 and NBW-9.0, respectively. As expected, the final cumulative CH_4 yield (Fig. 4-5b) was higher about 22%, 25%, and 17% in the DW-7.5 (268.54 ± 1.83 mL/g-VS_{added}), DW-8.0 (275.39 ± 2.85 mL/g-VS_{added}), and DW-9.0 (258.09 ± 4.12 mL/g-VS_{added}) than that in the $DW_{contorl}$-7.5 (220.83 ± 1.44 mL/g-VS_{added}), respectively. In the same way, the final cumulative CH_4 yield was higher about 19%, 26%, and 25% in the NBW-7.5 (281.40 ± 1.12 mL/g-VS_{added}), NBW-8.0 (297.21 ± 3.86 mL/g-VS_{added}), and NBW-9.0 (295.87 ± 7.29 mL/g-VS_{added}) than that in the $NBW_{contorl}$-7.5 (236.79 ± 10.30 mL/g-VS_{added}), respectively. These results indicate that the addition of K+ can effectively alleviate the inhibition of high salinity on methane production. This may be attributed to the fact that the supplementation of K^+ regulated the osmotic pressure in microbial cells and prevented the accumulation of Na^+ in the cells so as to maintain a high cell survival rate (Tang et al., 2018). Additionally, the methane yield showed a trend of decline with the increase of the K^+ dosage in the DW pre-domesticated inoculum reactors, which was consistent with the report by Li et al. (2018) that excessive K^+ would have a negative effect on methane production. This could be explained that the excessive concentrations of any salt are toxic to microbial activity (Ashfaq et al., 2020). However, the methane yield was no obviously decline when the K^+ dosage was increased by adjusting the pH to 9.0 in the NBW pre-domesticated inoculum reactors, indicating that the microorganisms domesticated by the Air-NBW have a higher tolerance to environmental toxicity.

The result of the ETS activity also confirmed this view as shown in Fig. 4-5c. As seen, the ETS activity in all reactors increased gradually during the first 24 days and was in agreement with the methane production. It is a remarkable fact that the ETS activity in the reactors with the K^+ addition was always higher than that in the control group, and the ETS activity of the NBW pre-domesticated inoculum reactors was always higher than that in the DW pre-domesticated inoculum reactors. In the DW group, the ETS activity was increased by 10%, 16%, and 15% in the DW-7.5 (122.26 ± 3.19 mg/(g·h)), DW-8.0 (128.89 ± 1.57 mg/(g·h)), and DW-9.0 (127.51 ±18.59 mg/(g·h))at the maximum value when compared with the $DW_{control}$-7.5 (111.15 ± 6.41 mg/(g·h)); in the NBW group, the ETS activity was increased by 15%, 31%, 17% in the NBW-7.5 (140.38 ± 1.55 mg/(g·h)), NBW-8.0 (159.33 ± 9.74 mg/(g·h)), and NBW-9.0 (142.22 ± 6.83 mg/(g·h))in comparison to the $NBW_{control}$-7.5 (121.58 ± 486 mg/(g·h)). Compared with the DW group, the ETS activity in the NBW group was increased about 9-23%. Results from this study demonstrate that the supplementation of K^+ and NBW pre-domesticated inoculum could improve the activity of microorganisms in the methanogenic stage AD of high salinity FW, overcoming the problem of low metabolic activity of microorganisms in the high-salt environment. Gagliano et al. (2017) drew a conclusion that the addition of K^+ (0.7 g/L) alleviated the negative effect on biofilm formation and the Up-flow Anaerobic Sludge Blanket (UASB) performance under the high salinity condition (20 g Na^+/L). In addition, the previous research indicated that the inoculum was pre-augmented by using N_2-NBW and O_2-NBW could increase by 10% and 22% of methane yield from the corn straw

(Wang et al., 2020).

(2) *Dissolved organic matters performance during the second stage*

Dissolved organic matters as the important intermediate products including the soluble proteins, soluble carbohydrates, lactic acid, and individual VFA are revealed in Fig. 4-6. In the first 6 days, the concentration of soluble proteins was dropped slowly and was increased in the DW-9.0, however, the concentration of soluble carbohydrates and lactic acid were decreased rapidly, especially in the NBW group, which was accompanied by a large accumulation of VFAs (Figs. 4-6a, b, and c). As known, lactic acid is one of the main by-products in the anaerobic fermentation of FW, and its initial hydrolytic product is propionic acid (HPr), which is detrimental to produce the methane (Cheng et al., 2020; Li et al., 2014). Thus, in this study, the higher accumulation of lactic acid in the DW group than that in the NBW group during the first stage (Fig. 4-4b) led to higher initial lactic acid concentration in the second stage, *i.e.*, 6.78 ± 0.20, 6.35 ± 0.03, 6.75 ± 0.30, 6.85 ± 0.07 g/L in the $DW_{control}$-7.5, DW-7.5, DW-8.0, DW-9.0 and 5.96 ± 0.10, 6.19 ± 0.07, 5.93 ± 0.00, 5.93 ± 0.00 g/L in the $NBW_{control}$-7.5, NBW-7.5, NBW-8.0, NBW-9.0, respectively. This would accompany by higher HPr rich VFAs production in the DW group, which may be the reason of the lower methane yield in the DW group than that in the NBW group. At the 24 days, the HPr was dominant in the DW group, which were 1,229.29 ± 15.39, 865.49 ± 0.83, 584.93 ± 28.63, 892.21 ± 11.57 mg/L in the $DW_{control}$-7.5, DW-7.5, DW-8.0, and DW-9.0, and the HAc was dominant in the NBW group, which were 873.93 ± 22.88, 787.09 ± 75.45, 607.87 ± 34.13, 202.75 ± 5.90 mg/L in the $NBW_{control}$-7.5, NBW-7.5, NBW-8.0, and NBW-9.0 (Fig. 4-6 d).

Moreover, in the K^+ added reactors, the degradation of organic matters was higher than that of control group, indicating that the higher methane yield in the K^+ added reactors may benefit from the rapid consumption of organic matters, especially the consumption of VFAs. Li et al. (2018) found that the COD removal efficiency, dehydrogenase activity and the viability of microorganisms were increased about 115.4%, 77.2%, and 20.3% with the addition of KCl when the NaCl content was 2% in the AD of organic wastewater. (Muñoz Sierra et al., 2018) also reported that the higher K^+/Na^+ obtained the higher removal efficiency of COD and methane production rates from the high salinity phenolic wastewater, highlighting the importance of K^+ to maintain the methanogenic activity under high salinity environment.

