

**Characteristics of Ultra-Fine Bubble Water and Its Effects
on Anaerobic Digestion of Waste Activated Sludge**

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Characteristics of Ultra-Fine Bubble Water and Its Effects on Anaerobic Digestion of Waste Activated Sludge

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Abstract

Energy shortage and solid waste pollution are hot issues nowadays, prompting researchers to investigate applicable technologies for renewable energy resources recovery and ecological environment protection. Anaerobic digestion (AD) is a widely applied technology for waste activated sludge (WAS) reduction, stabilization and renewable energy (methane) recovery. Recently, ultra-fine bubble water (UFBW) with its special physicochemical characteristics of stability and longevity has drawn intensive attention in many research fields like agricultural cultivation, medicine research and wastewater treatment. Few researchers, however, studied the application of UFBW to microorganisms, especially its effects on AD for methane production. In addition, little information is available about the influencing factor on AD performance, and the mechanisms involved in its possibly significant effects in UFBW-AD systems remain unknown. The main purpose of this research is to explore the effect of UFBW on AD and to reveal the mass transfer mechanisms involved in addition to the analysis of the distinctive characteristics of UFBW.

This study first explored the particle size distribution and concentrations as well as zeta potential of UFBW produced by respectively introducing air, N₂, CO₂ and H₂ gases under different preparation time and storage duration. Results show that the stability of ultra-fine bubbles (UFBs) follows a descending order as H₂ > Air > N₂ > CO₂ in these tested gases. The UFBs concentration increases with prolonging preparation time until 20 min and then the bubble size and its distribution remain relatively stable when preparation time is longer than 20 min. In the case of Air-UFBW produced by the designated UFBW generation machine, the number of Air-UFBs is around 2.25×10^8 particles/ml with 144.2 nm as the dominant size, which decreases to 1.87×10^8 and 1.00×10^8 particles/ml after one and two weeks' storage at room temperature, respectively. All the tested gas-UFBW reflect a similar phenomenon of the bubble number change. Results also show that UFBs could exist in the produced UFBW even after storage for several weeks. And the zeta potentials of the four kinds of UFBs would be affected by pH value. At a wide range of pH (from 3.0 to 12.0), all the zeta potential values of UFBW are negative, which increase with the increase of pH value from 3.0 to 10.0, and then decrease from pH 10.0 to 12.0. Considering colloids with high zeta potential (negative or positive) are electrically stabilized, so the most stable zeta potential of UFBW was achieved at pH 10.0. In the pH range (pH 7.0 - 8.0) that is most suitable for AD, stability follows a descending order as H₂ > N₂ > Air > CO₂ and all the zeta potentials at a range of -13 ~ -30 mV. The zeta potential value is positively correlated with the number and concentration of UFBs but there is no relation between the zeta potential and bubble diameter.

Then, a series of batch AD experiments were conducted at initial pH 7 and 37°C to investigate the effect of adding the above-mentioned UFBW on hydrolysis-acidification process and the whole process of AD of WAS. Total solids (TS), volatile solids (VS), pH, soluble total organic carbon (sTOC), volatile fatty acids (VFAs), alkalinity (ALK), proteins and polysaccharides, dehydrogenase, coenzyme F₄₂₀ (CoF₄₂₀), biogas production, etc. were tested during 3 days, 7 days, and 30 days AD experiments. Results show that in

the hydrolysis-acidification process, an average enhancement (32.4%) of biogas production from UFBW groups was observed in the hydrolysis-acidification process, which are remarkably higher than the DW group, especially during 7 days process. The reductions of proteins and polysaccharides in all the experimental groups were higher than those in the control group. The VS average reduction with UFBW addition increased to 7.05% and 11.53% on day-3 and day-7, which was higher than that in the control group, respectively. In the whole AD process, all the UFBW addition exhibited an enhancement effect on biogas and methane production, especially CO₂-, H₂- and Air-UFBW, achieving 20% higher of biogas and methane production than those from the control tests. The reductions of TOC and VFA in the UFBW groups were also faster than the control group. As for the enzymes examination, the contents of dehydrogenase and CoF₄₂₀ were slightly higher in the UFBW group than those in the control during the same processing time. It is speculated that the mobility of water molecules caused by the addition of UFBW that could promote mass transfer efficiency, which in turn indirectly enhanced the activities of microorganisms during the AD process of WAS. Furthermore, the VS reduction rate in first 3 or 6 days (6.7 ~ 11.88%, 16.03 ~ 22.44%) were faster and sharper than that in DW group (4.59 ~ 14.30%), but no obvious difference at the end of AD process.

Besides, acetate-enriched seed cultures were used to investigate the uptake and mass transfer of trace metals for methane producing bacteria. In this study, acclimated-methanogens were cultured in batch feed substrate acetate with the addition of nutrient solution at 37°C. The uptake of trace metals by acclimated-methanogens cell was tested by using ICP-MS after being washed with phosphate buffer and 10% HCl solutions and wet digestion. Results show that, as the only carbon source, acetic acid was fully utilized. In the 1st period, the great difference of biogas production was observed between the UFBW group and control with no obvious difference in the 2nd and 3rd period. All UFBW groups showed an enhancement effect in CH₄ production, especially Air-UFBW, achieving a 26.76% increase in comparison to the control group. In the case of other nutrients sufficiently provided, the addition of acetic acid effectively enhanced the biogas production. But the lack of other nutritional elements would become the limiting factor for CH₄ production even with sufficient carbon sources. Results from inorganic compounds analysis show that the contents of trace metals contained in the inoculum increased, which followed a descending order as Fe>Zn>Co>Ni>Cu in the cells. The uptake of trace metals in methanogens was higher in UFBW group, especially for the metal of Fe, which is about 48.97% higher than that in the control group. This may be because the addition of UFBs may have an indirect stimulatory effect and promote mass transfer into the organism cells.

This study shows the addition of UFBW was beneficial to the hydrolysis-acidification process and the whole AD process and it provides opportunities for the application of UFBW in the AD system based on the unique physicochemical characteristics of UFBW and mechanisms of promoting CH₄ production.

Key words: Ultra-fine bubble water; Particle size distribution; Anaerobic digestion; Methane production; Waste activated sludge; Gompertz model

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Acronyms and abbreviations

AD	Anaerobic digestion
ALK	Alkalinity
CH ₄	Methane
C/N	Carbon to nitrogen ratio
COD	Chemical oxygen demand
CO ₂	Carbon dioxide
DO	Dissolved oxygen
EPS	Extracellular polymeric substances
GC	Gas chromatography
H ₂	Hydrogen
LB-EPS	Loosely bound extracellular polymeric substances
sCOD	Soluble chemical oxygen demand
sTOC	Soluble total organic carbon
TA	Total alkalinity
TB-EPS	Tightly bound extracellular polymeric substances
TCOD	Total chemical oxygen demand
TF	Triphenylformazan salt
TS	Total solids
TTC	Triphenyltetrazolium chloride
TVFAs	Total volatile fatty acids
UFBs	Ultra-fine bubbles
UFBW	Ultra-fine bubble water
VFAs	Volatile fatty acids
VFA/TA	Volatile fatty acid to total alkalinity ratio
VS	Volatile solids
WAS	Waste activated sludge

Chapter 1 Introduction

The continuous development of the industrial society and the surge in population have led to an increase in energy consumption, a reduction in available resources, and a significant increase in solid waste pollution and wastewater discharge. Various crises are coming. Therefore, the world has turned its attention to the development of alternative energy, clean energy and energy recycling methods.

In this chapter, the various crises currently facing, the development and utilization of alternative and renewable energy, the new technology and its application fields generated in recent years, as well as the objective and purpose of this thesis will be discussed.

1.1 The crisis issues of energy and environment

1.1.1 The current issues

Energy and environment quality are both critical basic issues for the survival and development of human beings that we need to face. Nowadays, with the growing population of the world and vigorous development of industrialization, increasing demand for energy, especially fossil fuels, is causing a more significant and critical challenge [1]. The shortage of energy supply that our society constantly worried about is that the continued utilization of fossil fuels as a dominant energy source is not consistent with the long-term sustainability of our environment. Besides, pollutants emission during the combustion of fuels as well as unsanitary disposal of large quantities of energy-rich organic materials causing critical environmental problems, such as ozone layer destruction, greenhouse effect, air quality damage and especially the haze, constitutes serious threats to our living environment.

1.1.2 Alternative and renewable energy

In order to build a low-load global environment, the development of new ecology-protection technology has become a top priority research topic. For the early response to the constraints of the national economy caused by fossil energy depletion and low-carbon energy, renewable fuels as well as sustainable energy development and utilization have

become the main focus for many researchers. Alternative clean sources of energy are available [2]. Among those renewable energy sources, including biomass, solar, wind, hydroelectric, geothermal, etc., biomass energy is not only the most widely used traditional energy but also the largest use of renewable energy on earth and ranks fourth in the current energy consumption, only behind the coal, oil and natural gas the top three conventional energy [3].

1.2 Biomass energy and anaerobic digestion

1.2.1 Biomass and its conversion technologies

The term of biomass is defined as an organic substance that is formed directly or indirectly by plant photosynthesis, including animals, plants, microorganisms and organic materials which excreted and metabolized by all these organisms. Generally speaking, biomass mainly includes lignocellulosic materials, animal feces, urban solid organic waste living and industrial organic wastewater and energy biological.

Waste activated sludge (WAS), as one of the most common biomass produced from the wastewater treatment plant in the municipal and industrial sewage treatment industry, is rich in organic matter and nutrients that can be recycled and reused, whereas it brings about a lot of serious environmental problems due to its huge production amount, toxic and harmful substances contained and difficult to dispose of [4]. Due to the continuous reproduction of microorganisms, problem of the huge residual amount of WAS needs to be solved urgently. The high disposal cost of WAS and sanitation concerns have been attracting growing attention [5]. More importantly, wastewater contains a lot of organic matter and chemical energy, which are useful resources that enriched in WAS. There is thus a strong incentive to recover this resource/energy while reducing the cost and difficulty of disposal [5].

The current technologies of biomass conversion include direct combustion, thermal or thermochemical processes (vaporization, pyrolysis and liquefaction) and biological or biochemical method (the microbial conversion of biomass to obtain gaseous and liquid fuels by fermentation and anaerobic digestion (AD)) [6].

Among those biomass conversion methods, bioenergy recovery and pollution control through AD is a promising greenhouse gas mitigation option [7] and considered to be an efficient and sustainable way to treat waste sludge and wastewater [8]. It is

basically a microbiological degradation process, which offers the benefits of mass reduction, pathogen removal and most importantly, generation of methane (CH₄) [9].

1.2.2 AD and its mechanisms

CH₄, a biogas product, is a clean and renewable form of energy. CH₄ production from AD, sometimes called methane fermentation, has been confirmed as a suitable process for sludge treatment and bioenergy recovery [10]. AD is considered to be a microbiological degradation process used for nutrient and energy recovery from biomass and also to stabilize the sludge produced in wastewater treatment [2]. The process involves four major stages shown in Fig.1-1 [11]: hydrolysis, acidogenesis, acetogenesis and methanogenesis [12, 13].

Hydrolysis stage involves the transformation of complex macromolecule organics compounds such as proteins, polysaccharides, etc. into soluble and lower molecular organics like glucose, fatty acids, amino acids, etc. [11-14]. Hydrolysis refers to those biological reactions that occur before the process of the organic matter enters into microbial cells. Biocatalytic reactions can be driven by the microorganisms of hydrolytic like *bacteoides*, *selenomonas*, *streptococci* etc. via releasing the extracellular free enzyme or connecting the immobilized enzyme attached to the outer wall of the cell. The second stage named acidogenesis is a typical fermentation process. During this phase, dissolved organic compounds such as simple fatty acids, sugars and amino acids are converted into alcohols, aldehydes, organic acids, hydrogen (H₂) and carbon dioxide (CO₂) by the fermentation of facultative or obligatory bacteria [11-13, 15]. Sometimes, the two above stages can be combined into one stage, which called the hydrolysis and acidification stage, and the purpose of hydrolytic acidification is to provide substrates for methane fermentation during mixed AD process.

Among those products in acidogenesis, the produced alcohols and long chain organic acids cannot be converted directly by the methanogens except for formic acid, acetic acid and methanol. Hence, those intermediate metabolites such as propionic acid, butyric acid, etc. must be decomposed by acetogenic bacteria into acetic acid, H₂ and CO₂ in the third stage of acetogenesis. The acetic acid, which has been reported to be the main precursors for CH₄ production, and from 65% to 95% CH₄ is directly produced from acetic acid [15]. The final stage, methanogenesis, is a critical step in the entire AD process. In the

methanogenic phase, those acetic acid, H₂ and CO₂ are converted into biogas, a mixture of CH₄ and CO₂, by the methanogenic bacteria under strictly anaerobic conditions. The acetotrophic methanogens like methanosarcina, methanothrix, etc. transform the acetate produced in acetogenesis into CH₄ and CO₂ while the hydrogenotrophic methanogens like methanobacterium, methanococcus, etc. convert H₂ and CO₂ into CH₄ [11, 15, 16].

1.3 Current methods used to improve AD efficiency

Many kinds of substrates that rich in organic matters were used in the AD process. To improve the efficiency and CH₄ production, various methods have been adopted in the AD process. Table 1-1 shows different methods were used to enhance the effectiveness of the AD either in the hydrolysis and acidification steps or in the methanogenesis step.

1.3.1 Optimization of operating parameters

Always, considering that CH₄ production is restricted by some parameters, many researches focus on the various limitations of parameters, such as total solids (TS) content, temperature, the ratio of carbon to nitrogen (C/N), pH, organic loading rate (OLR), hydraulic retention time (HRT) and inoculum to substrate ratio to investigate how to increase bio-methane production through parameters adjustment because these process can affect the healthy fermentative bacterial growth directly or indirectly.

For example, Kumanowska et al. [17] explored the influence of the target pH-values in the acidification process, and got results of at pH 5 and 5.5, butyric acid production dominated, guided by H₂ production and at pH 6 acetic acid was the main product. The absence of H₂ and the highest substrate-specific CH₄ yield makes it favorable under practical aspects.

On the studies of swine manure in AD process, Lin et al. [18] adjusted the temperature at the range of 25 – 55 °C and found that daily CH₄ production increased with temperature up to 50 °C, but then decreased. And the results of the functional gene expression showed great difference at different conditions. But in laboratory-scale experiments of Deng et al. research [19], the CH₄ yield and volumetric CH₄ production rate at 25 °C were 6.24 times higher than those at 15 °C. However, the CH₄ yield and the volumetric CH₄ production rate at 35 °C were only 4.86% higher than those at 25 °C. The lower biogas production in dry AD compared with that in wet AD could be attributed to

the ammonia inhibition.

Hassan et al. [1] investigated a C/N optimization case on the CH₄ production from co-digestion of chicken manure and wheat straw with WAS. Results showed that the optimum treatment of a C/N value of 20/1 with the CH₄ enhancement of 85.11%, and VS removal of 66.83%. For other compositions of C/N value of 25/1, 30/1 and 35/1, the CH₄ enhancement showed 75.85%, 63.04% and 59.96%, respectively.

Chen et al. [20] did the test of comparison of high ratio of solids to liquid co-digestion of food waste and green waste to explore the effect of TS% content (5–25%) on CH₄ production. Results showed that CH₄ yields from AD of 15 – 20% of TS were higher than the AD of 5 – 10% of TS, while methanogenesis was inhibited by an increasing the TS content to 25%.

The inhibition may be caused by excess ammonia or more OLR. Agyeman et al. [21] tested the effect of OLR on CH₄ yield and found that CH₄ production was highest at the OLR of 2 g VS/L/d, being 0.63, 0.56, and 0.47 L CH₄/g VS with fine, medium, and coarse food waste, respectively. The rate of CH₄ production was highest at the OLR of 3 g VS/L/d.

Anbalagan et al. [22] investigated the influence of HRT on microalgae and WAS process. They found that an average TN removal efficiency of maximum $64.6 \pm 16.2\%$ and 81.5 ± 5.1 was achieved at 4 and 6 days HRT, and the AD process showed a higher biogas production of 349 ± 10 mL g VS⁻¹ with 2 days HRT.

Brown et al. [23] explored co-digestion and the ratio of inoculum to substrate on the feasibility of AD for contaminated rice straw inoculated with WAS. Results showed that the CH₄ production of food waste enhancement were 10% and 20% at F/E ratios of 2 and 1, respectively. The co-AD of food waste with yard waste at specific ratios can improve AD operating characteristics and better performance than the AD of yard waste alone.

Parameters adjustment can improve the efficiency of AD process to some extent and it can be known through the above examples. However, for various complex organic wastes, such as agriculture and forestry residues, and it is widely known that decomposition of lignocellulose is not easy because their structure hinders the process. Therefore, to accelerate carbohydrates like short chain fatty acid production obtained after degradation of those complex organics could further improve methane yield. This requires to through some pre-processing approaches.

1.3.2 Pretreatment methods

Pretreatment is reported to enhance the hydrolysis and degradation of macromolecules especially those complex organics in the first or first two stages of AD, which can change the chemical composition and physical structure of biomass waste substrate that can be more accessible to microorganism and hydrolytic enzymes [11]. Some reports pointed out that methods of pretreatment, such as acid or alkali treatment, mechanical, biological, hydrothermal, thermal, microwave and electro-hydrolysis pretreatment of substrates could improve the AD efficiency.

For example, Zhao et al. [24] explored the CH₄ yield from rice straw pretreated by a mixture of acetic-propionic acid and got a 35.8% higher of CH₄ production than control. Chen et al. [25] found a method for CH₄ production increase from municipal organic wastes via regulation of AD process is to use alkaline to pretreat to improve H₂ and volatile fatty acids (VFAs) enriched fermentation liquid in the first step in the AD process. Some environmentally friendly methods like fungal [26] or novel microbes [27] pretreatment were adopted to enhance CH₄ production due to the digestibility of macromolecular organic matter like lignocellulosic biomass improved by those biological methods. Some researches focus on the high-temperature treatment methods like thermal [28-30] and hydrothermal [31, 32] pretreatment to evaluate the AD performance of organic waste and obtained valuable research results of effectively solubilize organic-waste and increasing CH₄ yield. Recent years, microwave irradiation has become attractive with additional advantages such as rapid/selective heating, reduction in reaction times, ease of control, compactness and minimization of hazardous product formations [33, 34]. It also can be recognized as a thermal pretreatment for effective disruption of complex organic matter and subsequent AD enhancement. Veluchamy [33, 35] adopted a novel method of electro-hydrolysis pretreatment for CH₄ production enhancement from lignocellulose waste pulp and paper mill sludge. Results showed that CH₄ production enhancement was 13.8% and 301 ± 3 mL CH₄/ g VS. It is supported the potential for CH₄ recovery enhancement from waste organics using electro-hydrolysis as a pretreatment method.