(3) *pH and alkalinity during the second stage*

In the methane production stage, the initial pH was adjusted to 7.5, 8.0, and 9.0 by using NaOH or KOH, which would inevitably cause the change of alkalinity during the process. Notoriously, the alkalinity is one of the key factors to maintain the stability of the AD process and is better to indicate the imbalance of AD process than pH (Wang et al., 2018). Among them, IA/PA and VFA/TA were also nominated the preference indicators for monitoring the methanogenic process (Sun et al., 2019). Therefore, the changes of pH, TA, IA/PA, and VFA/TA are revealed in Fig. 7. The changes in pH were consistent with the results of VFAs accumulation and consumption, *i.e.*, the pH dropped to the lowest when VFAs was accumulated in large quantities, and then the pH increased with the consumption of VFAs (Fig. 4-7 a). Certainly, the pH value of the system returned to optimum pH range (7.5-8.0) of the methanogens more quickly when the initial pH was higher, and this phenomenon was more obvious in the DW group reactors. For instance, the pH value was changed from 6.38, 6.48, 6.63, 6.85, 6.51, 6.51, 6.73, and 6.82 to 6.9, 7.04, 7.12, 7.42, 7.45, 7.41, 7.51, and 7.52 in the $DW_{control}$-7.5, DW-7.5, DW-8.0, DW-9.0, $NBW_{control}$-7.5, NBW-7.5, NBW-8.0, and NBW-9.0 from the 6 days to 12 days. This indicates that the addition of KOH played a better pH regulating role in the DW group, which may also be one of the reasons for the increase of methane production. The result of TA evolution is displayed in Fig. 4-7b, and the increase in TA was more remarkable in the prophase of methane production in all reactors, which may be due to the degradation of protein-rich substrates responsible for alkalinity production (Adou et al., 2020). This agrees with the changes in the concentration of protein, and the increase in the TA was only 825.83 and 938.44 mg $CaCO_3$/L in the DW-9.0 and NBW-9.0 on the 6 days, which was lower than other reactors due to the lower degradation of protein. However, the TA in both reactors increased significantly after the 6 days, possibly due to the addition of a large number of alkaline matters (6 M KOH).

According to the description by lots of previous studies, PA and IA were used to distinguish the bicarbonate alkalinity and VFA alkalinity, and the IA/PA ratio has been certified to be better in monitoring the buffering capacity of AD process (Jantsch and Mattiasson, 2003; Sun et al., 2019). In the $DW_{control}$-7.5, the IA/PA ratio was reached to 4.85 on the 6 days (Fig. 4-8a), indicating extreme instability because sudden increase in the IA/PA ratio represented a decrease in pH buffering capacity (Banks et al., 2012). Interestingly, the IA/PA ratio in the NBW group and the reactors with the addition of KOH were lower than that in the corresponding DW group and the reactors without the addition of KOH, indicating that the buffering capacity of the NBW group was higher than the DW group, and the addition of KOH also enhanced the buffering capacity of the AD system. As the AD progresses, the IA/PA ratio continued to decline in all reactors and decreased to 0.25, 0.24, 0.24, 0.20, 0.17, 0.13,0.08, and 0.16. in the DWcontrol-7.5, DW-7.5, DW-8.0, DW-9.0, NBWcontrol-7.5, NBW-7.5, NBW-8.0, and NBW-9.0 at the end of the methane production stage, which foreboded that the reactors were gradually recovering to stability. And again, the VFA/TA ratio also showed a trend of increasing first and then decreasing in all reactors (Fig. 4-8b). Liu et al. (2019a) summarized the range of VFA/TA ratios for the stability of AD of different substrates for methane production, *i.e.*, the VFA/TA ratios were < 0.4 to suppose the stabilization in the co-digestion of WAS and FW or co-digestion of sewage sludge and fruit wastes; however, the VFA/TA ratios was 0.59 for the stable operation in the mono-digestion of FW. In this study, the VFA/TA ratios was lower than 0.5 in all reactors after the 12 days. The conclusion could be drawn from the above results that the combination of Air-NBW pre-domesticated inoculum and the supplementation of KOH could better maintain the pH buffer capacity in the process of methane production from the high salinity FW to make it run stably.

For the protein-rich substrates, the ammonia nitrogen would be released due to the degradation of proteins, amino acids, urea, and nucleic acids. Considering the addition of KOH in the methanogenic stage to supplement the alkalinity, the conversion of NH_4^+ to FAN in a slightly alkaline environment is known as one of the inhibitors of methanogens (Sposob et al., 2020). Thus, the concentration of TAN and FAN was summarized in Table 4-3. The TAN and FAN production in the NBW group was slightly higher than that in the corresponding DW group, and the increase of the FAN was more obvious in the DW-9.0 and NW-9.0 reactors because the FAN is mainly affected by TAN, pH, and temperature according to the Eq. (1). Multitudinous studies have proved the inhibition effect of TAN and FAN on AD, but it is difficult to determine the limit value of TAN or FAN concentration to inhibit methane production. Bi et al. (2020) reported that more than 3,000 mg N/L of TAN would reduce methane production and produce toxicity to methanogens. However, Dai et al. (2016) found, however, that the methanogenic activity was affected only when the TAN concentration was higher than 5,000 mg N/L. In any case, the concentration of TAN in this study (always < 1,700 mg N/L) was far less than the inhibition concentration reported above and thus indicated no inhibition on the methane production.

In addition, the concentration of the K^+ in the biogas slurry of the KOH added reactors (2,210.55 ± 53.46, 2,484.90 ± 24.40, 3,058.05 ± 41.58, 2,177.25 ± 49.21, 2,625.45 ± 37.55, 3,102.90 ± 30.12 mg/L in the DW-7.5, DW-8.0, DW-9.0, NBW-7.5, NBW-8.0, and NBW-9.0) was higher than that in the reactors without the addition of KOH (220.65 ± 34.37 and 273.15 ± 69.16 mg/L in the DWcontrol-7.5 and NBWcontrol-7.5) at the end of the second stage, indicating that the addition of KOH could remarkably improve the fertilizer value of the digestate. Certainly, the application and performance of this strategy in large-scale and long-term two-stage AD of actual high-salt FW will be the focus of future research.

4.4 Summary

This study investigated the two-stage AD performance of high salinity (20 g NaCl/L) FW by using Air-NBW pre-domesticated anaerobically digested sludge as inoculum and the effect of K^+ addition on methanogenic stage by adding KOH to adjust the initial pH. Results indicate that H_2 yield increased by 46% in the NBW group, and the addition of KOH also enhanced the CH_4 yield (increase by 17-25% and 19-26% in the DW group and NBW group compared with the control group) and ETS activity (increase by 10-16% and 15-31% in the DW group and NBW group compared with the control group) in the second stage. Additionally, the shorter start-up period and more stable performance were obtained in the NBW group reactors with the KOH added, especially in the NBW-8.0, and the fertilizer value of the digestate also improved.