1.3.3 Additives addition

In addition to those methods of operating parameters optimization, enhancement of

AD performance via the methods of pretreatment before or in the hydrolytic process of AD, additives added to the AD system are also effective ways to increase the biogas production [15, 23].

In recent years, the addition of zero valent iron (ZVI) to AD system has been reported by several researchers. Feng et al. [36] achieved increased biogas productivity by 43.5% at ZVI of 20 g/L. Jia et al. [37] pointed out that ZVI could enhance the degradation of chemical oxygen demand (COD) and promote volatile fatty acids (VFAs) generation, and the results from Puyol et al. [38] indicated that ZVI addition could increase CH₄ production and accelerate the formation of siderite and vivianite. Besides, the addition of ZnO nanomaterials [39], metallic wastes [40], ferroferric oxide [41], CaO₂ [42] and bentonite [43, 44] has also been reported to promote AD process because of the enhanced microbial hydrolysis, acidification and metabolism under the ambient conditions. In recent years, some researchers have tested the effect of the addition of emerging carbon-based functional materials on AD, including graphene, biochar, activated carbon and carbon cloth due to the key roles of carbons in improving cell growth, enrichment and activity, and accelerating their co-metabolisms [45]. In the research work of Dang et al. [46], carbon cloth was used as an electron transfer mediator to promote the direct interspecies electron transfer, therefore achieving faster recovery of soured reactors and stimulated CH₄ production than the control. The addition of biochar, on the other hand, might provide temporary substrates to support microbial metabolism and growth that could shorten the lag phase by 21.4% and increase CH₄ production potential by 9.6% [47]. In addition, ozone was found to initially and temporarily reduce biomass viability and activity, while after the lag phase, CH₄ yield from the ozone-treated sludge could be enhanced by 52% [48].

Also, up to the present, for some metal-contained materials, probably due to the large amount of chemicals consumption and secondary pollution it caused and thus low environmental-friendliness, its application is not realistic at present. However, for those materials that are cheap and easy to obtain and are not burdened by the environment, like some carbon-based materials, its application prospects are brighter and broader.

1.4 Ultra-fine bubble technology

1.4.1 Bubbles and the classification

In recent years, ultra-fine bubble (UFB) technologies, also known as microbubble, nanobubble or micro-nano bubble technologies, have drawn great attention due to its special physicochemical characteristics and have been reported to be effectively applied in many fields of science and technology although the mechanism is not yet well understood. Ultra-fine bubble water (UFBW) refers to the water with bubbles of particle size at micro-nano scale and nano-scale.

Bubbles are defined as hollow globules of gas. Bulk UFBs is used to describe nanoscopic gas-filled cavities with internal equilibrium pressures equal to the external environment that have a diameter of less than 1 μm in bulk liquids [49]. Figure 1-2 shows bubble classification and its trajectory of motion in water [50].

By vigorous mixing of water and gas, solutions contain a large number of bubbles with a wide range of diameters can be well produced. Macrobubbles ($>50 \mu\text{m}$) can rise up quickly and burst at the surface of aqueous solution due to their high buoyancy by a theory of Hadamard–Rybczynski (H–R) equation [51]. For microbubbles, or called it micro-nano-bubbles (1~50 μm), the situation can be separated. For the larger bubbles, buoyancy will lead to them rise to the surface of the liquid quickly than smaller bubbles via the Stokes' equation as follows (Eq. 1-1):

$$R = \rho g d^2 / 18\mu \quad (1-1)$$

where R is the rising speed of spherical bubbles (m/s), ρ is the density of the liquid (kg/m^3), g is the gravity acceleration (m/s^2), d is the bubble diameter (m) and μ is the dynamic viscosity of liquid (Pa·s). According to this relationship a 40 μm diameter bubble rises 100 times faster than a 4 μm diameter bubble [52].

The degree of saturation next to a bubble depends on the gas pressure within the bubble. According to the widely accepted Young-Laplace expression, the pressure in the interior of gas bubbles is inversely proportional to their diameter. The pressure of bubbles is given by the Young-Laplace expression [53] as follows (Eq. 1-2):

$$\Delta P = 2\gamma/r \quad (1-2)$$

where $\Delta P = P_{\text{vap}} - P_{\text{liq}}$ is the difference between the pressure inside (vapor phase) and outside (liquid phase) of the bubble (Pa), γ is the surface tension (N/m) and r is the cavity

radius (m). Under atmospheric conditions, the surface tension of pure water against air is 72 mN/m [54], and at these circumstances, small bubbles have higher internal pressure and release gas to dissolve into the surrounding solution whereas larger bubbles grow by taking up gas from the solution. During this process, small bubbles shrink until they disappear while larger bubbles grow and burst. But for UFBs, because of their very small particle size ($< 1\mu\text{m}$) and their low buoyancy, they rise much more slowly than their random Brownian motion that not can be determinable. So the actual behavior of UFBs is more complex than macro or micro bubbles, and the kinetic stability of UFBs is not yet clear.

1.4.2 UFB characterization

In general, the significant history of UFBs can be traced back to the fifties of the last century, the Epstein-Plesset theory, which is used to study the bubble dissolution and growth [55]. Afterwards, to fully exploit the active effects and potential utilization of bulk UFBs, numerous basic researches were carried out to study their existence, behavior and characteristic. In this direction, over the last decades, the emerging field of UFBs in bulk water has drawn great attention and been a subject of intensive research in the area of science and technology due to their special physicochemical properties.

Water that is enriched with UFBs has completely different physicochemical characteristics compared with water which does not contain UFBs [54]. The most notable characteristic of UFBs is their stable extended lifetime in suspension, being able to last for weeks. Although according to classical thermodynamic theory, UFBs should dissolve extremely quickly [56], many researchers have reported the longtime existence of UFBs in aqueous solution on the basis of the experimental results [18, 54, 57, 58].

One of the most important theories indicated that the stability of UFBs can be explained by the charging mechanism, which can be reflected by the spin-spin relaxation time measurement and the zeta potential value of UFBW. One explanation is that the hydration energies of hydrogen and hydroxyl ions are different and then hydrogen ions are more likely to remain in the bulk liquid phase than hydroxyl ions [59, 60]. Another explanation is that an electric double layer is formed because of the orientation of water dipoles at the interface, with hydrogen and oxygen atoms respectively pointing toward the water and gas phase [61]. It is believed that the developed double layer directly leads

to the attraction of anions to the interface and plays a critical role in the formation and stability of UFBs in liquid [57].

Apart from the charging mechanism, Ohgaki et al. [54] reported that the stability of UFBW may be related to hydrogen bonding structures around UFBs formed by water molecules. They gave a point of view that the surface of the UFB contains hard hydrogen bonds, which may reduce the diffusivity of gas through the interfacial film, and the greater surface tension of the hard interface may also help maintain a kinetic balance against high internal pressure.

The experiments of Oshita et al. [62] further claim that UFBs in water could increase the mobility of water molecules in bulk, thus it could influence the physical properties of water. From the results of the ζ -potential of UFBW, it can be inferred that the negatively charged bubbles can adsorb hydrogen ions onto their surface, and the presence of those ions can modify the hydrogen bonding network in water. Although the hydrogen bonding around the UFB surface might enhance, for the rest of the large quantity of water, UFBs contributed to the weakening of the hydrogen bonding network of water so the mobility of water molecules would be accelerated.

1.5 Application of UFB technology

1.5.1 Current application of UFB technology

Despite the limited amount of theoretical research on bulk UFBs, the intensive attention and the corresponding published results in the different application fields become increasingly. In recent years, as shown in Table 1-2, as an emerging technology, UFBW has been reported to be effectively applied in many fields such as water treatment [63, 64], industry [65-67], food and agriculture [62] and biomedicine [68, 69], most probably owing to their special physicochemical characteristics including stable extended lifetime in water, reduced friction, increased solubility of gases in liquid and charged surface [64].

For example, Ebina et al. have studied the effect of O₂ nanobubble water, less than 200 nm in diameter, on the growth of plants, fishes and mice [70]. Both length and weight of test objects were treated with O₂ nanobubble water increased more than those were treated with normal water, which can be assumed that O₂ nanobubble water may affect the growth of life by changing oxygen condition. Liu et al. cultivated barley seeds for

germination by using N₂ nanobubble water and found that the germination rates of barely seeds dipped in nanobubble water were 15-25% greater than those in distilled water. This verified the clear effect of nanobubble water on the physiological activity, which could be explained by the increased mobility of the water molecules in the bulk nanobubble water as shown by the increase in proton NMR relaxation time T₂ [62], also the moderately level exogenous ROS produced by nanobubble water plays an important role in seed germination [71]. Some other researchers have pointed out cytoplasmic streaming rates inside the cells could be accelerated after treated by nanobubble water. The nanobubbles may affect transmembrane proteins or the membrane structure and provide a transport mechanism for nutrients delivery through the membrane into the cell [72, 73]. Brian et al. who produced a nanosized bubble population for medical applications as they can be used to target the capillaries outside the pulmonary bed [68]. Hydrogen nanobubbles are also present in health drinks [69] and have been commercialized, especially in Japan. Mineral separations are generally carried out by foam flotation. Nanobubbles are successfully being assessed for application in this field [74] due to the higher specific surface area and the surface energy. Nano bubbles also can be applied in water treatment [75] or as a material for surface cleaning [76] because of their significant surface area and high interfacial tension that can adsorb contaminants. Also, nanobubble solutions can provide a new cleaning mechanism free from detergents or chemicals [77].

1.5.2 Prospective the application of UFB technology in the future

As mentioned above, in the past ten years, UFB technology has experienced a leap from initial exploration to practical application and has flourished in many basic fields of industry, agriculture and manufacturing. According to this momentum of development, we have reason to believe that in the next few decades, UFB technology will not only continue to grow in these basic fields but also continue to explore and develop its application in those high-tech fields, such as medicine, imaging technology, pharmaceutical manufacturing, animals and plants cell culture fields. And our research will begin to study the effect and application potential of UFBW in a new unexplored field, the microbial and anaerobic fermentation field.

1.6 Objective of research and thesis structure

Although UFBs have been reported to be effectively applied in many areas of science and technology, especially in the field of planting and breeding, still, very little information is available on the microorganism and its influence on AD performances as well, and the mechanisms involved in its possibly significant effects in UFB-AD systems remain unknown. The effect of UFBs on microbes, as well as the mechanism is also not yet clear. So it is not only a challenge but also a chance for us to study the effect of UFBW on AD for methane fermentation to broaden its application fields. To the best of our knowledge, this is the first trial on the effect of UFBW on the AD process of WAS.

The objective of this research is (1) to explore the characteristics of UFBW produced by introducing different kinds of gases into the UFBW generator and (2) to explore its potential for promoting CH₄ production, aiming to provide a simple and effective method for organics degradation and bioenergy recovery.

The thesis structure is displayed in Figure 1-3. This dissertation is divided into 5 chapters. In Chapter 1, the research background and significance were proposed based on the literature review. It is introduced disposal and conversion methods for biomass energy and the current technologies for improving AD efficiency whereas the existed problems of secondary pollution and low environmental-friendliness for application. Then the development of the novel UFBW technology as well as its successfully application in various fields was introduced. At last, the objectives and framework of the thesis were presented. In Chapter 2, the characteristics of UFBW produced by using different kinds of gases were explored. Particle size distribution and concentrations as well as zeta potential of UFBW produced by respectively introducing Air, N₂, CO₂ and H₂ gases under different preparation time and storage duration, were investigated. Then in Chapter 3, a series of batch AD experiments were conducted at initial pH 7 and 37°C to investigate the effect of adding the above-mentioned UFBW on AD of WAS in both hydrolysis/acidification stage and methanogenesis stage. The test of adding DW was used as the control group. Total solids (TS), volatile solids (VS), pH, soluble total organic carbon (sTOC), volatile fatty acids (VFAs), alkalinity (ALK), proteins and polysaccharides, enzymes of dehydrogenase and coenzyme F₄₂₀ (CoF₄₂₀), H₂, CH₄ and biogas production were tested during 30 days' AD experiments. Besides, in Chapter 4, acetate-enriched seed cultures were used to investigate the uptake and mass transfer of

trace metals in CH₄-producing bacteria. Finally in Chapter 5, the major conclusions of the thesis were summarized, and also the future research directions were pointed out.

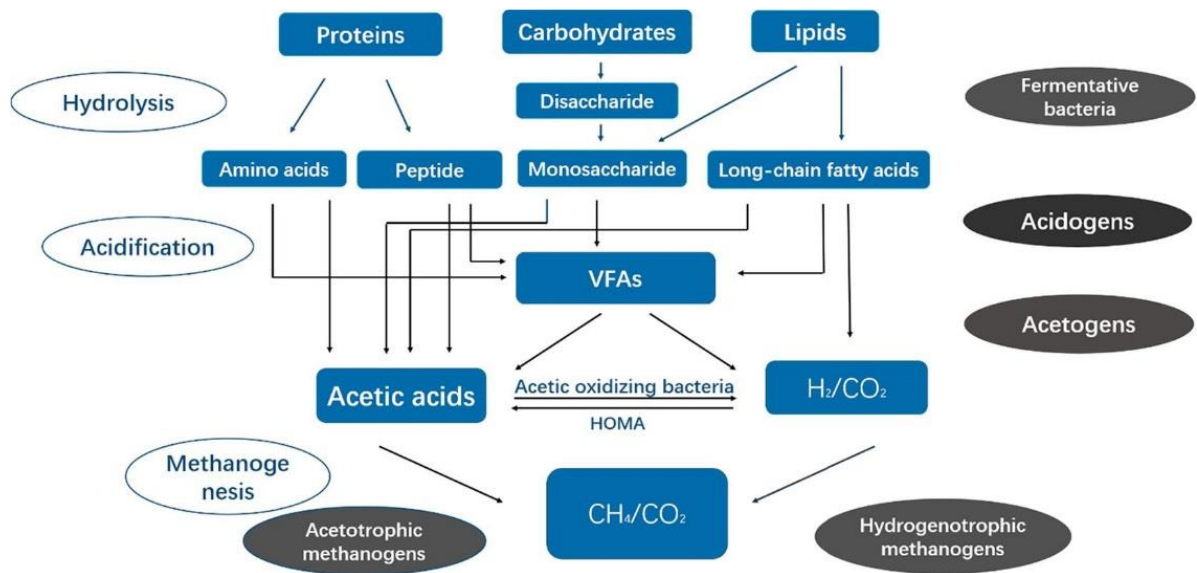


Figure 1-1. Flow chart of anaerobic digestion [11].

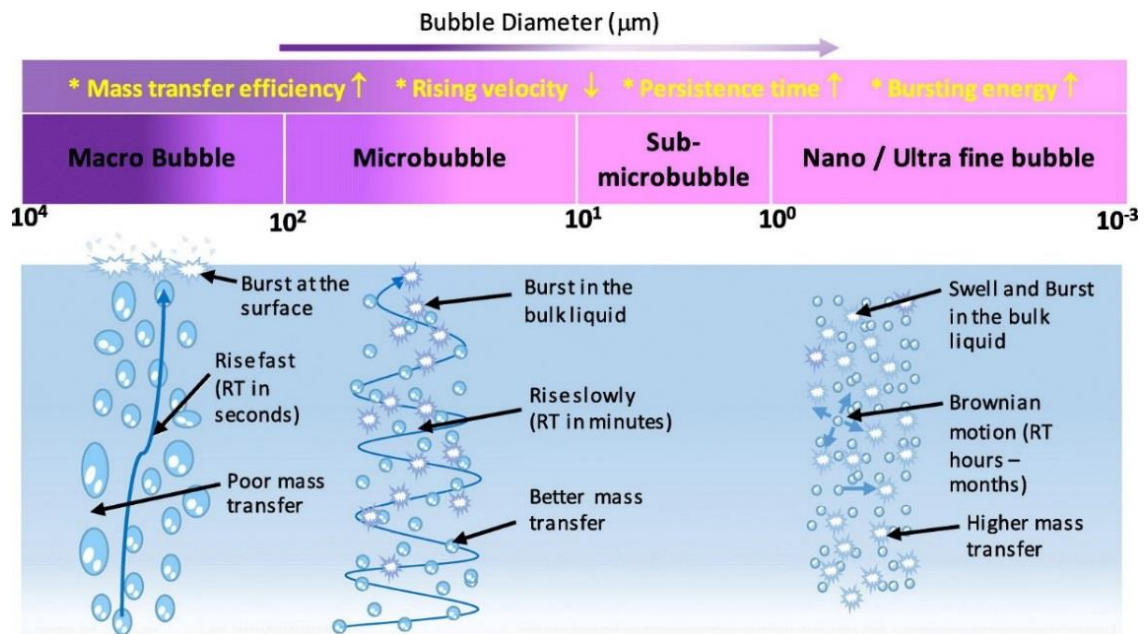


Figure 1-2. Bubble classification and its trajectory of motion in water [50].

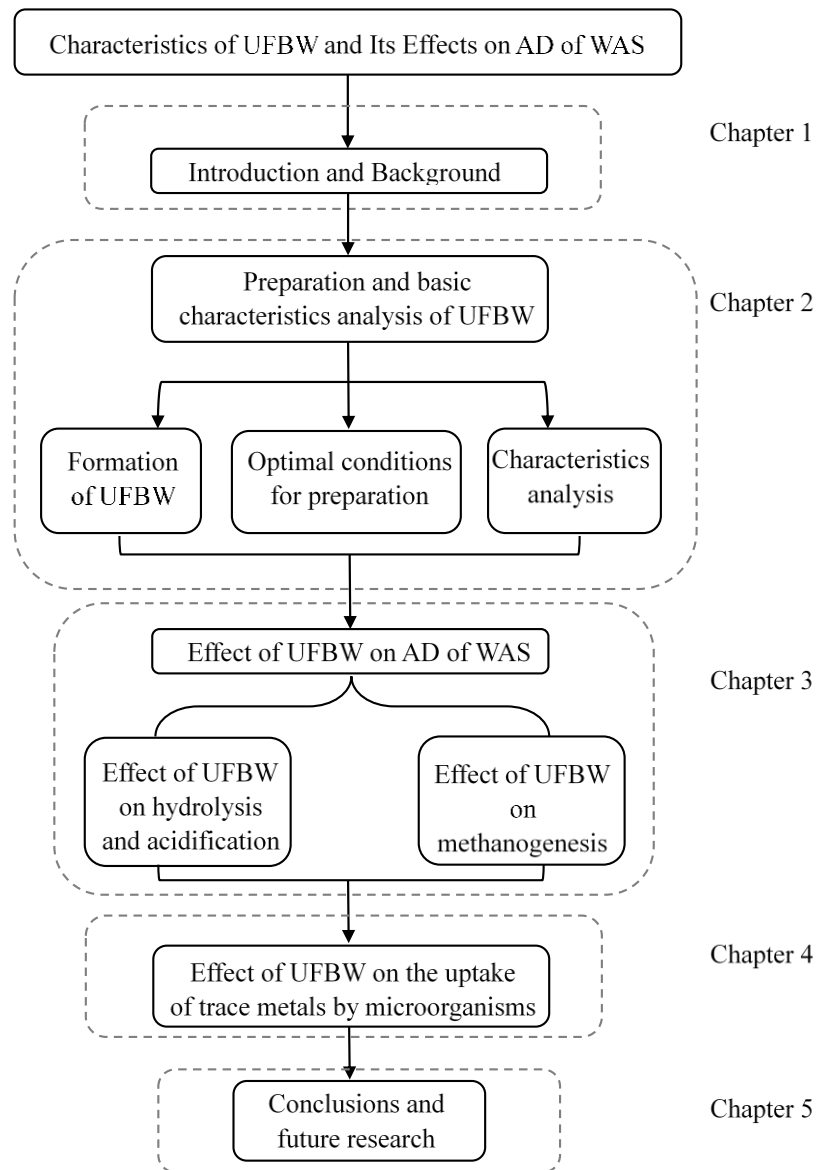


Figure 1-3. Experimental framework of this study. UFBW: ultra-fine bubble water, AD: anaerobic digestion, WAS: waste activated sludge.

Table 1-1. Different methods for AD efficiency increase and CH₄ production enhancement.

Substrate	Treatment	Characteristics of substrate	CH ₄ yield	CH ₄ production enhancement	Ref.
Sugar beet silage	pH adjustment (4, 5, 5.5, 6)	COD solid 266.75 g/L COD leachate 209.31 g/L	256.47 ±28.78 NL /kg COD/d	82.4 %	[17]
Swine manure	temperature adjustment (15, 25, 35 °C)	TS: 27.4%, VS: 77.8% of TS, TKN: 2.28% of TS, NH ₄ ⁺ -N: 0.4 g/L.	0.226 L/g VS	6.24 times	[18]
Wheat straw and chicken manure	C/N adjustment (35/1, 30/1, 25/1, 20/1)	WS: TS 98.20%, VS 95.91%, C/N 61.35. CM:TS 29.56%, VS 67.04%, C/N 8.51	385.28 mL/g VS	85.11%	[1]
Food waste and green waste	total solids content (TS%) adjustment	FW: TS 26.9%, VS 25.2%, C/N 22. GW: TS 86.8%, VS 74.3%, C/N 41.2	390.2 mL/g VS	45.1%	[20]
Food waste and dairy manure	mechanical pretreatment, organic loading rate adjustment	FW: TS 16.9%, VS 29.3%, DM: TS 16.9%, VS: 9.68%.	0.63 L/g VS	37.0%	[21]
Yard waste and food waste	co-digestion, the ratio of inoculum to substrate (I/S) adjustment	YW: TS 94.3%, VS 91.7%, C/N 55.3, FW: TS 15.2%, VS 13.8%, C/N 11.4	125L/kg VS	127%	[23]
Rice straw	a mixture of acetic-propionic acid pretreatment	Particles between 0.36 - 0.45 mm	604.5 mL	35.8%	[24]
Yard waste	fungal pre-treatment, I/S adjustment	TS 94.3%, VS 98.9%	44.6 mL/g VS	141.1%	[26]
Food waste	novel microbes pretreatment	VS 71.7%, COD 19.6 g/L	102 mL/g VS	14.6%	[27]
Primary and secondary sludge	thermal pre-treatment	TS 48.47 g/L, VS 32.33 g/L	130.0 mL/g VS	54.5%	[28]
Fruit and vegetable waste	hydrothermal pretreatment	TS 9.15%, VS 7.72%, Proteins 12.9%TS,	326.0 mL/g VS	16.1%	[32]
Waste activated sludge	microwave irradiation, NaOH	TS 29,615 mg/L, VS 17,120 mg/L,	296.9 mL/g VS	102.7%	[34]
Lignocellulose waste pulp	electro-hydrolysis pretreatment	TS 38.97 g/L, VS 28.87 g/L	303.0 mL/g VS	13.8%	[35]
Waste activated sludge	zero valent iron (ZVI)	TSS 13.4 mg/L, VSS 8.57mg/L	276.4 mL/VSS	43.5%	[37]
Primary and secondary sludge	metallic wastes	VTS 33800 mg/L, VSS 31900 mg/L	120 mL/g VSS	100%	[40]
Tryptone-based synthetic wastewater	ferroferric oxide	SS 6.29 g/L, VSS 3.96 g/L	114.1 mL/g VS	78.3%	[41]
Chicken manure	thermally modified bentonite	TS 27.92%, VS 18.93%	291 mL/g VS	41%	[43]
Dairy manure	low-cost composited	TS 15.95%, VS 12.35%, C/N 8.23	361.9 mL/g VS,	59.4%	[44]
Artificial complex waste	carbon felt	COD 1336 ±61 mg/g	110.8 mmol/d	10.8%	[46]
Aqueous carbohydrates food waste	biochar	TS 61.2%, VS 59.5%, C/N 18.7	160 mL	9.6%	[47]
Anaerobic digested sludge	ozone	Particles size < 5 mm	123 mL/g COD	52%	[48]

Table 1-2. Progress in the practical application of bulk UFBW in different fields.

Employ	Type	Field	Purpose	Result	Ref.
Nanobubbles	the gas mixture contained nitrogen and air	food and agricultural	to test the germination rates of barley seeds	germination rates 15-25% greater than those in distilled water	[62]
Ozone MNBs	ozone micro-nano-bubbles	groundwater treatment	for groundwater remediation.	show remarkable cleanup efficiency in situ remediation	[63]
Microbubble	seven kinds of gases distributors	wastewater treatment	to test oxygen absorption performance into water and organic waste water treatment	the microbubble generators showed better oxygen transfer rates than the typical gas distributors	[64]
Micro and nanobubbles	special gas not mentioned	industry	separation of emulsified crude oil in saline water by dissolved air flotation	increase overall efficiency of the flotation process from 92 to 95%,	[65]
Nanobubbles	the gas of air	industry	ceramic membrane defouling (cleaning)	the surface of the ceramic membrane was superbly cleaned by air NBs,	[66]
Microbubbles	containing perfluorocarbon, sulfur hexafluoride or air	biomedicine	nano-scale contrast agent	the in vitro dose response curves gave a maximum of 23–27 dB enhancement	[68]
Nanobubbles	hydrogen	biomedicine	for dietary supplement	Produce long-lived hydrogen nanobubbles. for dietary supplement	[69]
Nanobubble Water	the gas of oxygen and air	food and agricultural	promote the growth of plants, fishes, and mice	both length and weight of test objects increased	[70]
Nanobubbles	special gas not mentioned	physical biological	test explicit and implicit modeling in hydrophobic confinement	affect transmembrane proteins or the membrane structure	[73]
Nanobubbles	air	industry	study applications in flotation	collectors coated nanobubbles will broaden options in mineral flotation.	[74]
Nanobubbles	electrochemical process of electrolyzed solutions	industry	cleaning of contaminated surfaces	nanobubbles are able to prevent the fouling of both hydrophilic and hydrophobic surfaces.	[76]
Nanobubble	electrochemical process of NaCl solution	industrial	cleaning	NBs can aid a new cleaning mechanism free from detergents or chemicals	[77]

Chapter 2 Basic characteristics analysis of ultra-fine bubble water

2.1 Introduction

From Chapter 1, a lot of researches were conducted to do the study about the application of UFBW and investigate its special characteristics and its effect in various fields. What about the UFBW we employed in this study. We need to understand the way it is produced and the basic characteristics it has.

In this chapter, as shown in Figure 2-1, we explored the characteristics of UFBW produced by using different kinds of gases. First different preparation time were adopted and determined the optimal production condition for UFBW generation, and then the basic characteristics such as particle size distribution and concentrations, bubble lifetime as well as dissolved oxygen (DO), pH and zeta potential of UFBW produced by respectively introducing Air, N₂, CO₂ and H₂ gases that we will employ in the followed experiments under different preparation time and storage duration were investigated.

2.2 Materials and methods

2.2.1 Production of UFBW using an UFBW generator

The combination of pressurized mechanical cyclic, rotational flow and cavitation, an efficient and low-cost approach and widely used in generating UFBs in Japan, was adopted in this study. The photograph and schematic structure of UFBs generator (HACK FB11, JAPAN) is shown in Figure 2-2 and Figure 2-3. For the formation of UFBW, the liquid was injected into the cylinder together with the specific gas. The liquid in/out flow and gas inflow were manually adjusted to keep the pressure at around 0.25 MPa. A strong shear force produced by increasing pressure was applied on the gas-liquid mixed fluid to make it spin at a high speed. The cavitation could be produced through shearing while pressurizing inside the generation and mixing header, and then bubbles would be generated through a bubbling nozzle. In the current experiment, 1.5 L of DW in a transparent glass container was recycled into the generator circulation system by respectively introducing Air, N₂, CO₂ and H₂ gases. This generator can produce 8-11 L/min of UFBW generally. Freshly prepared UFBW is a milky white colored opaque

liquid, and then becomes colorless and transparent after standing for 5 to 10 minutes (shown in Figure 2-4). This phenomenon may be explained as a large number of visible microscopic bubbles in the bulk water rise to the surface and disappear.

2.2.2 Preparation and storage of UFBW under different durations

The UFBW samples of Air-UFBW, N₂-UFBW, CO₂-UFBW and H₂-UFBW were prepared under different generation time of 5, 10, 20 and 30 min and then storage at different durations of 0, 7 and 14 days. All samples were used to explore the characteristics of particle size, size distribution, concentration, lifetime and surface charge of the bubbles in the following experiments.

2.2.3 Analytical methods

After UFBW samples being prepared, they were kept in serum bottles for 10 min during which they turned from a turbid to clear liquid. Then it was divided equally (by volume) into the centrifuge tubes and stored in an incubator at 20°C for further tests. The bubble size distribution was determined by the nano-particle tracking analysis method (MALVERN, NanoSight LM10, UK) at a standard temperature of 20°C. The bubble size distribution and its concentrations were recorded as a function of storage time.

The DO in DW and different UFBW were measured with a DO meter (DKK-TOA DO-31P, JAPAN). The pH value was determined by a pH meter (METTLER TOLEDO FE20, SWITZERLAND). Previous research indicates that zeta potential possesses a key role in bubble stability, which is affected by pH [78]. In this study, the pH value of prepared UFBW was adjusted from 3.0 to 12.0 by adding 0.1 M NaOH or HCl solution when measuring the zeta potential of UFBW by the Zeta Potential Analyzer (MALVERN, Nano ZS, UK).

2.2.4 Statistical analysis

All the experiments were performed in triplicate. Average value was taken for all the determinations and used for results and discussion.

2.3 Results and discussion

2.3.1 Determination of UFBW generation time

Four kinds of gases, Air, N₂, CO₂ and H₂, were introduced respectively into DW to produce UFBW at different generation time (5, 10, 20 and 30 min) of the UFBW generator. The results of bubble-particle concentration are shown in Figure 2-5. Under the condition of 5 and 10 min generation time, almost the similar particle concentrations were detected in the Air-, N₂- and CO₂-UFBW, averagely around 0.5 or 0.75 ×10⁸ particles/ml, respectively. However, after the generation lasted 20 min, the particle concentration in the Air-UFBW was much higher, almost two times that of N₂- or CO₂-UFBW. For the H₂-UFBW, the bubble concentration exceeded 100 million particles/ml when the generation lasted for 5 min, and then kept increasing with the generation time. In this study, the bubble-particle concentration in all the UFBW samples exceeded 100 million particles/ml after the generation lasted for 20 min, much higher than that of the generation for 10 or 5 min. Even though the UFBs concentration increased with the prolonging of generation time, the bubble size and its distribution remained relatively stable when the generation time was longer than 20 min (Figure 2-6), therefore 20 min was determined as the generation time for the UFBW production in the following experiments.

2.3.2 Bubble size distribution and life time analysis of UFBW

A comparison was conducted in Figure 2-6 in regards to the bubble size distribution of the UFBW (with Air-UFBW as an example to compare), Tap water (TW) and DW in addition to the change of bubble size distribution and concentration in the different UFBW after different storage durations. Higher bubble density and more particles in the nanometer scale were detected in the UFBW than those in TW or DW, and the major particle size was around 144 and 223 nm in the Air-UFBW right after generation (Figure 2-6). In the case of Air-UFBW, the bubble number density decreased along with the storage duration, from 3.0 to 1.75 ×10⁶ particles/ml with a major particle size around 140 nm. A clear decline tendency in bubble concentration was also observed (Figure 2-7). Interestingly, however, during the 14 days' storage, little change was found in the major particle size (~140 nm) of the UFBs.

Figure 2-8 shows that the total bubble number concentrations in the produced UFBW

exceeded 100 million particles/ml at the generation day (0~3 h storage), and the highest concentration, 2.88×10^8 particles/ml was achieved in the Air-UFBW, followed by H₂-, N₂- and CO₂-UFBW. With the increase of storage time, the number of bubble-particles gradually decreased and dropped to around 1×10^8 particles/ml after storage for 336 hours (14 days). Results from Figures 2-8 and 2-9 show that the bubble number density decreased along with the storage time, either for the concentration of main bubbles or for the total number of bubbles. As the results indicated, UFBs could exist in the produced UFBW even after storage for longer than two weeks. After storage for 14 days, the UFBs concentration (which might be indicative of bubble stability) followed a descending order as H₂-UFBW > Air-UFBW > N₂-UFBW > CO₂-UFBW, which may be related to the zeta potentials of the different UFBs (Figure 2-10). Among the four kinds of UFBW, the concentration of bubble-particles in the CO₂-UFBs was always the lowest with the shortest lifetime of nanoscale bubbles, indicating that the CO₂-UFBs in the bulk water was not so stable compared to other tested UFBs. This observation could be attributable to the stability and solubility of CO₂, which is soluble in water and can combine with water to form carbonic acid in the aqueous solution.

2.3.3 DO concentration and pH value analysis of UFBW

During the AD process, the stepwise addition of DW, TW and four UFBW was performed on days 0, 4, 8 and 12, respectively. The usage of air to prepare UFBW did not increase the concentration of DO in the aqueous solution. The DO in the prepared Air-UFBW was 6.32 ± 0.3 mg/L, which is similar to that in DW or TW. Minamikawa et al. [79] also obtained similar experimental results. However, when N₂, CO₂ or H₂ was used to prepare UFBW, the DO in the prepared UFBW was reduced significantly. Especially in N₂-UFBW, the DO dropped to 2.86 ± 0.7 mg/L, which is probably due to the degassing effect caused by N₂ gas in the aqueous solution.

As for the pH value in the produced UFBW, since CO₂ is soluble in water, the pH of the CO₂-UFBW was decreased to 5.88 ± 0.4 , most probably due to the formation of carbonic acid. The pHs of other UFBW were basically maintained at neutral with little variation.

2.3.4 Zeta potential analysis of UFBW

Zeta potential is a key indicator of the stability of colloidal dispersions. A high zeta potential (negative or positive) could create repulsion forces that would avoid the coalescence and contribute to the electrical stabilization while colloids with low zeta potentials tend to coagulate or flocculate. In order to further explore the stability of UFBW, zeta potentials of UFBs under different pH values were measured (Figure 2-10). Results show that the zeta potentials of UFBs were generally negative at a wide range of tested pH (3-12), which was affected by pH as well as the gas introduced. In a previous report, Ushikubo et al. [57] pointed out that the zeta potentials of micro- and nano-bubbles were all negative and their absolute value was in the range between 34-45 mV (O₂), 17-20 mV (Air), 29-35 mV (N₂) and 20-27 mV (CO₂). The negative value is attributed to the predominance of hydroxide ions in the first molecular layers of water at the gas-liquid interface, thus the different zeta potential value may indicate the different stability of UFBs in the different gas-based UFBW, and their different structures at the gas-liquid interface as well. In this study, the negative zeta potential increased with the increase of pH value from 3.0 to 10.0 and reached the maximum at pH 10.0 and then decreased when pH was further increased to 12.0. This observation is in agreement with the findings of previous researchers [52, 74, 80]. In the range of pH from 7.0 to 8.0, which is the most suitable pH condition for methane fermentation, the stability of the different UFBs followed a descending order as H₂ > Air > N₂ > CO₂ in the tested UFBW with the zeta potentials being higher than -20 mV.

2.4 Summary

In this chapter, we explored the characteristics of UFBW produced by using different kinds of gases. First different preparation time were adopted and determined the optimal production condition for UFBW generation, and then the basic characteristics such as particle size distribution and concentrations, bubble lifetime as well as DO, pH and zeta potential of UFBW produced by respectively introducing Air, N₂, CO₂ and H₂ gases that we will employ in the follow-up experiments under different preparation time and storage duration were investigated. Results show that:

The UFBs concentration increases with prolonging preparation time. However, the bubble size and its distribution remain relatively stable when preparation time is longer

than 20 min. A higher bubble density and more particles in nanometer scale can be detected in the UFBW than that in TW or DW which almost no nanoscale bubbles were detected.

In the case of Air-UFBW produced by the designated UFBW generation machine, the number of Air-UFBs is around 2.25×10^8 particles/ml with the most distributed size of 144.2 nm, which decreases to 1.87×10^8 and 1.00×10^8 particles/ml after one and two weeks' storage at room temperature, respectively. All the tested gas-UFBW reflect a similar phenomenon about the bubble number change. UFBs could exist in the produced UFBW even after storage for several weeks. After storage for 14 days, the UFBs concentration followed a descending order as H_2 -UFBW > Air-UFBW > N_2 -UFBW > CO_2 -UFBW.

Zeta potential of UFBs is affected by pH value. At a wide range of pH (from 3.0 to 12.0), all zeta potential values of UFBW are negative. It increases with increasing the pH value from 3.0 to 10.0 and then decreases from pH 10.0 to 12.0. In the pH range (pH 7.0 - 8.0) which is most suitable for AD, stability follows a descending order as H_2 > N_2 > Air > CO_2 in these tested gases and all the zeta potential are higher than -20 mV. The zeta potential values are positively correlated with the number and concentration of UFBs but there is no significant relation between zeta potential and bubble diameter.