Table 4-1 Physicochemical characteristics of the food waste and inoculum.

Parameter	Food waste	Inoculum
Total solids (TS, %)[a]	18.77 ± 0.40	0.88 ± 0.04
Volatile solid (VS, %)[a]	18.01 ± 0.03	0.58 ± 0.05
Total volatile fatty acids (VFAs, mg/L)	1,211.16 ± 5.77	43.21 ± 0.19
Lactic acid (g/L)	4.30 ± 0.17	/
Soluble proteins (mg/L)	13,810.88 ± 112.77	295.51 ± 8.90
Soluble carbohydrates (mg/L)	23,100.80 ± 86.55	22.66 ± 0.45

[a] Wet weight basis.

Table 4-2 Parameters estimated from the first-stage and second-stage AD tests by using the Modified Gompertz model.

Stage/Reactors	Parameters in the modified Gompertz model			
The first stage AD of FW				
	P_{H2} (mL/g-VS_{added})	$R_{H2, max}$ (mL/(g-VS_{added}·h))	λ (h)	R^2
DW	40.76 ± 0.09	17.52 ± 0.40	3.72 ± 0.06	0.9999
Air-NBW	59.71 ± 0.03	21.26 ± 0.09	2.90 ± 0.02	1.0000
The second stage AD of FW				
	P_{CH4} (mL/g-VS_{added})	$R_{CH4, max}$ (mL/(g-VS_{added}·d))	λ (d)	R^2
$DW_{control}$-7.5	226.88 ± 3.08	68.43 ± 3.07	12.25 ± 0.20	0.9980
DW-7.5	276.27 ± 2.67	72.42 ± 3.77	11.03 ± 0.09	0.9986
DW-8.0	281.41 ± 2.49	74.56 ± 2.75	9.38 ± 0.09	0.9985
DW-9.0	261.86 ± 2.61	70.99 ± 2.49	9.09 ± 0.18	0.9986
$NBW_{control}$-7.5	237.32 ± 3.17	70.24 ± 2.10	9.69 ± 0.14	0.9962
NBW-7.5	282.17 ± 3.06	73.26 ± 5.65	9.27 ± 0.11	0.9978
NBW-8.0	299.52 ± 2.50	75.15 ± 2.75	8.41 ± 0.22	0.9977
NBW-9.0	295.58 ± 3.19	81.08 ± 3.13	9.14 ± 0.19	0.9983

Notes: AD-anaerobic digestion; VS-volatile solids; FW-food waste; DW-deionized water; NBW-nanobubble water; P_{H2} or P_{CH4}-maximum hydrogen or methane production potential; $R_{H2, max}$ or $R_{CH4, max}$ -maximum hydrogen or methane production rate; λ-lag time.

Table 4-3 The concentrations of TAN and FAN in each reactor during the second stage AD.

Reactors		$DW_{control}$-7.5	DW-7.5	DW-8.0	DW-9.0	$NBW_{control}$-7.5	NBW-7.5	NBW-8.0	NBW-9.0
TAN (mg N/L)	6 d	1,454± 42	1,376 ± 16	1,365 ± 24	1,397 ± 2	1,450 ± 24	1,441 ± 28	1,448 ± 29	1,416 ± 19
	12 d	1,368 ± 28	1,394 ± 11	1,439 ± 8	1,566 ± 206	1,348 ± 68	1,410 ± 28	1,610 ± 28	1,553 ± 196
	18 d	1,194± 38	913 ± 3	1,204 ± 43	1,312 ± 8	1,278 ± 57	1,258 ± 200	1,215 ± 4	1,356 ± 23
	24 d	1,214 ± 18	1,076 ± 16	1,065 ± 24	1,183 ± 36	1,329± 53	1,396 ± 13	1,410 ± 3	1,416 ± 19
	30 d	1,449 ± 12	1,501 ± 30	1,497 ± 15	1,530 ± 26	1,576± 17	1,516 ± 20	1,528 ± 14	1,510 ± 15
FAN (mg N/L)	6 d	4.81 ± 0.25	5.73 ± 0.20	7.92 ± 0.23	13.39 ± 0.17	6.40 ± 0.33	6.42 ± 0.13	10.67 ± 0.03	12.82 ± 0.18
	12 d	14.88 ± 0.65	20.59 ± 0.08	25.77 ± 0.44	54.83 ± 6.02	50.64 ± 3.68	48.43 ± 0.97	68.21 ± 1.78	68.23 ± 10.10
	18 d	35.06 ± 0.34	30.00 ± 0.77	52.78 ± 3.05	78.90 ± 1.36	44.36 ± 1.49	46.18 ± 7.36	50.36 ± 0.70	75.58 ± 2.90
	24 d	53.18 ± 1.57	109.91 ± 2.81	115.85 ± 6.22	112.41 ± 5.76	67.95 ± 4.20	119.40 ± 1.12	124.48 ± 1.08	197.98 ± 5.14
	30 d	155.82 ± 1.26	182.35 ± 3.77	237.66 ± 4.55	259.87 ± 0.61	152.84 ± 1.49	168.13 ± 3.98	201.26 ± 2.19	215.35 ± 2.07

Notes: TAN-total ammonia nitrogen; FAN-free ammonia nitrogen; AD-anaerobic digestion; DW-deionized water; NBW-nanobubble water.