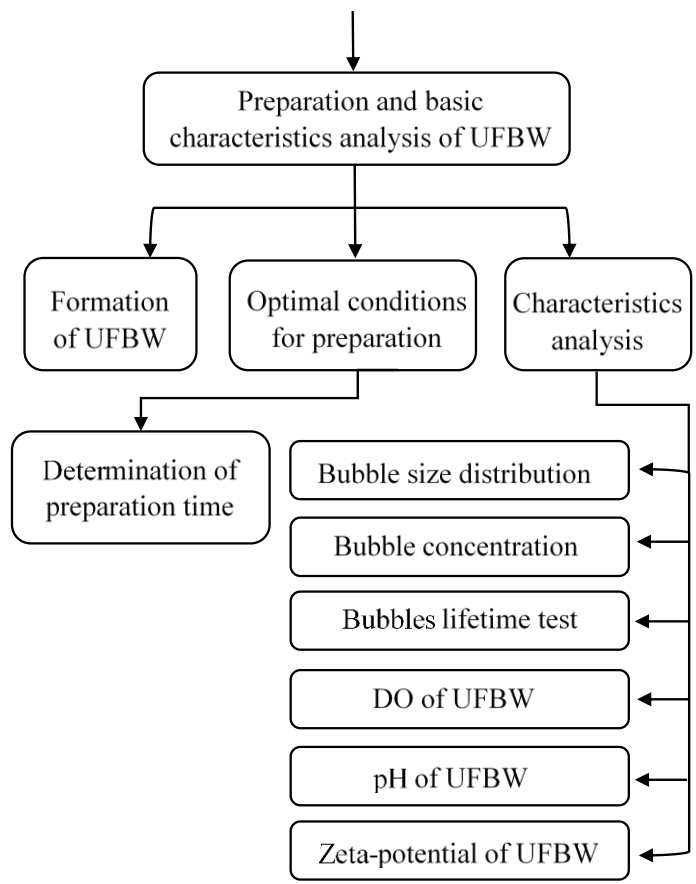


Figure 2- 1. Experimental framework of Chapter 2.

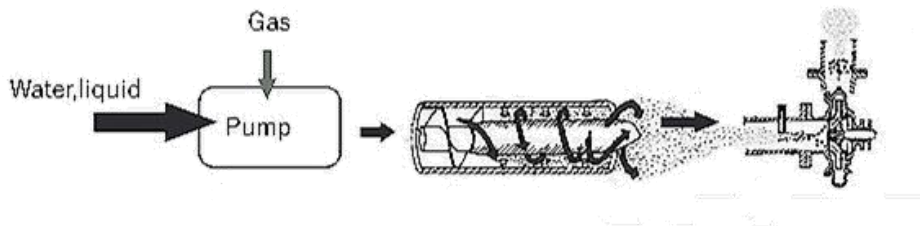


Figure 2-2. Physical photograph and schematic of UFBs generator provided by the company (HACK UFB Co., Ltd. JAPAN).

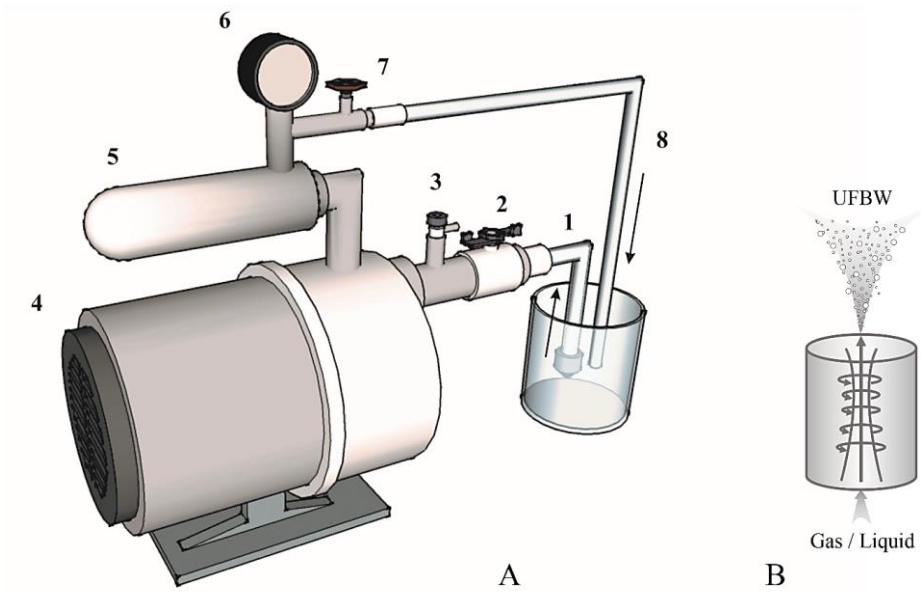


Figure 2-3. Diagrammatic sketch of the ultra-fine bubble (UFB) generator. 1. Water inlet, 2. Water inflow valve, 3. Gas inlet (can be connected to the gas bag) and intake valve, 4. Pump, 5. Bubble generating part, 6. Pressure meter, 7. Water outflow valve, 8. Water

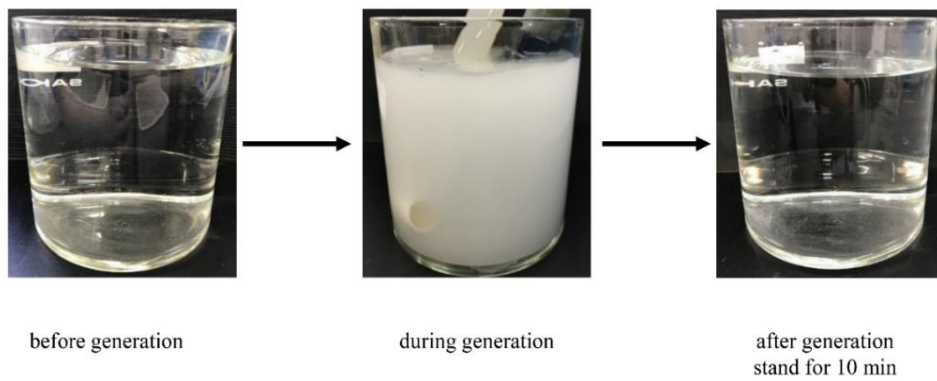


Figure 2-4. Photographs of UFBW before generation, during generation and after generation for 10 min standing.

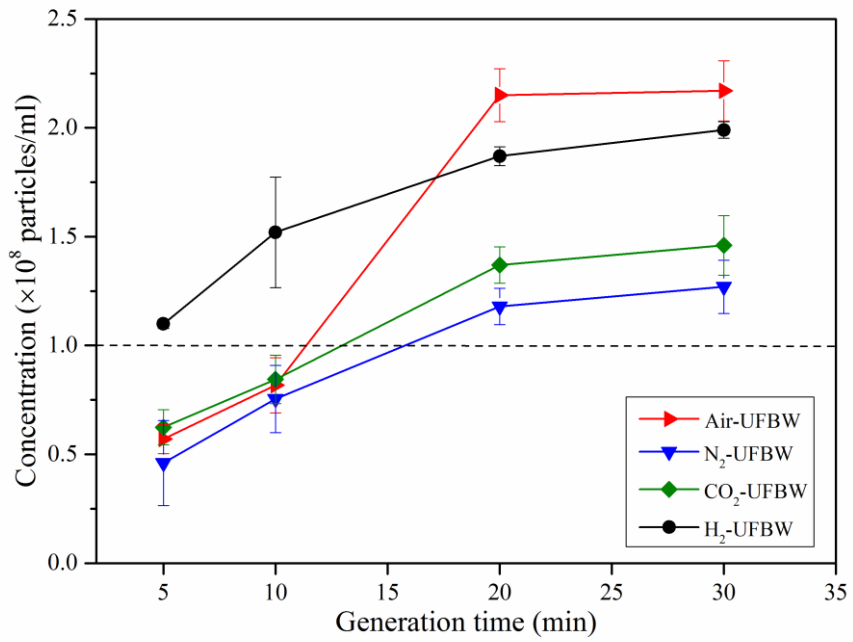


Figure 2-5. Particles concentrations in UFBW using different gases prepared for different generation time.

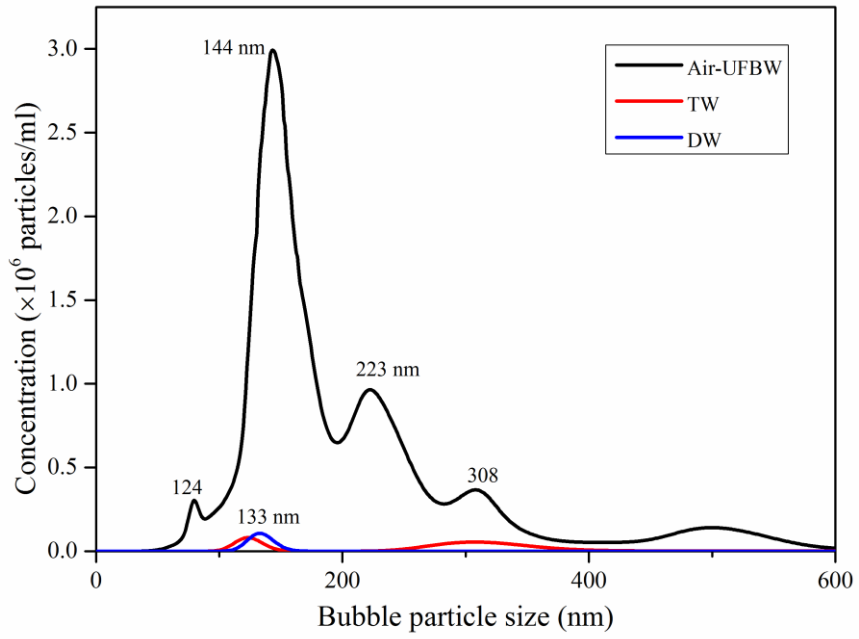


Figure 2-6. Comparison of bubble size distribution among the Air-UFBW right after generation, TW and DW.

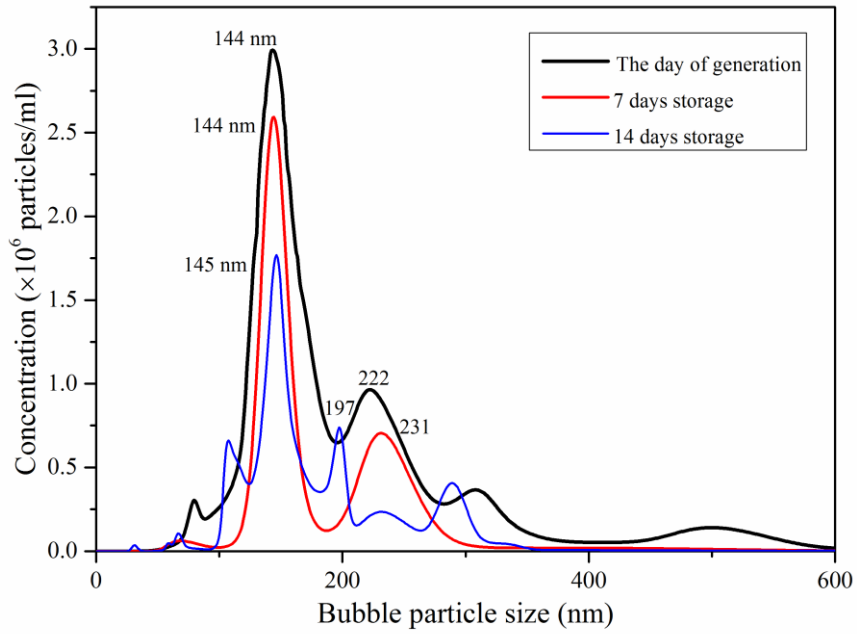


Figure 2-7. Changes in bubble size distribution in Air-UFBW along with storage time.

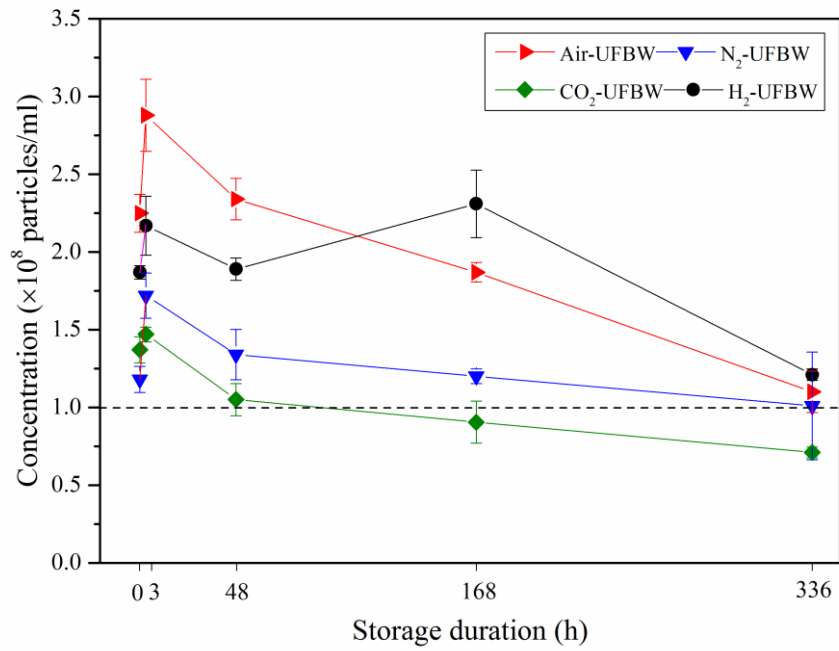


Figure 2-8. Variations of particle concentration in different UFBW after different storage time.

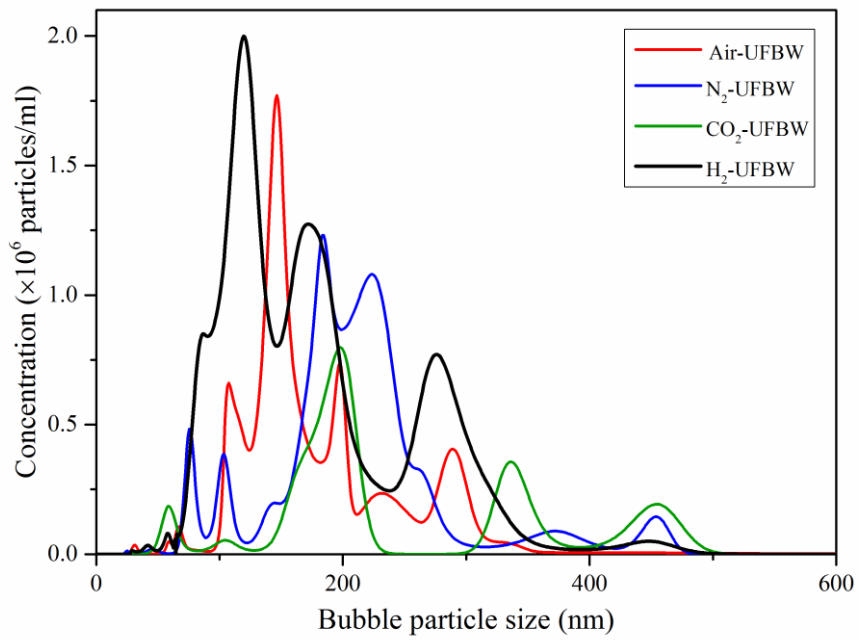


Figure 2-9. Bubble size distribution in different UFBW after storage for 14 days.

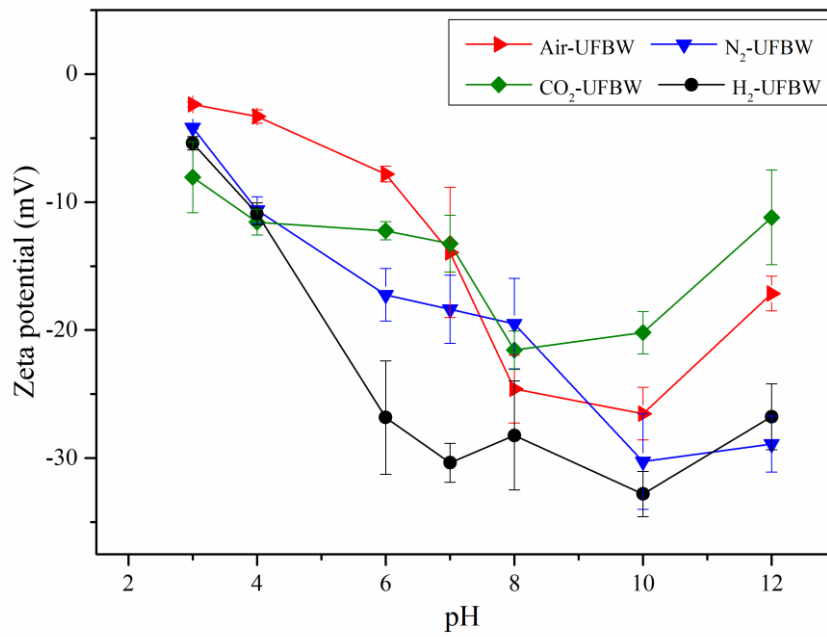


Figure 2-10. Changes in zeta potential of UFBs in different UFBW with variation of solution pH.

Chapter 3 Effects of ultra-fine bubble water on anaerobic digestion of waste activated sludge

3.1 Introduction

In Chapter 2, the basic properties of UFBW including particle size and distribution, bubble concentration, zeta potential, etc. were analyzed. If it has been proven and applied to the growth of plants and animals, what effect does it have on microorganisms, especially on the process of AD which requires the coordination and interaction of multiple microorganisms. This is the subject that we need to do in the next step.

Therefore, in this chapter, as shown in Figure 3-1, a series of batch AD experiments were conducted at initial pH 7 and $36 \pm 2^\circ\text{C}$ to investigate the effect of adding the above-mentioned UFBW on AD of WAS. The test of adding DW was used as the control group. The experiments were divided into the two process. The hydrolysis process has been recognized as the rate-limiting stage for macro-organic matter disintegration. With the consideration of it, the first experiment process was lasted for 7 days to investigate the effect of UFBW on the hydrolysis-acidification process. In this process, total solids (TS), volatile solids (VS), pH, H_2 , soluble total organic carbon (sTOC), volatile fatty acids (VFAs), protein and polysaccharide were tested on day-3 and day-7 in this stage. The second experiment process was lasted for about 30 days in order to explore the effect on the whole AD process of WAS. TS, VS, pH, sTOC, VFAs, alkalinity (ALK), dehydrogenase, coenzyme F_{420} (CoF_{420}), biogas and CH_4 production were tested during 30 days' AD experiments.