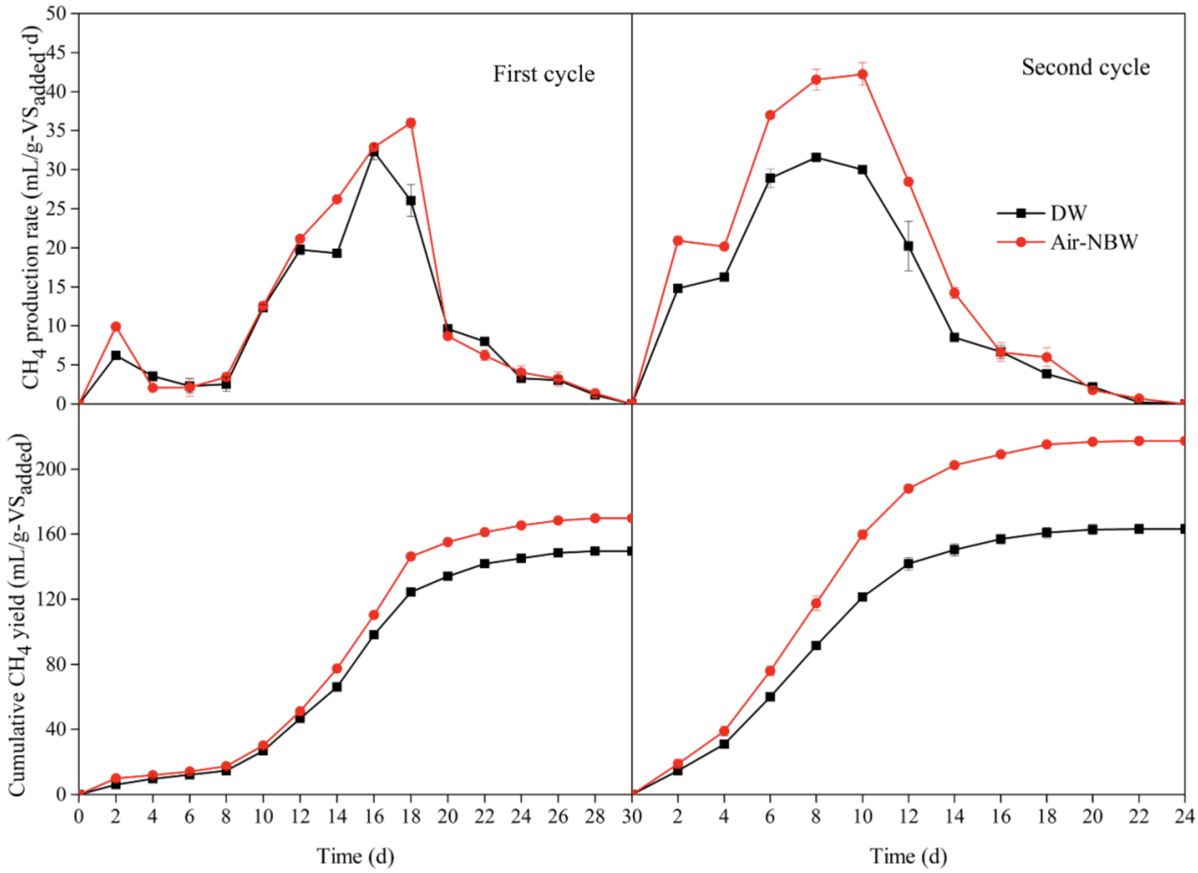

Fig. 4-1 CH_4 production rate and cumulative CH_4 yield during pre-domesticated two cycles. (VS-volatile solids; DW-deionized water; NBW-nanobubble water)

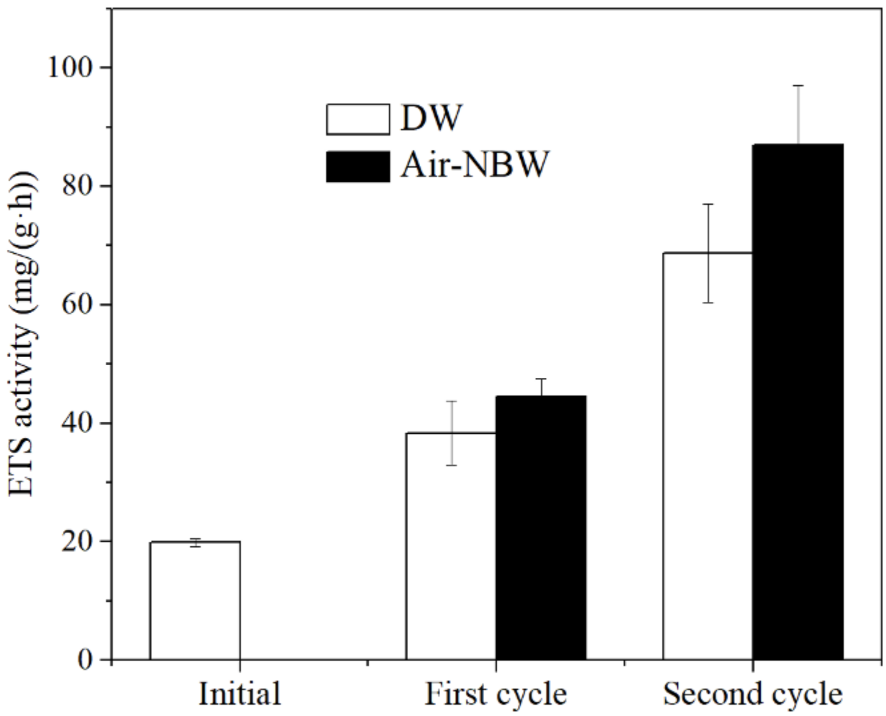

Fig. 4-2 Changes in ETS activity at the end of each cycle of anaerobically digested sludge pre-domesticated by DW or Air-NBW. (ETS-electron transport system; DW-deionized water; NBW-nanobubble water)

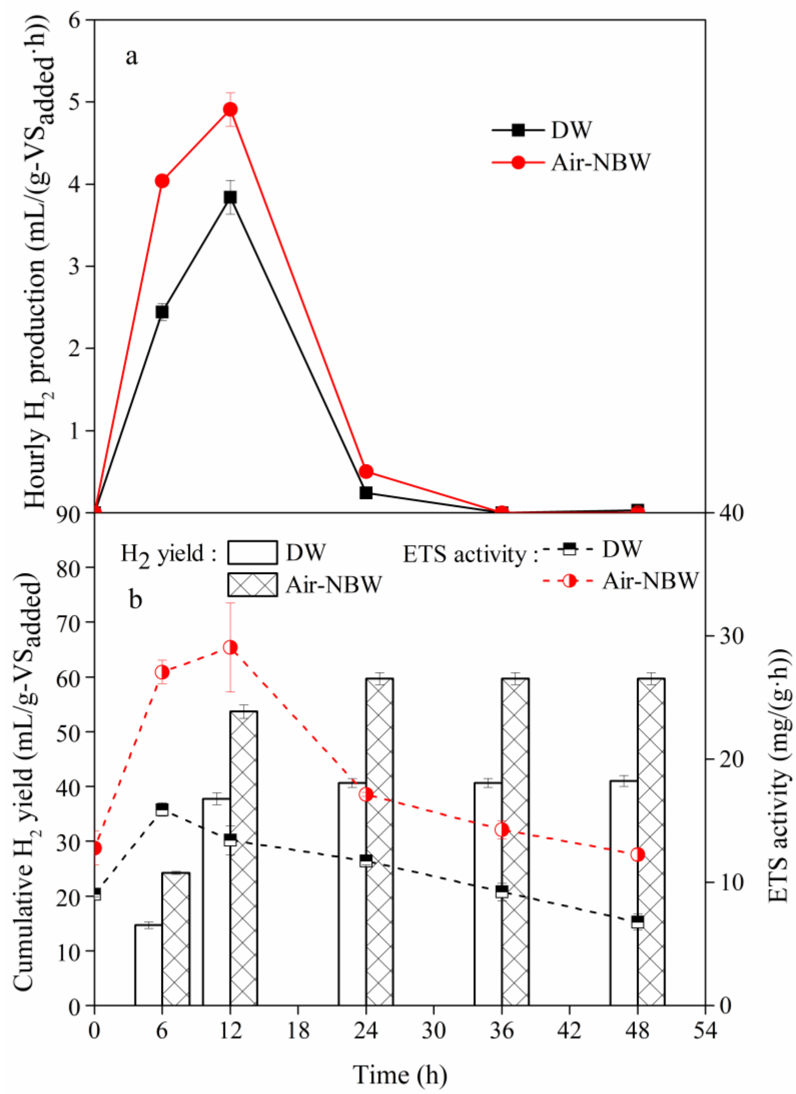

Fig. 4-3 Hourly H_2 production (a), cumulative H_2 yield and ETS activity (b) during the first stage AD of high salinity FW using anaerobically digested sludge pre-domesticated by DW or Air-NBW. (VS-volatile solids; ETS-electron transport system; DW-deionized water; NBW-nanobubble water)

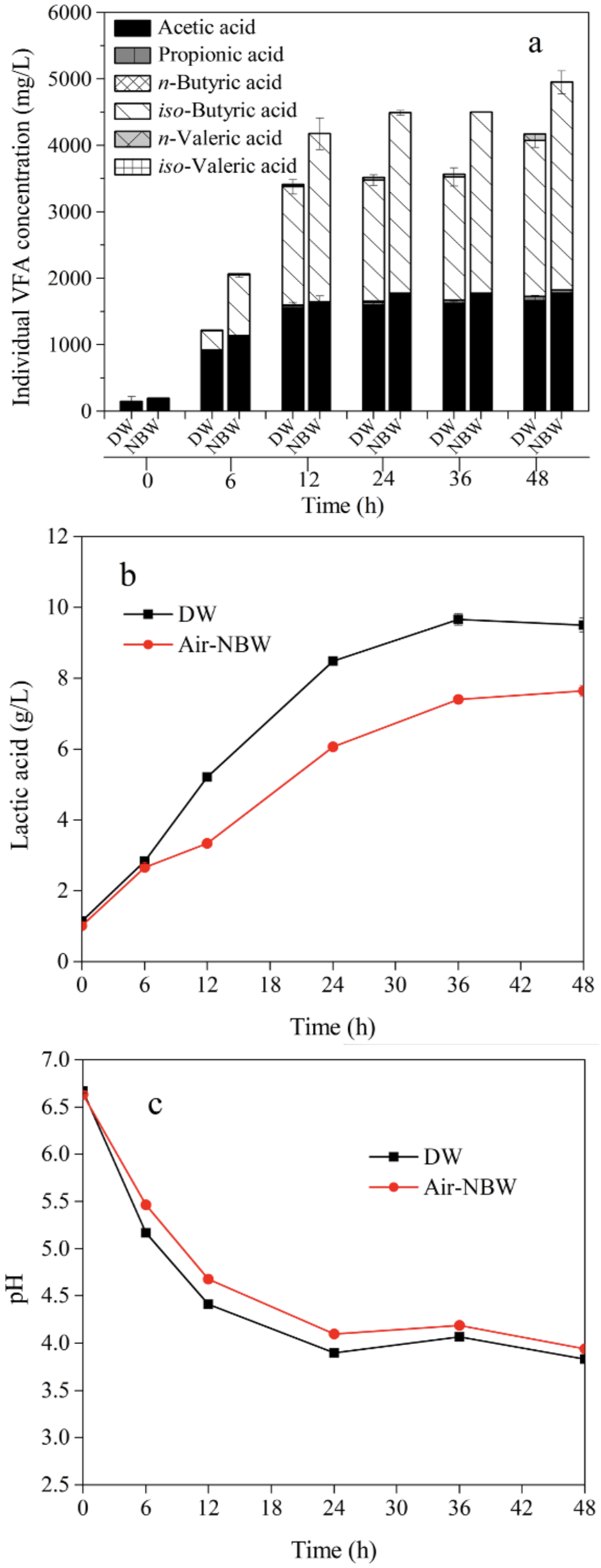

Fig. 4-4 Changes of the individual VFA concentration (a), lactic acid (b), and pH (c) during the first stage. (VFA-volatile fatty acid; DW-deionized water; NBW-nanobubble water)