3.2 Materials and methods

3.2.1 Raw materials

The water samples used in the experiment were DW (as the control) and different UFBW (including Air-UFBW, N_2 -UFBW, CO_2 -UFBW and H_2 -UFBW). Digested sludge and WAS were sampled from a wastewater treatment plant in Ibaraki (Japan) and stored in the refrigerator (4°C) prior to the experiment.

3.2.2 Batch experiments on hydrolysis-acidification process

Schott Duran serum bottles in two sizes were used in the fermentation experiments. A 1 L bottle was used for preparation and acclimation of inoculum and 250 mL ones were used for anaerobic fermentation. Digested sludge was added into the 1 L reactor and the tested sludge (WAS) was acclimated in this reactor under mesophilic condition ($36 \pm 2^\circ\text{C}$) for about one month. After the above acclimation step, the digestate was used as inoculum. The TS, volatile solids (VS) of the inoculum and WAS were $2.21 \pm 0.50\%$, $1.24 \pm 0.02\%$ and $3.18 \pm 0.29\%$, $2.76 \pm 0.13\%$, respectively. In each 250 mL anaerobic digester, 35 mL of inoculum and 65 mL of WAS were mixed and added to each serum bottle to keep the inoculation ratio (inoculum/WAS, VS basis) in all the reactors around 20%. From references, heating [81] and drug (2-bromoethanesulfonic acid, (BESA)) [82] treatment have been applied to get rid of methanogens in the AD system. With the consideration of it, in the hydrolysis-acidification process, the mixture raw materials including WAS and seed sludge was heated at $105 \pm 2^\circ\text{C}$ in an oven for about 30 min. After that, when the mixture was cooled down to the room temperature, the drug of BESA with a concentration of 50 mM was added into each reactors to mix with sludge and inhibit the activity of methanogens. In this study, 100 mL of DW and four kinds of gas-UFBW addition were performed on day-0, and the total volume of added DW or UFBW (100 mL) and the mixed sludge (100 mL) was 200 mL of the working volume for each reactor. The initial pH was adjusted to 7.0 ± 0.1 by adding 0.1 M NaOH or HCl solution. Prior to the experimental run, all the reactors were carefully sealed and flushed with high purity nitrogen gas for 2 min to create an anaerobic environment. The reactors were then placed in a temperature-controlled incubator ($36 \pm 2^\circ\text{C}$) for 7 d. During the digestion, the bottles were shaken once per day at the fixed time prior to sampling the biogas for composition measurement. A 60 mL volume gas-tight syringe was used to measure the biogas production by directly reading the scale after being connected to the reactor for 2 min and then normalized to the standard temperature and pressure conditions. One mL of biogas was also sampled and used for biogas composition analysis. After the digestion process, the mixture of sludge was centrifuged at 9,000 rpm and 4°C for 15 min and then filtered through a $0.22 \mu\text{m}$ microfiber filter. Its supernatant and remaining sludge were analyzed, respectively.

3.2.3 Batch experiments on the whole AD process

The experiment conducted for the whole AD process was same operated with the hydrolysis-acidification process but without heating and drug treatment. The process was lasted for about one month till the biogas production stopped (approximately 30 days) to ensure the complete AD.

3.2.4 Analytical methods

TS content was measured by drying the raw and final sludge samples at 105°C for 24 hours till constant weight and VS content was determined by incinerating the dried sludge samples at 600°C for 3 hours. The calculation of TS, VS and ALK were determined in accordance with standard methods (APHA, 2012). The pH value was determined by a pH meter (METTLER TOLEDO FE20, SWITZERLAND). Biogas composition was determined using a gas chromatography (SHIMADZU GC-8A, JAPAN). The sTOC in digestate was analyzed by a TOC-V_{CSN} analyzer (SHIMADZU, JAPAN). The digestate supernatant obtained after centrifuging filtering in Chapter 3.2.2 was acidified by 3% phosphoric acid solution before VFAs analysis by the gas chromatography equipment (SHIMADZU GC-8A, JAPAN) packed with Unisole F-200 30/60 column. For the extracellular polymeric substances (EPS) tests, loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) were extracted by modified heat extraction method [83]. Proteins were measured with Lowry's method using bovine serum albumin as a standard solution [84]. Polysaccharide was measured with phenol-sulfuric acid method using glucose as a standard solution [85]. In this chapter, the concentration of VFAs (according to the order in which the peaks appear is HAc: acetic acid, HPr: propionic acid, iso-HBu: iso-butyric acid, nHBu: n-butyric acid, nHV_a: n-valeric acid and iso-HV_a: iso-valeric acid) and EPS were presented as equivalent COD values calculated from the theoretical formula of each VFA component. The equivalent relationships between COD and organic substrates were as follows: 1.07 g-COD/g HAc, 1.51 g-COD/g HPr, 1.82 g-COD/g HBu, 2.04 g-COD/g HV_a, 1.5 g-COD/g protein and 1.06 g-COD/g carbohydrate. Slurry samples were periodically collected every 3 days and analyzed for the content of the CoF₄₂₀ to represent the variation of methanogen activity. Ultraviolet spectrophotometry was used to determine the content of CoF₄₂₀ [86]. The total dehydrogenase activity assay was based on the reduction rate of triphenyltetrazolium

chloride (TTC) to triphenyl formazan. One enzyme unit was defined as the amount of enzyme producing 1 µg of triphenylformazan salt (TF) per hour [87]. Zeta potential of the raw water (DW and different UFBW) and the mixture (sludge with DW and different UFBW) was determined by the zeta potential analyzer (MALVERN, Nano ZS, UK) The proton spin-spin relaxation time (T_2) was determined via nuclear magnetic resonance (NMR) spectroscopy (JNM-MU25A, JEOL, JAPAN).

3.2.5 Calculations and kinetics models

The Logistic function is a classical population growth model, which is widely used in the kinetics analysis of anaerobic fermentation because of its good performance in the description of biogas production from the AD process [88]. The following Logistic function (Eq. (3-1)) was used in this study:

$$P = \frac{P_{max}}{1 + \exp\left[\frac{4R_{max}(\lambda - t)}{P_{max}} + 2\right]} \quad (3-1)$$

where P (ml CH₄/g-VS) is the cumulative CH₄ production at time t , P_{max} (ml CH₄/g-VS) is the maximum CH₄ production potential at the end of incubation time, R_{max} (ml CH₄/g-VS-d) is the maximum CH₄ production rate, t (d) is the duration time and λ (d) is the lag phase defined as a delayed period of a culture in responding to a new environment and starting to produce CH₄.

Due to the successful modeling of CH₄ production by the modified Gompertz model [89, 90], this equation was also used in this study to fit to the experimental data of cumulative CH₄ production under different conditions. The following modified Gompertz equation (Eq. (3-2)) was employed in this study:

$$P = P_{max} \exp\left\{-\exp\left[\frac{R_{max}e}{P_{max}}(\lambda - t) + 1\right]\right\} \quad (3-2)$$

where e is 2.71828, and all the other parameters are the same as in Eq. (3-1). The correlation coefficient (R^2) was calculated to indicate the kinetic model fitting to the experimental results from CH₄ production.

3.2.6 Statistical analysis

The batch fermentation experiments were performed in triplicate. Average value was taken for all the determinations and used for results and discussion. For data analysis, one-way analysis of variance (ANOVA) was used to analyze the statistical differences

among the experimental scenarios by using Microsoft Office Excel 2013. Significant difference was assumed at $p < 0.05$.

3.3 Results and discussion

3.3.1 Hydrolysis-acidification process

(1) Hydrogen and carbon dioxide production

Figure 3-2 shows the cumulative biogas production in the 3 days and 7 days hydrolysis-acidification process. The highest 3 days biogas production was observed in the Air-UFBW group (26.74 ml/g-VS), followed by H₂- (26.56 ml/g-VS), N₂- (25.34 ml/g-VS) and CO₂-UFBW group (24.95 ml/g-VS). The difference is not obvious, but the results of all the experimental groups are dramatically higher than that of the DW group (19.98 ml/g-VS). For the results of the 7 days process, the difference between the experimental group and the control group is more significant. An average enhancement (32.4%) of biogas production in UFBW groups was observed compared to that in the DW group. As shown in Figure 3-3, both H₂ and CO₂ production of the experimental groups in the hydrolysis-acidification process are remarkably higher than that of the control group, especially in the 7 days process. It means that the efficiency of hydrolysis-acidification for biogas production was improved by introducing UFBW in the AD system. The accumulated H₂ and CO₂ would act as the feedstock for CH₄ production in further methanogenesis process.

(2) Composition of VFAs in the digestate

The accumulation of VFAs in the stage of hydrolysis-acidification could be recognized as an important monitor in the AD process. VFAs are the raw materials for the production of H₂ and CO₂. VFAs can be gradually degraded into acetic acid which can be directly utilized by microorganisms, thereby producing methane. However, the accumulation of excessive VFAs is likely to cause the low pH of the system that would affect the activity of microbes and cause a long lag phase, which has a negative impact on the subsequent methanogenesis. In the 3 days and 7 days hydrolysis-acidification process, cumulative VFAs variation was tested in each period with results shown in Figure 3-4. The VFAs concentration increased sharply in the first 3 days with the obvious

difference between the UFBW groups and the DW group, and then increased gradually from day-3 to day-7, in which period the difference is not obvious. For the components of TVFAs, the acetate accounts for almost half of the proportion, growing gradually without decrease during the 7 days process. This is because the complex organic matter is continuously degraded into simple organic matter, and the long-chain VFAs are continuously degraded into the short-chain ones, which just accumulates without consumption in a large amount due to the inhibition of methanogens by drugs. During day-3 to day-7, several detected volatile fatty acids appear to grow but the reducing of butyrate concentration was observed. This might be because comparing to others, butyrate could be more easily degraded to acetate.

(3) sTOC component variation

Figure 3-5 shows the curves of sTOC concentration variation on 3 days and 7 days process. Results show an increasing trend of sTOC concentration in the hydrolysis-acidification stage, and it is consistent with the results of VFAs. In the hydrolysis stage, sTOC values increased due to the relatively high conversion rates from macromolecules to VFAs and from a solid state to a soluble state by acidogens. sTOC can be utilized by methanogens to generate CH₄ and CO₂ in further methanogenesis process. sTOC increased higher on day-3 and day-7 comparing with the sTOC of raw material and the difference between the experimental group and the control group on day-7 is obviously higher than that on day-3, which means the conversion rate of macro organic matter to soluble organics was improved with the addition of UFBW.

(4) Total protein and polysaccharide variation

EPS of different layers extracted from sludge, namely soluble EPS (S-EPS), loosely bound EPS (LB-EPS), and tightly bound EPS (TB-EPS), were used to determine the variation of protein and carbohydrates in the sludge. Fig. 3-6 shows the EPS concentration after the 3 days and 7 days hydrolysis-acidification process with and without UFBW addition.

The protein concentration of the raw sludge was 2,694 mg COD/L which decreased at the range of 2,067 – 2,289 mg COD/L during the 7 days process in both the experimental groups and the control group, this may be due to the degradation and change

of protein to small molecule compound which could be easily used by microbes. The decrease of TB-EPS was relatively obvious compared to soluble- and LB-EPS. The reason can be contributed to that the structure of TB-EPS becomes loose, and TB-EPS will change into LB-EPS then further soluble-EPS, resulting in no significant increase of LB-EPS, to make itself easier to be utilized for other microorganisms during this hydrolysis-acidification process, resulting in sludge disintegration. Besides, the reduction of protein in all the experimental groups is higher than that in the control group which indicates that the UFBW addition can accelerate the transportation of protein to small molecule compounds.

Compared to the reduction of protein, the decrease of polysaccharide is not so obvious for day-3. However, a relatively higher degradation of polysaccharide was observed on day-7. Among them, the TB-EPS shows a slightly decreased trend during this 7 days process while the LB-EPS increased on day-3 and then decreased on day-7. This may be because the alternation of TB-EPS to LB-EPS was dominant in the first 3 days, and then the change of LB-EPS to soluble-EPS further to VFAs and other small molecule compounds was the main process from day-3 to day-7. Besides, on day-7, both TB-EPS and LB-EPS in the experimental groups except H₂-UFBW group are lower than that in the control group, which demonstrates the addition of UFBW would be beneficial to the degradation of polysaccharide.

As the TB-EPS maintains the sludge stability, and the decline of both TB-EPS to the LB- and soluble-EPS means sludge could be easily degraded by microorganisms, especially for the UFBW addition group.

(5) Sludge reduction

Sludge reduction (VS reduction) is usually acted as an indicator to characterize the efficiency of the AD process [91]. Figure 3-7 shows the reduction of VS after the hydrolysis-acidification process. With UFBW addition, the VS average reduction increased to 7.05% and 11.53% on day-3 and day-7, respectively, while those in control group were 5.23% and 6.79%, respectively, which means a faster hydrolysis and reduction rate of organic matter was caused by UFBW addition.

3.3.2 The whole anaerobic digestion process

(1) Biogas and CH₄ production

The daily and cumulative biogas production during the AD process with the addition of DW or different UFBW were determined and shown in Figure 3-8. The corresponding daily and cumulative CH₄ production were shown in Figure 3-9. The AD reactors were run for about 30 days till almost no biogas production was detected. During most of the testing period, the daily biogas or CH₄ production from the AD reactor with UFBW addition was higher than that from the reactor with DW addition, especially during the first 10 days can be observed clearly.

In regard to the daily biogas production shown in Figure 3-8A, peak value was observed on day-8 with the production of 139.40 ml/g-VS d (mean value, the same below) in reactors that with CO₂-UFBW addition. Followed by Air-, N₂- and H₂-UFBW group, which peak value was 128.70, 126.37 and 124.25 ml/g-VS d, respectively and no significant difference. However, for the DW group, the peak value was observed on day-10 with the production of 99.58 ml/g-VS d. Both rate and peak of production are lagging and decreasing in the early stage of AD process including the hydrolysis-acidification stage. This might be due to the improved hydrolysis efficiency for the decomposition of macromolecular organic matter into simple organic by adding the negatively charged UFBW. After the hydrolysis and acidification stage, the slight improvement of daily biogas production can be observed almost in every day results while the difference is not so obvious each day. This might be because more simple organic matter hydrolyzed in the early stage that can be continuously decomposed into biogas by microbial utilization in the subsequent stage. The results of accumulated biogas yield show quite different trends (Figure 3-8B). All the UFBW groups show higher production, at the range of 20.95% - 27.56%, than that of the control group with the addition of DW. It means that the addition of UFBW can effectively increase the production and accumulation of biogas.

For the daily and cumulative CH₄ production shown in Figure 3-9, the trend is similar to the curve of biogas production mentioned above. Peak value at the range of 87.44 - 103.46 ml/g-VS d was noted on day-8 in reactors added with UFBW, in comparison to 71.11 ml/g-VS d from the reactor with DW addition. The time reached to the peak value and the corresponding CH₄ concentration of the DW group were also

delayed and declined compared with the experimental groups. While almost no significant difference was detected in daily CH₄ production among the reactors added with different UFBW. Besides, an average significant difference ($p = 0.0009 < 0.05$) in the cumulative CH₄ production was observed between the reactors with UFBW addition and with DW addition. The average cumulative CH₄ yields were obtained in the reactors added with UFBW followed a descending order of 387.84 (CO₂-UFBW) > 372.77 (H₂-UFBW) > 369.47 (Air-UFBW) > 361.24 (N₂-UFBW) ml/g-VS, about 27.55%, 22.59%, 21.50% and 18.80% higher than that from the reactor with DW addition (304.08 ml/g-VS), respectively. An average increase of 22.61% can be observed in UFBW group and the relatively higher CH₄ accumulation (increase more than 20%) was obtained from the CO₂-, H₂-, and Air-UFBW added reactors compared to that of control group. The addition of N₂-UFBW was slightly worse than other UFBW, which however also demonstrated some positive effect on CH₄ production in comparison to the control group.

During day-8 to day-10, around 74.23 - 78.93% and 71.40 - 78.40% of CH₄ content were detected in the biogas from the CO₂-UFBW and DW reactors, respectively (Figure 3-10). During the whole process of AD, there was a slight improvement in the CH₄ content between the experimental group and the control group but the difference was not significant.

This observation mentioned above may be attributable to the existing nanoscale UFBs in the aqueous solution, which plays an important role in the AD process. UFBs may affect the physical properties of the aqueous environment and improve biological activity, thereby promoting the mass transfer efficiency of nutrients and enhancing the utilization and conversion efficiency of organic substrates. In addition to the effect of these ultra-fine structured bubbles, the introduced gases might also play important roles in CH₄ production. CO₂- or H₂-UFBs in the UFBW might be easily utilized by the microorganisms for anaerobic fermentation, while N₂-UFBs can ensure a strict anaerobic environment. Most interestingly, the presence of air (or partial O₂) is generally regarded as toxicant to anaerobic methanogens. However, the Air-UFBW addition can also promote methane fermentation with obviously more beneficial effect than DW condition. Probably the presence of limited micro-oxygen could facilitate the hydrolysis of the substrates in the hydrolysis and acidification stages of AD [92]. The environment could rapidly become anaerobic as the limited oxygen was quickly consumed by aerobic or

facultative anaerobic bacteria. On the other hand, some methanogens may have some certain extent of intrinsic tolerance to oxygen exposure, and their bioactivity could be recovered soon after the inhibition of oxygen exposure was removed [93, 94]. The synergistic effects of the UFBs, as well as the micro-oxygen environment, may promote the AD process. This part of the study first proved that Air-UFBW is beneficial for the AD process, which still needs further in-depth research to shed light on the mechanisms involved.

(2) VFAs production and composition in the digestate

Volatile fatty acids (VFAs) are very important intermediates in the AD process that generated mainly from the stage of hydrolysis-acidogenesis and acetogenesis [95]. Figure 3-11 shows the total VFAs, VFA-Acetic acid and VFAs concentration variation during the whole AD process of WAS with and without UFBW addition. Total VFAs concentration first up to the peak value because of the degradation of macro-organics in the first several days by acidogens, and then gradually decreased to the minimum till the last day due to the short-chain acid consumption and CH₄ production by methanogens. The occur of peak value of total VFAs (3,534.38 – 4,320.09 mg-COD/L) in the reactors with UFBW addition were observed on day-3, while in control group, the peak value (3,770.19 mg-COD/L) that with DW addition was delayed and detected on day-6. These results agreed with the tendency of daily biogas or CH₄ yields in the control and experimental group trials. Since the lag phase is needed for the conversion from VFAs to CH₄. It is the reason why the occurrence of peak CH₄ yield delayed 4 to 5 days than the peak VFAs. Relatively high production of VFAs can increase CH₄ production in subsequent stages. The faster degradation of organics into VFAs and then into biogas might be correlated to the enhancement of acidogens and methanogens activity in the presence of UFBs.