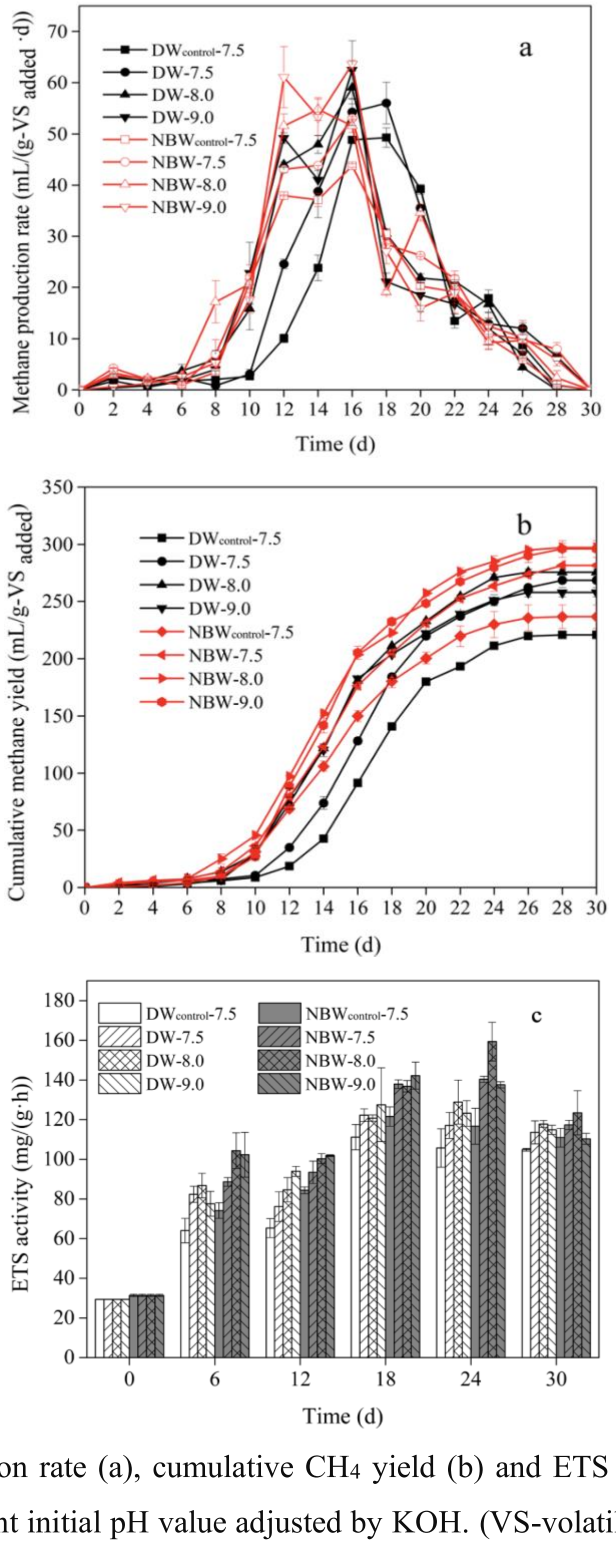

Fig. 4-5 CH_4 production rate (a), cumulative CH_4 yield (b) and ETS activity (c) during the second stage at different initial pH value adjusted by KOH. (VS-volatile solids; ETS-electron transport system; DW-deionized water; NBW-nanobubble water)

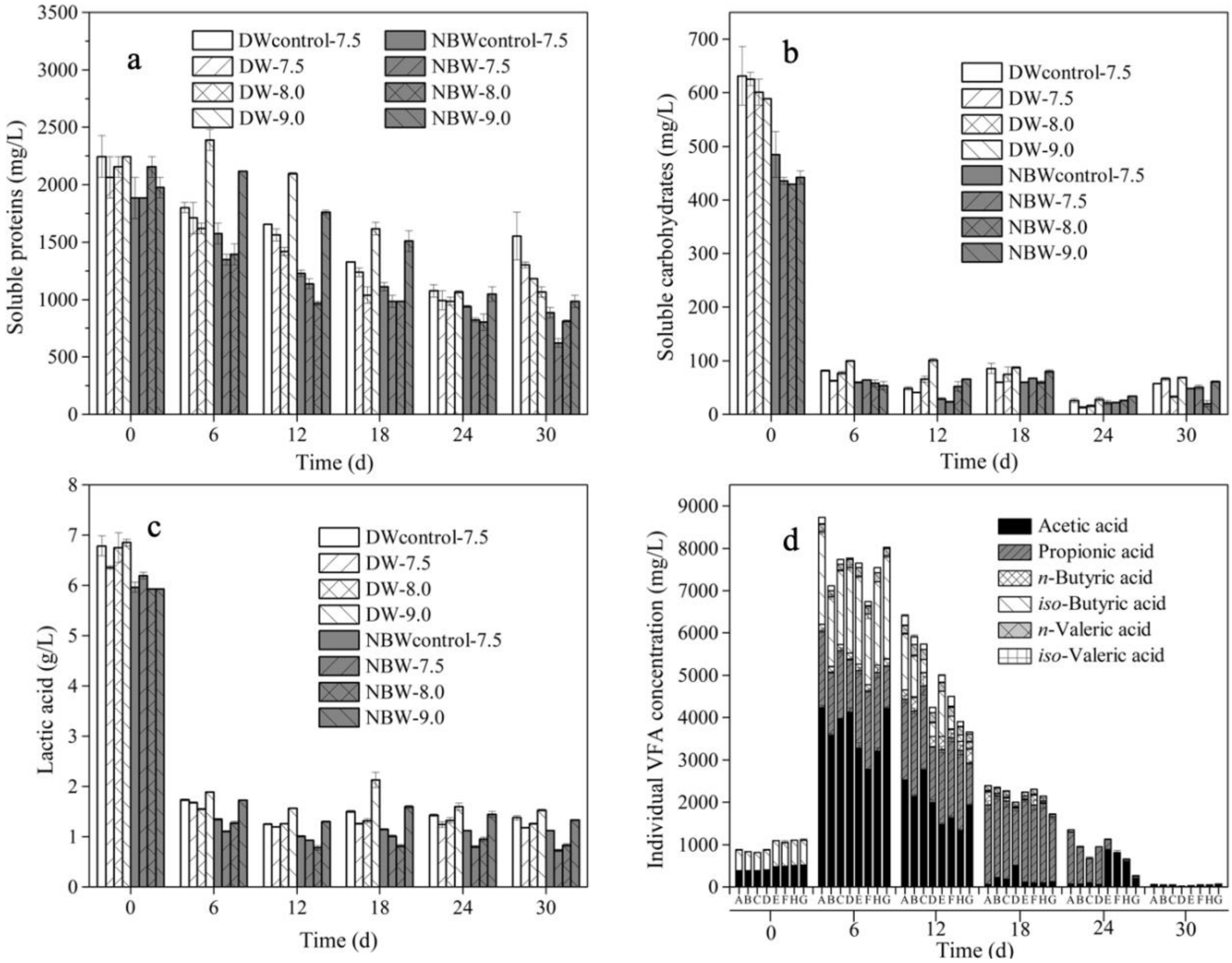

Fig. 4-6 Soluble proteins (a), soluble carbohydrates (b), lactic acid (c), and individual VFA concentration (d) (The letters near the X-axis are: A, DWcontrol-7.5; B, DW-7.5; C, DW-8.0 D, DW-9.0; E, NBWcontrol-7.5; F, NBW-7.5; G, NBW-8.0; H, NBW-9.0) during the second stage AD of high salinity FW. (DW-deionized water; NBW-nanobubble water; VFA-volatile fatty water; AD-anaerobic digestion; FW-food waste)

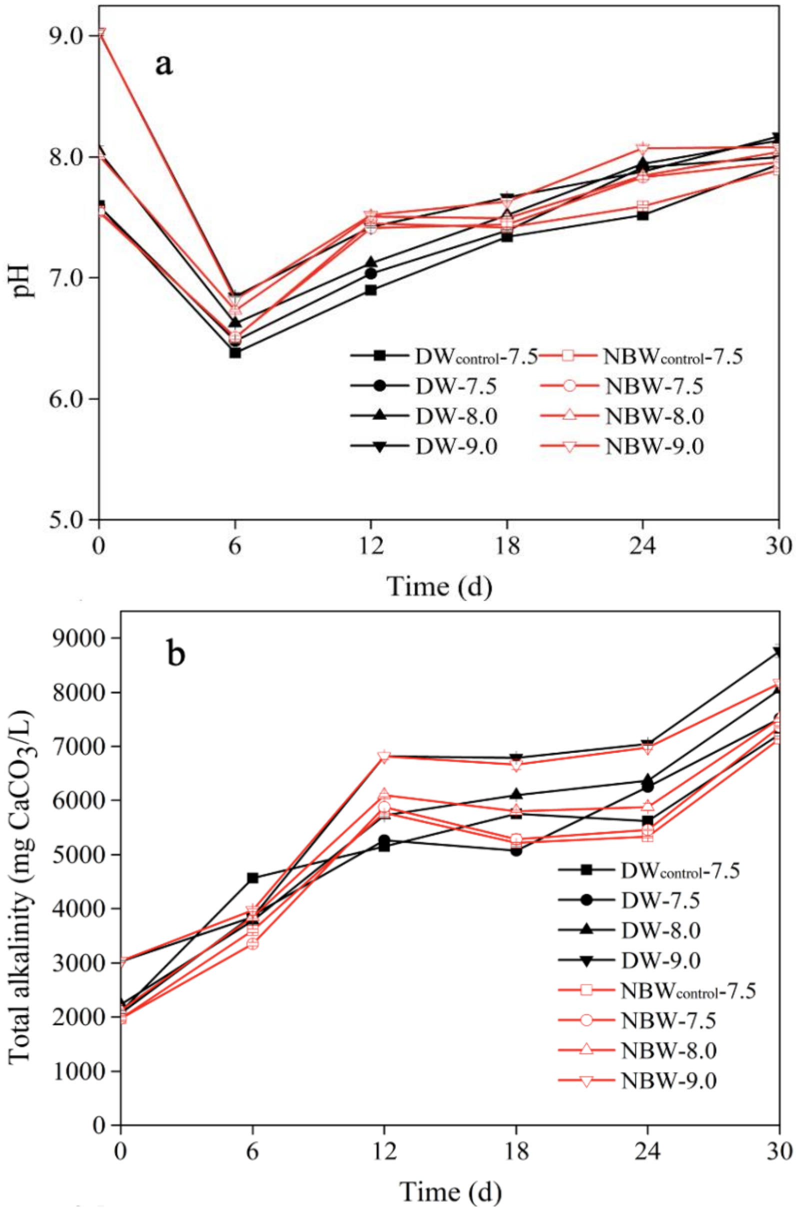

Fig. 4-7 pH (a) and total alkalinity (b) during the second stage AD of high salinity FW. (DW-deionized water; NBW-nanobubble water; AD-anaerobic digestion; FW-food waste)

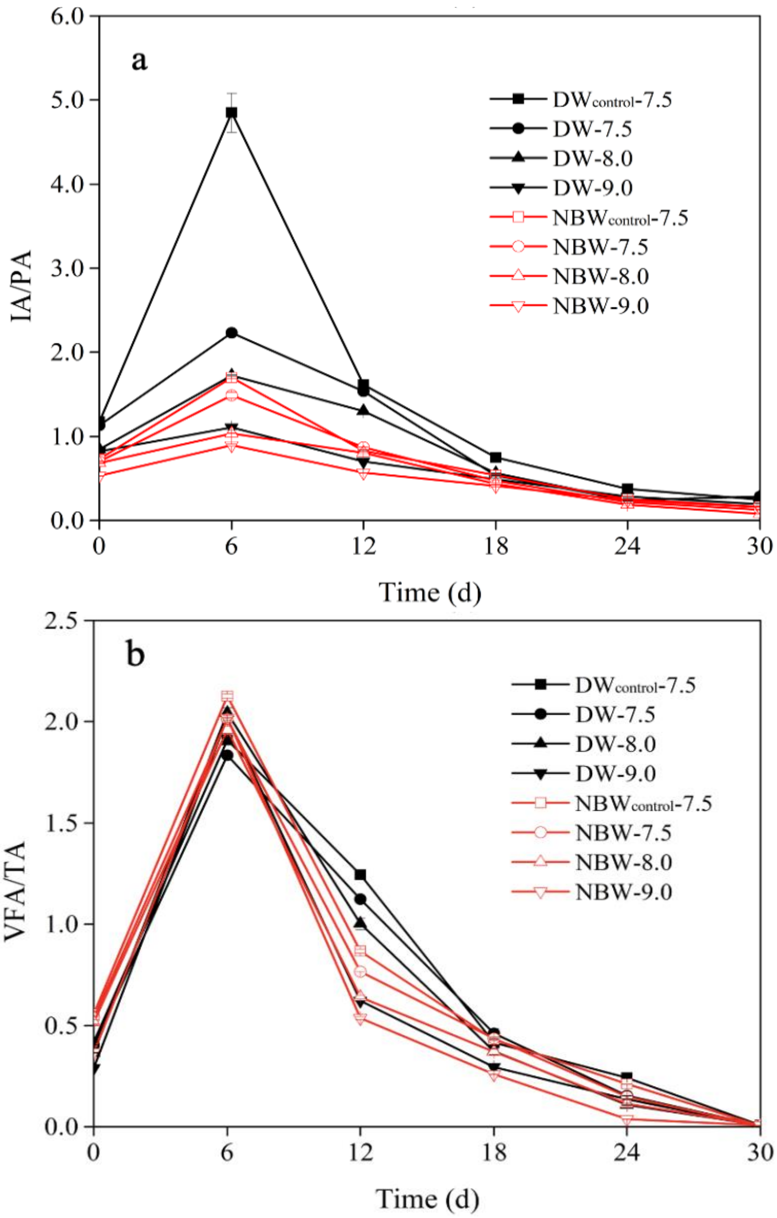

Fig. 4-8 IA/PA (a) and VFA/TA (b) during the second stage AD of high salinity FW. (IA-intermediate alkalinity; PA-partial alkalinity; VFA-volatile fatty acid; TA-total alkalinity; AD-anaerobic digestion; FW-food waste)

Chapter 5 Conclusions and future research works

5.1 Conclusions

In this thesis, NBW-based AD is regarded as a promising and environmentally friendly approach, targeting the maximization of renewable energy recovery from FW, which opens a new path for the improvement of hydrogen and methane recovery from high salinity FW with zero pollution. The main results are summarized as follows.