Besides, the main components of VFAs observed in all groups were acetate, propionate, butyrate and valerate, and acetate was the prevailing product. For the reactors with UFBW addition, the acetate concentration reached the peak value of 1,041.92 mg-COD/L on day-3 and then decrease while the acetate concentration of the DW group reached to peak (739.21 mg-COD/L) on day-6 and lower than UFBW group. Noticeably, in all groups, the concentration of propionate increased until day-12 and then decreased

with the consumption of acetate and butyrate. Between day-7 and day-9, the concentration of butyrate decreased quickly, and the peak of daily CH₄ yield was also observed at the same time. Previous studies have pointed out that butyrate could be more easily degraded to acetate than propionate at the low hydrogen partial pressure and the propionate was an unfavorable substrate for microbes [96].

(3) pH variation

Figure 3-12 shows the pH values variation. It was opposite to the trend of VFAs concentrations mentioned above. pH values in all digesters dropped to around 6.0 at the beginning of AD and this was consistent with the accumulation of VFAs in the reactor. Then it increased and maintained around 7.0 due to the consumption of VFAs. pH values of all reactors with UFBW addition or DW addition are similar and have a slight but no significant difference. pH values remained at the range of 5.97 – 7.37 which means no acid inhibition occurred and was appropriate for microbes in AD process because fermentative microorganisms can function in a wide range of pH between 4.0 and 8.5 [97]. It agreed with the results of VFAs in Chapter 3.4.2. In addition, less fluctuation of pH in this trials were observed after day-12, which was related to the higher stability in reactors in this stage.

(4) Alkalinity variation and VFA/TA

As a result of the high amount of VFAs produced during the early stage of AD, it is very important to monitor the AD process stability. ALK and the ratio of VFA to total alkalinity (TA) are effective parameters to measure the stability of an AD process. The ALK of a steady state AD system ranged between 2,000 and 4,000 mg CaCO₃/L [98] and for another criterion for judging digester stability, the critical values for the VFA/TA ratio are: $VFA/TA \leq 0.40$ stable digester, $0.40 < VFA/TA < 0.80$ some instability signs, and $VFA/TA \geq 0.80$ significant instability [99]. Figure 3-13 shows the variations of ALK and VFA/TA ratio during the whole AD trails of WAS with and without UFBW addition. The ALK in all digesters at the range of 2,700 and 4,200 mg CaCO₃/L. It means the anaerobic system maintained a relatively stable state. For the UFBW groups, the ALK gradually increased from the first day until day-15 and then basically remained stable at around 4,000 mg CaCO₃/L, and for the DW group, the ALK gradually growth to around 4,100

mg CaCO₃/L throughout the fermentation duration. The VFA/TA ratio of UFBW groups increased sharply in the first 3 days, and then a significant decrease was also observed. For the DW group, VFA/TA ratio always increased until day-6. Fast increase of VFA/TA manifests an unstable system in the start-up stage. In hydrolysis-acidification stage, more VFAs formation and the environment is acidic thus microbes were trying to adapt to the new environment in this stage. All the digesters including the UFBW groups and the DW group kept a stable system ($VFA/TA \leq 0.40$) or only a little unstable ($0.40 < VFA/TA < 0.80$) for most of digestion duration and significant instability ($VFA/TA \geq 0.80$) in the early stage.

(5) sTOC variation

Figure 3-14 shows the curves of sTOC concentration variation. Results shows an increasing and then decreasing trend of sTOC concentration, and it is consistent with the results of VFAs. In the early stage, because of higher conversion rates from macromolecules to VFAs and from a solid state to a soluble state by acidogens than the utilization rate of VFAs by methanogens, sTOC values increased. Then with the accumulation of simple organic matter and the gradual increase of methanogens activity, sTOC was utilized by methanogens to generate CH₄ and CO₂. For the UFBW group, sTOC increased to a peak value on day-3 or day-6 and higher than that in DW group, which reached the peak value on day-9. It indicated that with the addition of UFBW, the conversion rate of macro organic matter to soluble organics was higher than that with the addition of DW.

(6) Key enzymes variation

The activities and content of enzymes secreted from microorganisms bring additional information of microbes. Although various enzymes participated in the AD process, two key enzymes of dehydrogenase and CoF₄₂₀ were essential and responsible for methanogenesis. The activity of dehydrogenase can reflect the microbial dehydrogenation capability of organics which indicates the microbial activity indirectly. Higher activity of dehydrogenase means the substrate can be efficiently converted to VFAs, which can be utilized to produce CH₄ [100]. The enzyme of CoF₄₂₀ is an electron carrier linked to the reduction pathway of bicarbonate by hydrogen into CH₄. The content

of CoF_{420} in the sludge can be used as a biological indicator to monitor and estimate the methanogenic potential activity [86, 101]. Figure 3-15 shows activities of dehydrogenase and CoF_{420} in the whole AD process of WAS with and without UFBW addition. When compared with the scenario by adding DW, obvious enhancement of dehydrogenase activity was observed by adding UFBW. The peak activity of dehydrogenase was noted on day-12 in the experimental group and was an average 17.62% higher than that in the control. It exposed that the addition of UFBW could increase the activity of dehydrogenase in the AD process. This might be associated with the degradation of macro organics during the early stage that promotes organics bioavailability for biogas production. The content of CoF_{420} in all groups increased steadily, but higher in the experimental group than that in the control group. It might be due to the addition of UFBW that could promote the growth and biological activity of methanogens. The increase in CH_4 productivity was closely related to the increase content of CoF_{420} [102]. In the reactors with UFBW addition, the higher CH_4 production was observed, which could prove the above deduction. Besides, the steady increase content of CoF_{420} also might be due to the growth of methanogens, which would finally become the dominant microbial group in AD system.

(7) Sludge reduction

Due to the limitation of AD duration, anaerobic fermentation can only partially decompose organics. Thus reduction of sludge also can be used as a monitor to characterize the performance of AD system. Figure 3-16 shows the reduction of VS after the whole AD process of WAS with and without UFBW addition. The content of VS was 2.029 (%) before the digestion, and after the whole AD process its content decreased to 1.218 – 1.203 (%) (39.97 – 40.71% of reduction) at UFBW group and 1.260 mg/L (37.90% of reduction) at DW group. However, the reduction rate of UFBW group in the first 3 or 6 days (6.7 - 11.88% in 3d and 16.03 - 22.44% in 6d) were faster and sharper than that in DW group (4.59% in 3d and 14.30% in 6d), which means faster hydrolysis and reduction rate of sludge was caused by UFBW addition.

(8) Kinetic models analysis

Under the steady state, the fitting curves and relevant parameters (mean standard

deviation) obtained by fitting the modified Gompertz and the Logistic models are shown in Figure 3-17 and Table 3-1, respectively. The experimental data well fitted to Gompertz ($R_2 = 0.99$) with the maximum CH_4 production predicted being consistent with the actual experimental results, which is better than the Logistic models ($R_2 = 0.98$). Compared to the AD reactor with DW addition, the reactors with UFBW addition showed relatively higher CH_4 productions and shorter lag phase at significant difference $p < 0.05$, which could be also indicated by the higher P_{\max} , R_{\max} value and lower λ value, further confirming that the addition of UFBW is beneficial for CH_4 production. The good simulation results obtained by these two kinetic models in this study reflect that these models can be applied in the actual AD process to predict the biogas production from WAS under UFBW addition.

(9) Preliminary analysis on mechanisms involved

Figure 3-18 shows the zeta potential of the raw water (DW and different UFBW) and the mixture (sludge with DW and different UFBW), the proton-nuclear magnetic resonance spin-spin relaxation time (T_2) of the raw water (DW and different UFBW). Results show that for the raw water, the zeta potential of DW is around 0 mV. But for UFBWs, the zeta potential is at a range of -11.87 ~ -23.30 mV and higher than that of DW. After mixed with sludge, zeta potential of all samples is negative and increases to some extent. And for each of the UFBW group (-22.67 ~ -26.23 mV), it was higher than that of DW group (-17.63 mV). The proton relaxation time is widely used to study the mobility and diffusion of the water molecules in various fields. As shown in Figure 3-18, the T_2 values of UFBW (2,711.51 ~ 2,911.83 ms) are higher than that of DW (2,497.87), indicating the existence of UFBs will weaken molecular interactions such as hydrogen bonding and then result in a higher molecular mobility.

Results from the multiple parallel experiments showed that the introduction of UFBW enhanced CH_4 production from WAS via AD. Not only CO_2 - and H_2 -UFBs, but also Air- and N_2 - UFBs in water can enhance the efficiency of biogas production. The results suggest that the resultant nanoscale UFBs other than the type of gas play a major role in the AD reactor. Why do UFBs have such effects? One explanation is attributable to the changes of the microscale water environment due to the addition of UFBW, like the acceleration of the mobility of water molecules in the liquid phase. This could promote

the mass transfer of nutrients from the liquid phase to the microbial cells, and thus enhance the bioactivity of microorganism in both hydrolysis-acidification stage and methanogenesis stage. At a wide range of pH, the zeta potentials of UFBs are negative, which can be explained by the excess of OH^- relative to H^+ ions at the gas-liquid interface, or adsorption of ions in the electrolyte solutions [103]. The negatively charged UFBs may contribute to the weakening of the hydrogen bonding network in the bulk water. Bubbles can adsorb H^+ ions onto their surface, and the shifting or absence of protons in water could trigger the modification or destruction of the hydrogen bonding network [104]. Thus for the larger quantity of bulk water that is far away from the surface of UFBs, the hydrogen bonds become weak due to the absence of protons. The weakening of the hydrogen bond network will reduce the water viscosity and accelerate the mobility of water molecules [105]. Also, the formation, shrinkage and disappearance of UFBs would affect the number of water molecules and the structure of water. A previous study [62] shows that the addition of nanometer bubbles prolonged the instability time of proton when measured by the proton spin-spin relaxation time, which has been shown to be related to the increase of the mobility of water molecules [106, 107]. The increased mobility of water molecules would accelerate the rate of mass transfer of nutrients from liquid to bacterial cells, which further has an indirect stimulatory effect on improving the activity of microorganisms and enzymes, facilitating the decomposition of the substrates with improved biogas production. In any case, research on the real mechanisms involved in this complex process was worthy to be investigated in detail.

3.4 Summary

In this part, the effects of UFBW on AD of WAS in both hydrolysis stage and the whole stage were investigated.

In hydrolysis stage, an average enhancement (32.4%) of biogas production in UFBW groups was observed compared to that in the DW group. Both H_2 and CO_2 production of the experimental group are remarkably higher than that of the control group, especially on 7-day process. The VFAs concentration increased sharply in the first 3 days with the obvious difference between the UFBW groups and the DW group, and then increased gradually from day-3 to day-7. sTOC increased higher on day-3 and day-7 comparing with the sTOC of raw material. The reduction of protein and polysaccharide in all the

experimental groups is higher than that in the control group. With UFBW addition, the VS average reduction increased to 7.05% and 11.53% on day-3 and day-7, respectively, which is higher than that in the control group.

In the study of the whole AD process, the UFBW addition group achieved 20% higher of biogas and methane production than that in the control tests. Both production rate and the peak of daily production are higher and the lag phase is shorter than that of the DW group. The rate of accumulation and reduction of VFAs, especially acetate in UFBW groups, were higher and faster than the control group, leading to the higher production of CH₄ in 4 to 5 days later. During day-7 to day-9 of the AD process, the butyrate concentration decreased quickly, and the peak value of daily CH₄ yield was also observed at the same time. The pH values showed at first decrease and then increase trend and were opposite to that of VFAs concentrations. In addition, less fluctuation of pH were observed after day-12 means the higher stability in reactors. The ALK in all digesters at the range of 2,700 and 4,200 mg CaCO₃/L, which shows a relatively stable state in reactors. All the digesters including the UFBW groups and the DW group kept a stable system ($VFA/TA \leq 0.40$) or only a little unstable ($0.40 < VFA/TA < 0.80$) for most of digestion duration. The sTOC peak accumulation and sTOC reduction rate of UFBW group were also higher and faster than that of the DW group. As for the enzymes in the substrate, the contents of dehydrogenase and CoF₄₂₀ were slightly higher in the UFBW group than that in the DW group at the same processing time. The reduction rate of UFBW group in the first 3 or 6 days (6.7 - 11.88% in 3d and 16.03 - 22.44% in 6d) were faster and sharper than that of the DW group (4.59% in 3d and 14.30% in 6d).

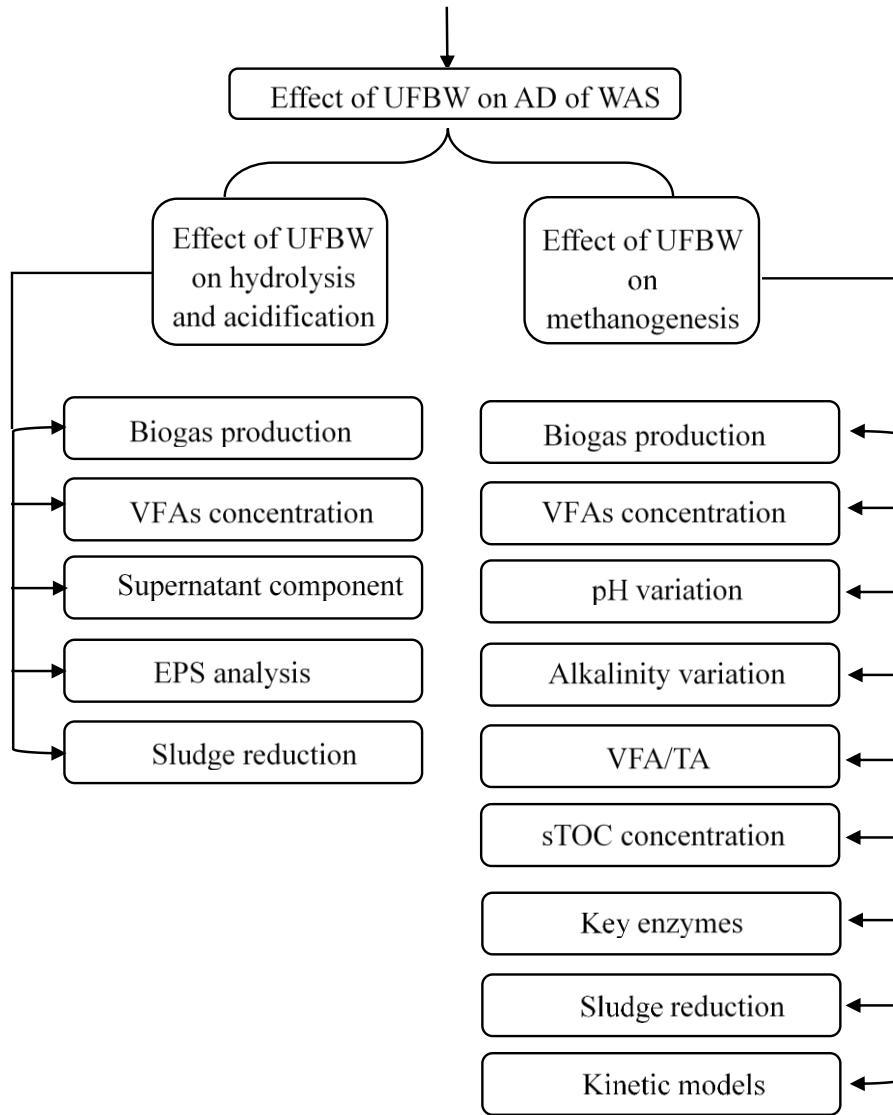


Figure 3-1. Experimental framework of Chapter 3.

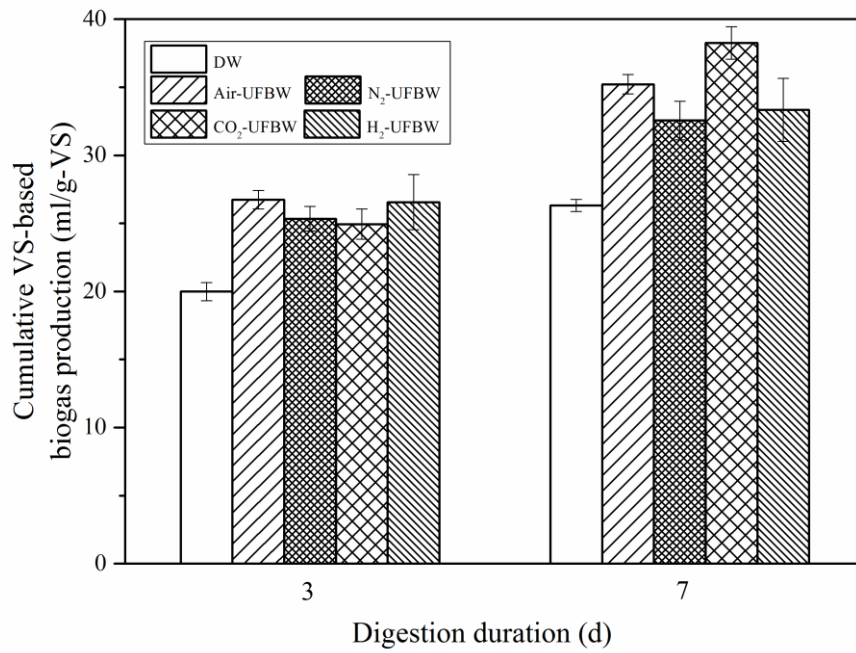


Figure 3-2. Cumulative biogas production from the 3 days and 7 days AD trails of WAS with and without UFBW addition.