5.1.1 Comparison of the effects of N_2- and Air-NBW addition on the two-stage AD of FW

The highest cumulative H_2 yield and CH_4 yield were obtained from the FW+Air-NBW, increasing by 38% and 24% compared to the control (FW+ DW), respectively. Further investigations indicate that different gas NBW may positively impact the different stages of AD process. Addition of N_2-NBW only enhanced the hydrolysis/acidification of FW with no significant effect on methanogenesis. By comparison, addition of Air-NBW promoted both hydrolysis/acidification stage and methanogenesis stage, reflecting by the enhanced activities of four extracellular hydrolases at the end of hydrolysis/acidification and coenzyme F_{420} at the end of methanogenesis, respectively. Total energy recovery was 24% higher in FW+Air-NBW reactor, with 23% increase in electricity generation from the FW+Air-NBW reactor when compared to the FW+DW reactor. These results indicate that Air-NBW is more promising than N_2-NBW for the two-stage AD of FW to produce hydrogen and methane sequentially. Because the air is preferable and free everywhere, which is easier to obtain than nitrogen. Thus, the manufacturing of Air-NBW can reduce cost and operation difficulty compared with that of N_2-NBW. Additionally, addition of Air-NBW showed superior effectiveness than that of N_2-NBW in the two-stage AD of FW tests either in terms of hydrogen production or methane production.

5.1.2 Effects of Air-NBW addition on the two-stage AD of FW under the different salt concentration (0-30 g NaCl/L)

High salinity strongly inhibited the both stage in the two-stage AD of FW, especially in the methane production with the long adoption period when the salt concentration was ≥ 20 g NaCl/L. According to the conclusion in chapter 5.1.1, Air-NBW was selected to mitigate the inhibition effect of high salt on two-stage AD of FW. Results show that Addition of Air-NBW can effectively alleviate the inhibition of salt on H_2 and CH_4 production from the two-stage AD of high salinity FW. When compared with the corresponding DW group, H_2 and CH_4 yield was higher 21-65% and 14-43%, and ETS activity increased by 5-92% during the first stage and 9-30% during the second stage under the addition of Air-NBW at salt concentration of 0-30 g/L.

Two-stage AD of FW used in this study may add some mitigation effect of salt inhibition on methane production, as the microorganisms in the first stage have been exposed and got accommodated to the high salinity conditions. Additionally, an efficient approach for hydrogen and methane recovery from two-stage AD of FW under high salinity was proposed through improving microbial electron transfer and corresponding enzyme activities at each stage via Air-NBW addition.

5.1.3 Optimization of NBW application in AD and improvement on the tolerance of methanogens to high salt in combination with "salt in" strategy

To further optimize NBW application in AD of high salinity FW, using Air-NBW to pre-domesticated anaerobically digested sludge as inoculum for the two-stage AD of high salinity FW. During the entire DW or Air-NBW pre-domestication of AD sludge, the methane yields and ETS activities in all the reactors were enhanced in the second cycle tests. After the second pre-domestication cycle, ETS activity of AD sludge pre-domesticated by Air-NBW was increased about 27% when compared to the DW pre-domesticated inoculum reactor. In the first stage, H_2 yield was increased by 46% in the Air-NBW pre-domesticated inoculum reactor compared with the DW pre-domesticated inoculum reactor. Supplementation of K^+ in the methanogenic stage could resist the inhibition of salt on methanogens by adding KOH to adjust the initial pH, increasing by 17-25% and 19-26% in the DW group (DW-7.5, DW-8.0, and DW-

9.0) and NBW group (NBW-7.5, NBW-8.0, and NBW-9.0) compared with the control group ($DW_{control}$-7.5 and $NBW_{control}$-7.5). The shorter start-up period and more stable performance were obtained in the NBW group reactors with KOH addition, especially in the NBW-8.0, and the fertilizer value of the digestate also improved.

This strategy could optimize NBW-based AD of high salinity FW. On the one hand, the volume of the reactor could be reduced, and on the other hand, the start-up of each stage was accelerated under the inhibition of high salinity. In addition, from an economic perspective, this study substituting KOH for NaOH in the methanogenic stage to adjust the initial pH would not increase the cost because it is normally necessary to adjust the pH in the second stage of the two-stage AD of FW. Compared with the addition of KCl to improve the inhibition of salt on methanogens not only achieved higher methane production and a more stable AD process, but also avoided excessive K^+ and Cl^- addition.

5.2 Future research works

Although the application of NBW technology for improving the energy recovery from the two-stage AD of FW system in adverse environment was realized in this thesis, the application of NBW technology in practice need be continuously optimized.

(1) NBW technology's application and performance in large-scale and long-term two-stage AD of FW will be the focus of future research and fully evaluate the economy of NBW-based AD system.

(2) The subsequent treatment of digested residues from the wet two-stage AD of FW and the fertilizers value should be investigated in the future.

(3) Consideration should be given to improving the inhibition of hydrogen and methane production due to the large accumulation of lactic acid during FW storage and transportation stage or AD system's hydrolysis/acidification stage.

(4) Different regions produce FW with different compositions and characteristics due to the differences in food culture. In the followed-up research, the effects of NBW on AD of FW with different compositions should be tested. For instance, FW contains a high amount of oil, which coats the surface of microorganisms and prevents microorganisms from degrading organic matter into energy. Pretreatment of high oil FW with NBW to improve oil degradation efficiency is also the focus of future research.

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Publications

1. **Hou T.**, Zhao J., Lei Z., Shimizu K., Zhang Z., 2021. Addition of air-nanobubble water to mitigate inhibition of high salinity on co-production of hydrogen and methane from two- stage anaerobic digestion of food waste. Journal of Cleaner Production. 314, 127942. (IF=9.297)

2. **Hou T.**, Zhao J., Lei Z., Shimizu K., Zhang Z., 2021. Enhanced energy recovery via separate hydrogen and methane production from two-stage anaerobic digestion of food waste with nanobubble water supplementation. Science of the Total Environment. 761, 143234. (IF=7.963)

3. **Hou T.**, Zhao J., Lei Z., Shimizu K., Zhang Z.,2020. Synergistic effects of rice straw and rice bran on enhanced methane production and process stability of anaerobic digestion of food waste. Bioresource Technology. 314, 123775. (IF=9.642)

4. **Hou T.**, Chen N., Tong S., Li B., He Q., Feng C., 2019. Enhancement of rice bran as carbon and microbial sources on the nitrate removal from groundwater. Biochemical Engineering Journal. 148, 185-194. (IF=3.978)

5. Zhao J., **Hou T.**, Wang Q., Zhang Z., Lei Z., Shimizu K., Guo W., Ngo H.H., 2021. Application of biogas recirculation in anaerobic granular sludge system for multifunctional sewage sludge management with high efficacy energy recovery. Applied Energy. 298, 117212. (IF=9.746)

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7. Zhao J., **Hou T.**, Zhang Z., Shimizu K., Lei Z., Lee D., 2020. Anaerobic co-digestion of hydrolysate from anaerobically digested sludge with raw waste activated sludge: Feasibility assessment of a new sewage sludge management strategy in the context of a local wastewater treatment plant. Bioresource Technology. 314, 123748. (IF=9.642)

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