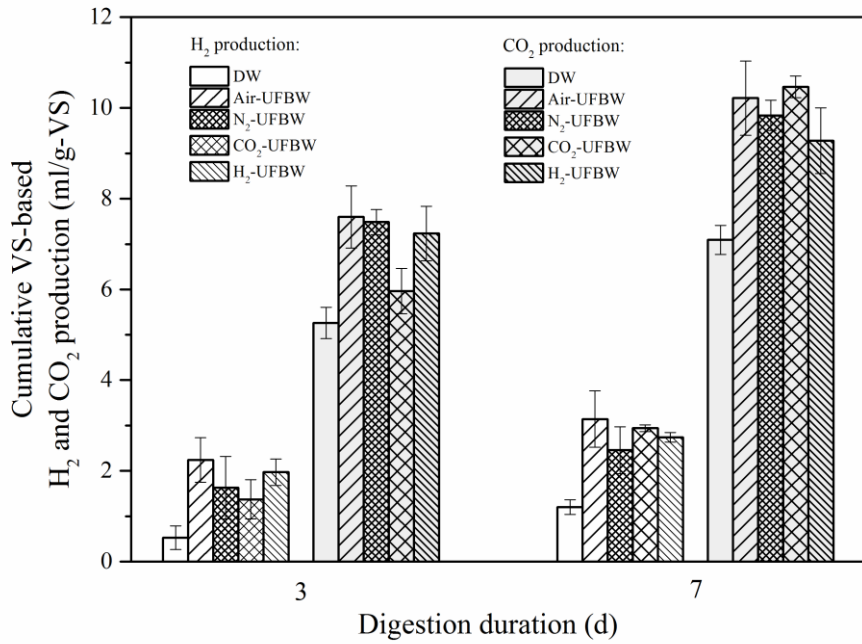


Figure 3-3. Cumulative H₂ and CO₂ production from the 3 days and 7 days AD trails of WAS with and without UFBW addition.

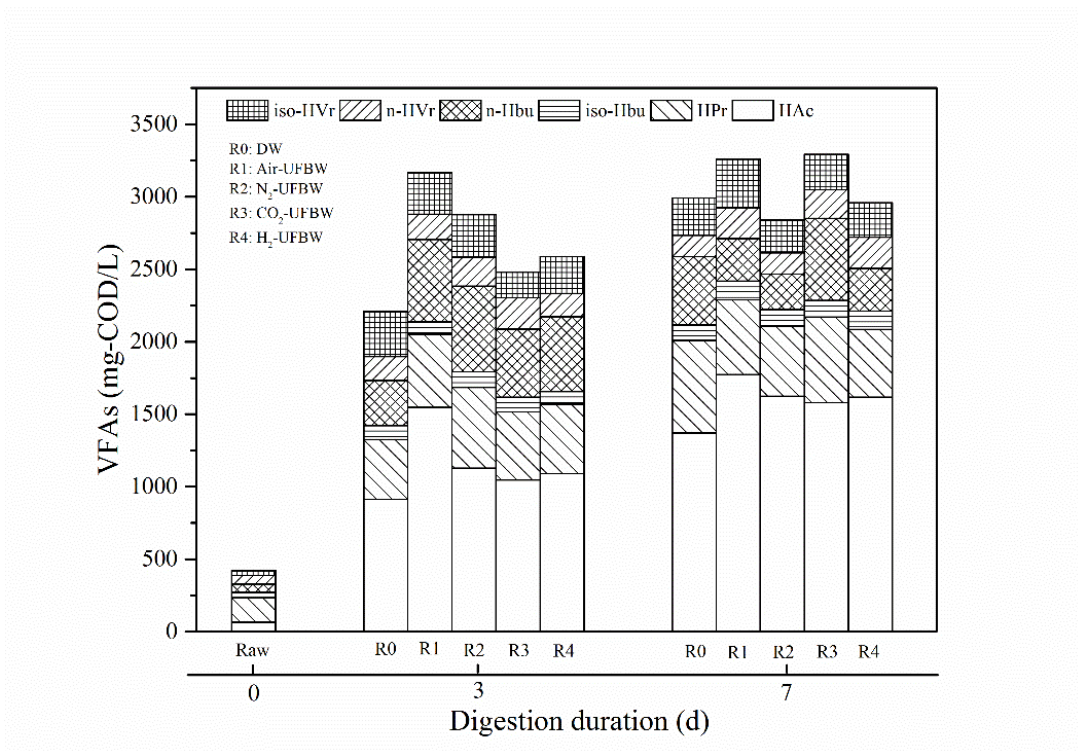


Figure 3-4. Cumulative VFAs variation during the 3 days and 7 days hydrolysis-acidification process of WAS with and without UFBW addition.

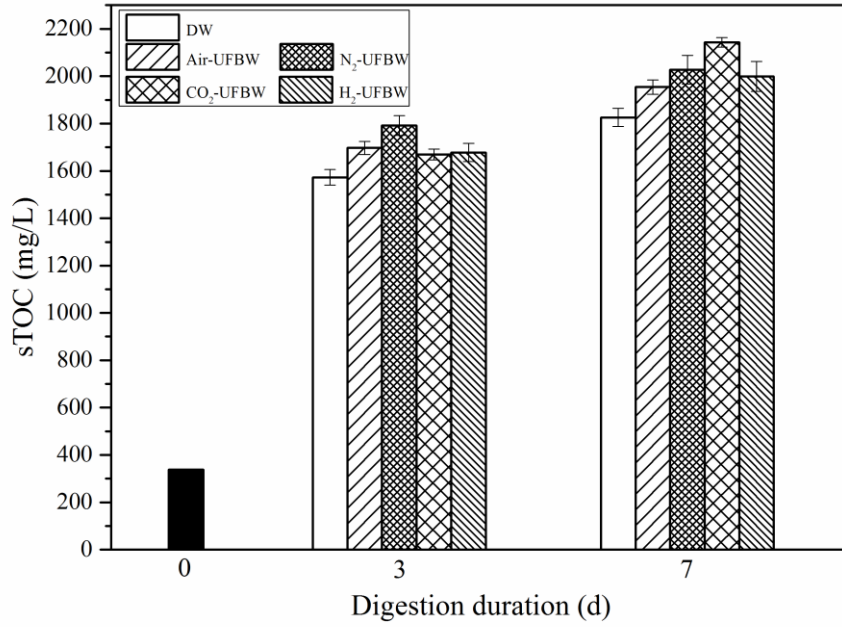


Figure 3-5. Soluble total organic carbon concentration in digestate after fermentation for 3d and 7d.

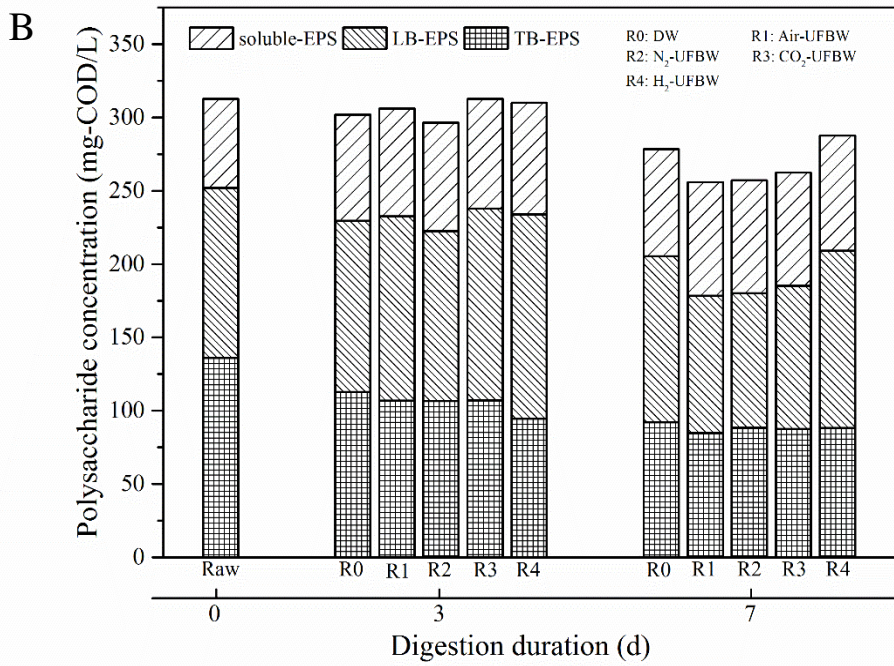
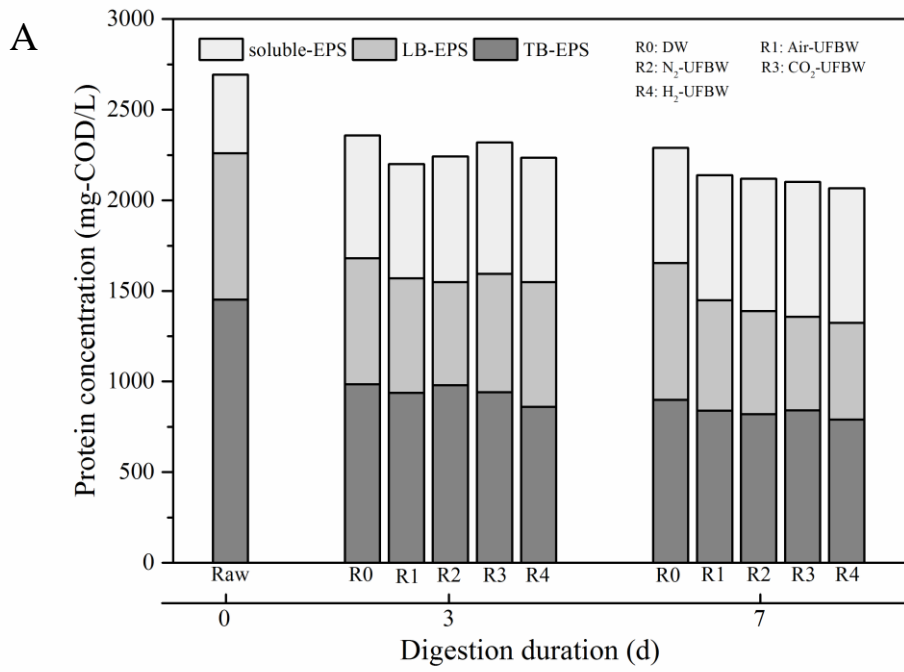


Figure 3-6. Protein (A) and polysaccharide (B) concentration after 3 and 7 days hydrolysis-acidification process with and without UFBW addition.

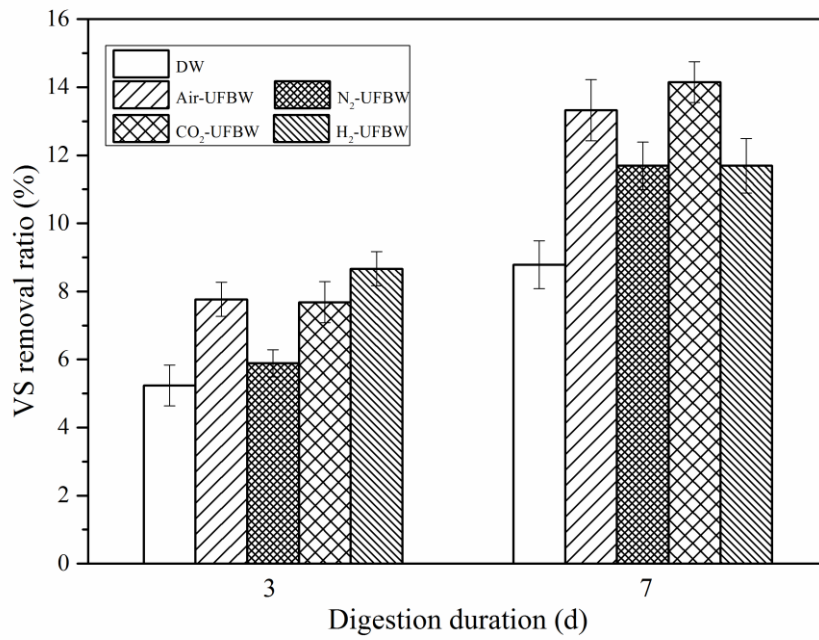


Figure 3-7. Reduction of VS in the hydrolysis-acidification of AD process with and without UFBW addition.

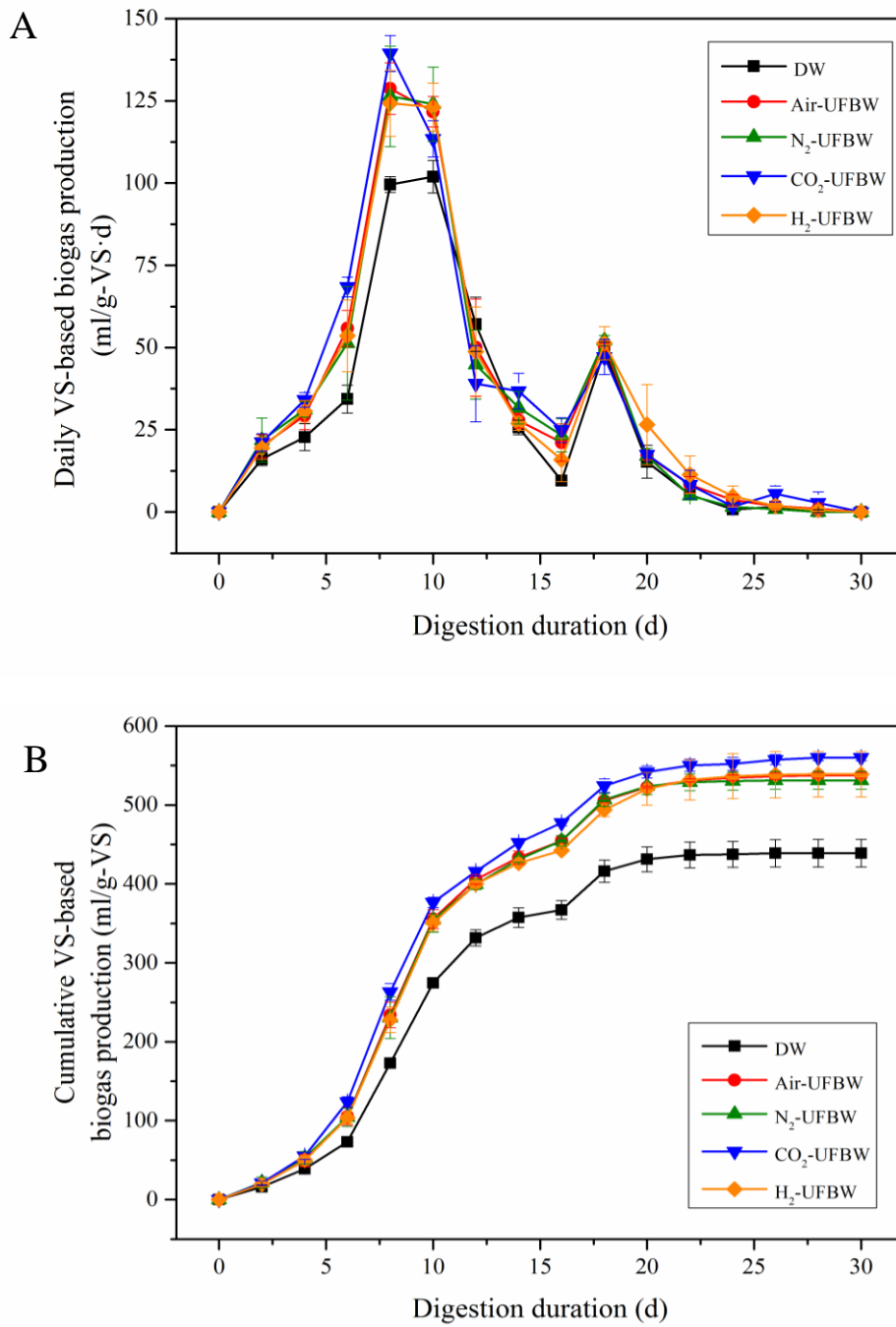


Figure 3-8. Daily (A) and cumulative (B) biogas production from the whole AD trails of WAS with and without UFBW addition.

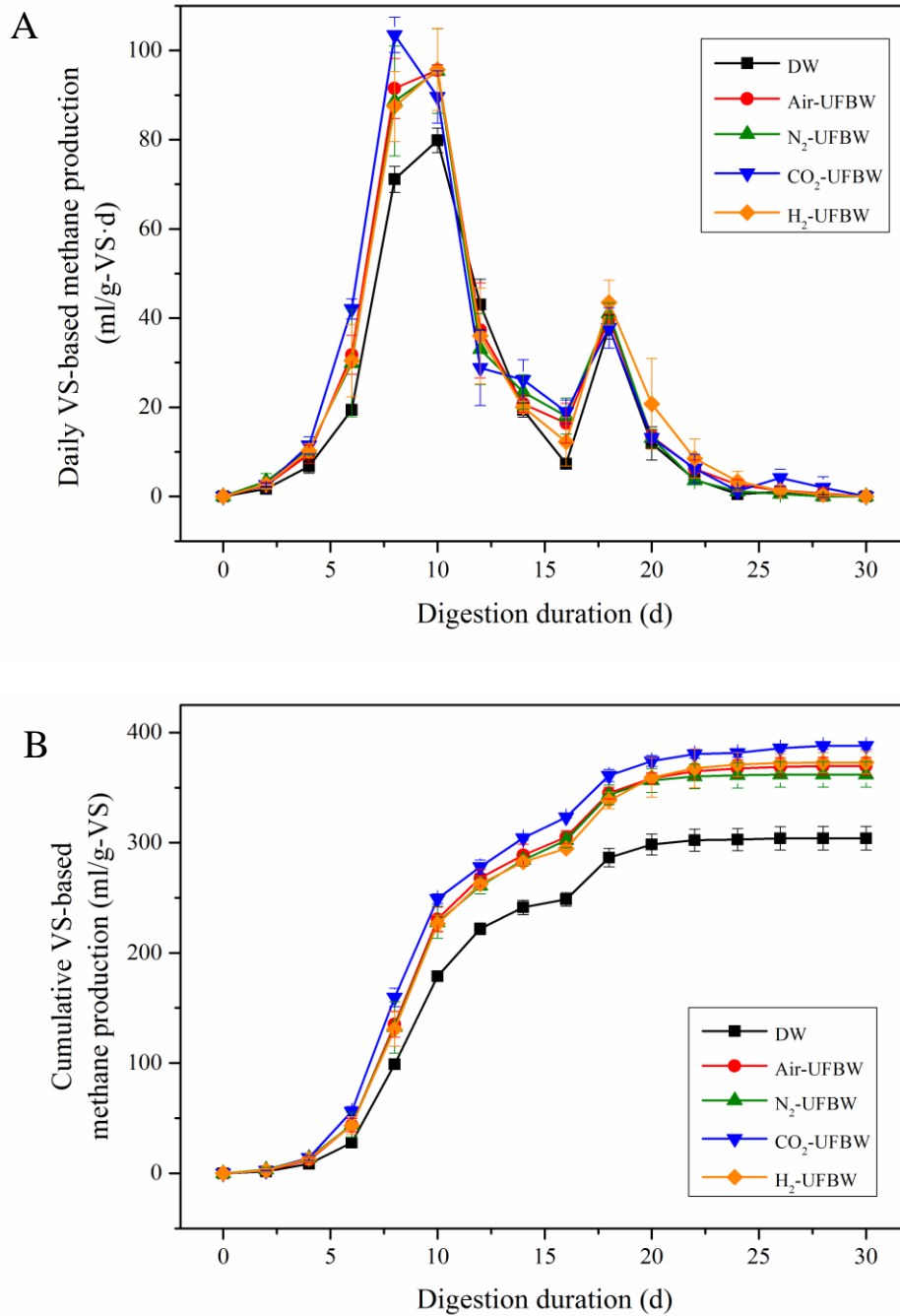


Figure 3-9. Daily (A) and cumulative (B) CH₄ production from the whole AD trails of WAS with and without UFBW addition.

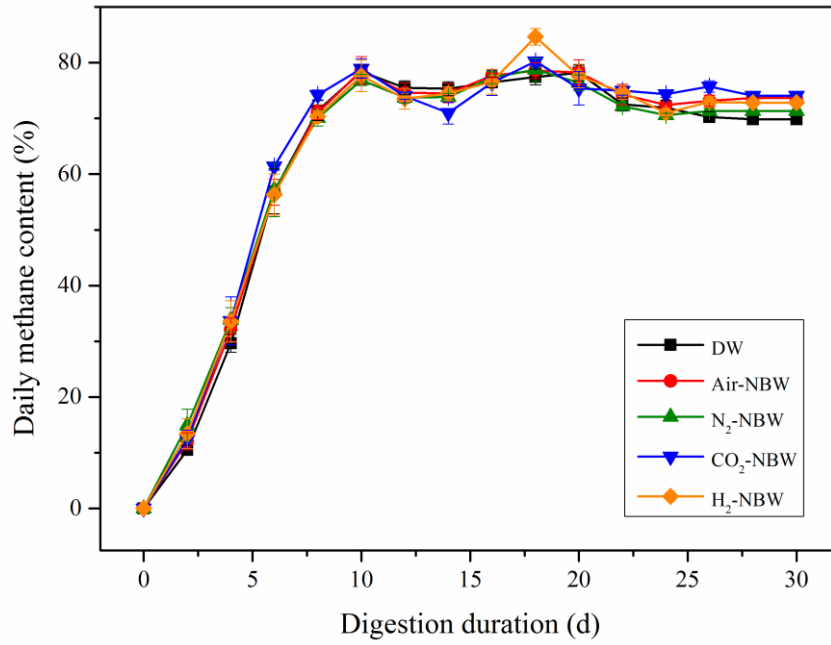


Figure 3-10. CH₄ content (%) of each reactor during the whole AD trails of WAS with and without UFBW addition.

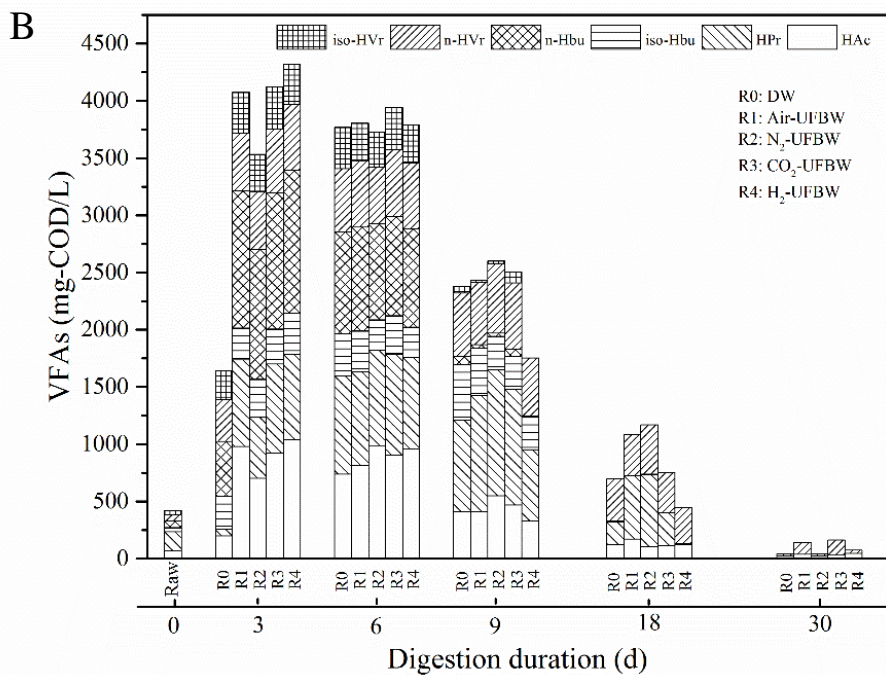
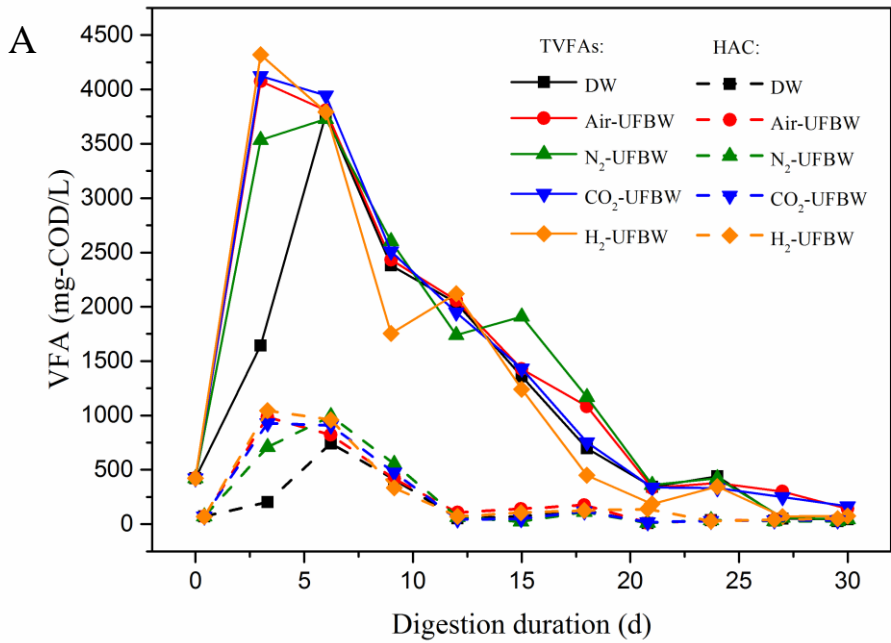


Figure 3-11. Total volatile fatty acids (TVFAs), VFA-Acetic acid (A) and VFAs (B) variation during the whole AD process of WAS with and without UFBW addition.

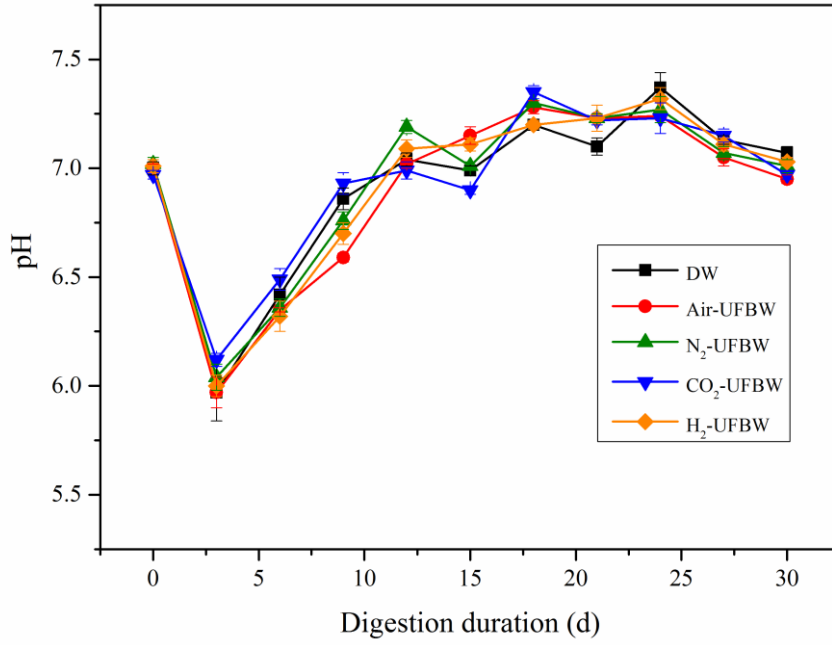


Figure 3-12. pH variation during the whole AD trails of WAS with and without UFBW addition.

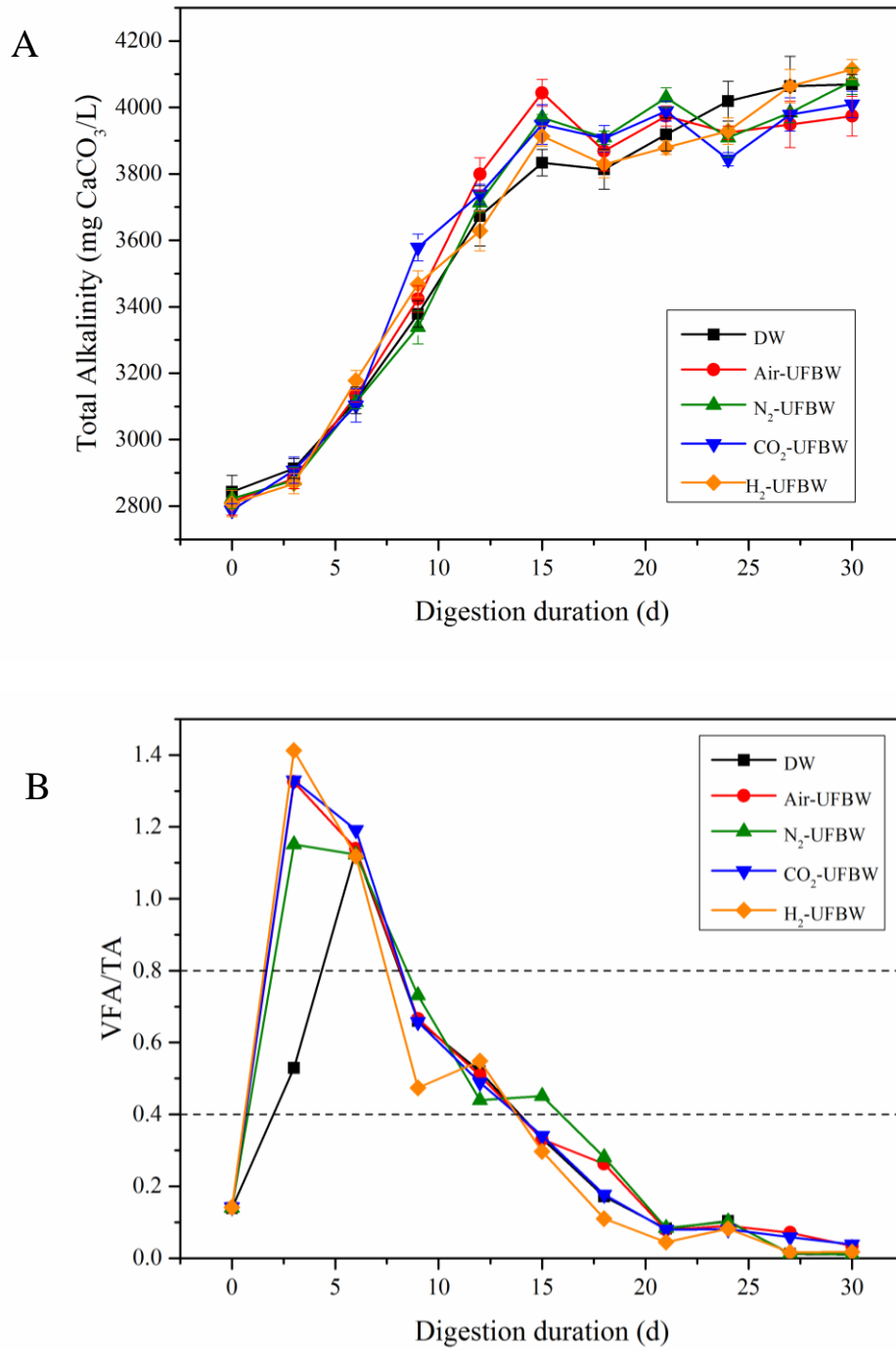


Figure 3-13. Alkalinity variations (A) and the VFA/TA ratio (B) during the whole AD trails of WAS with and without UFBW addition.

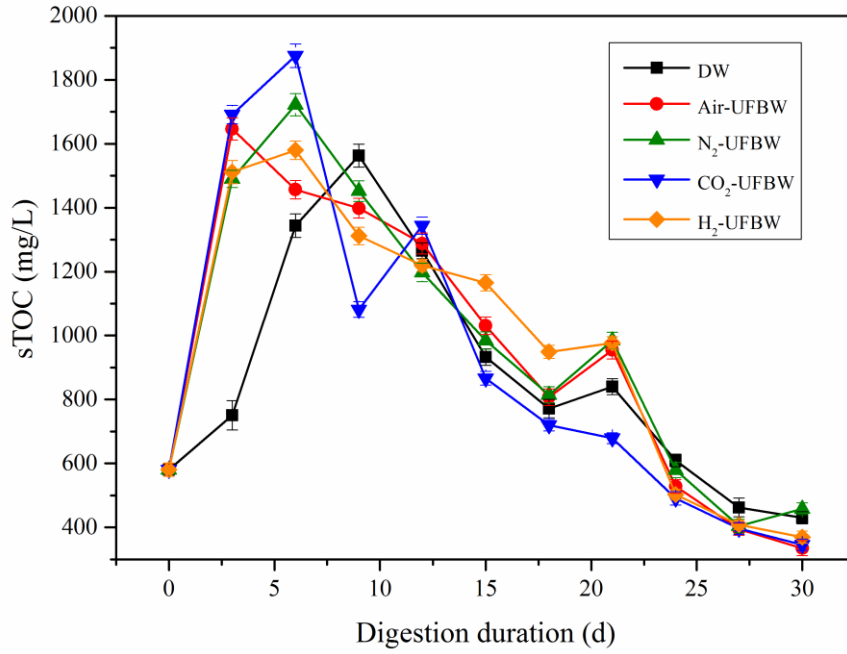


Figure 3-14. sTOC variation curves during the AD process of WAS with and without UFBW addition.

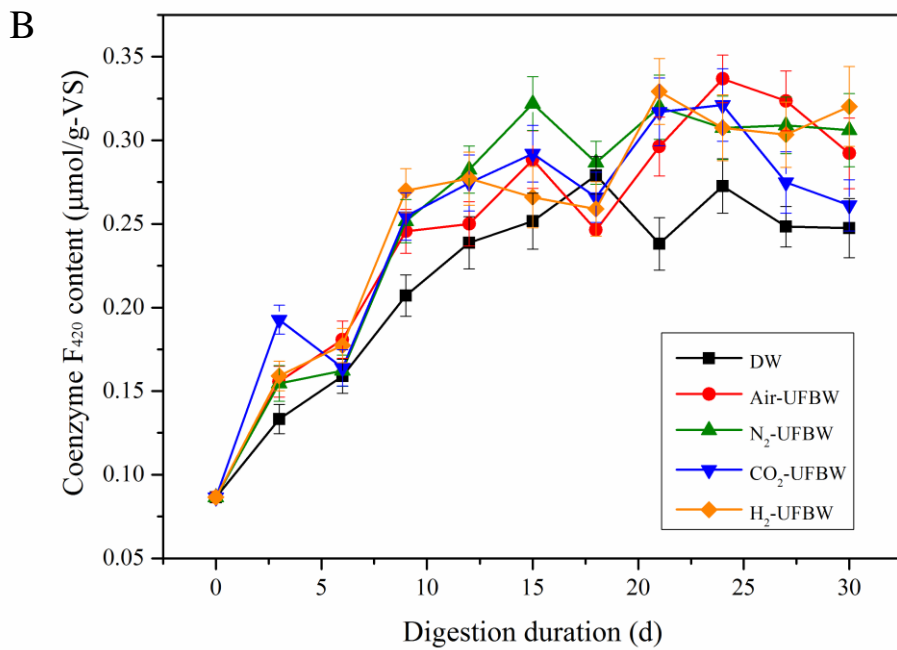
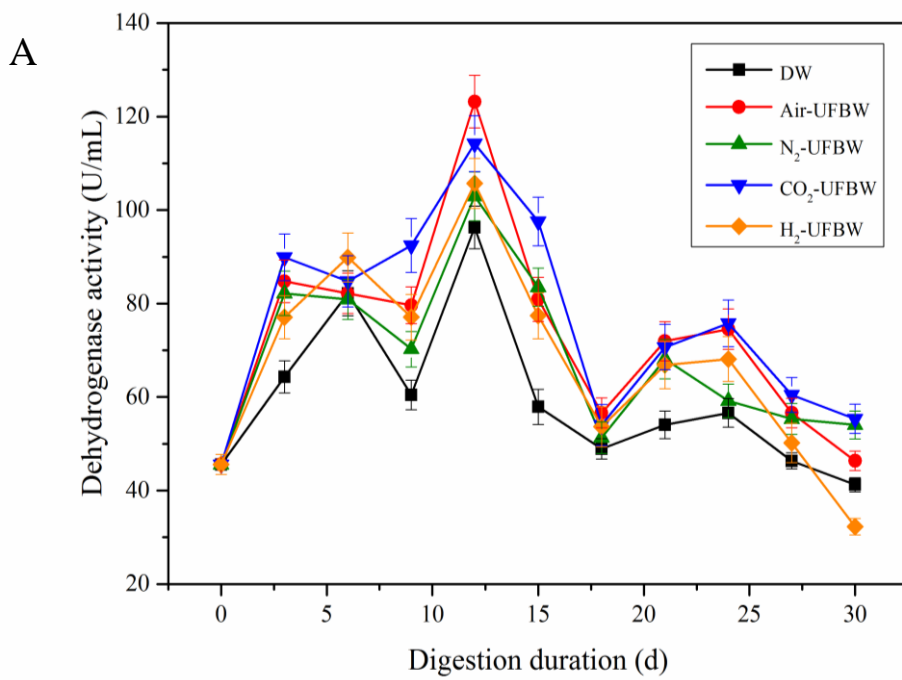


Figure 3-15. Activities of dehydrogenase (A) and content of CoF₄₂₀ (B) in the whole AD process of WAS with and without UFBW addition.

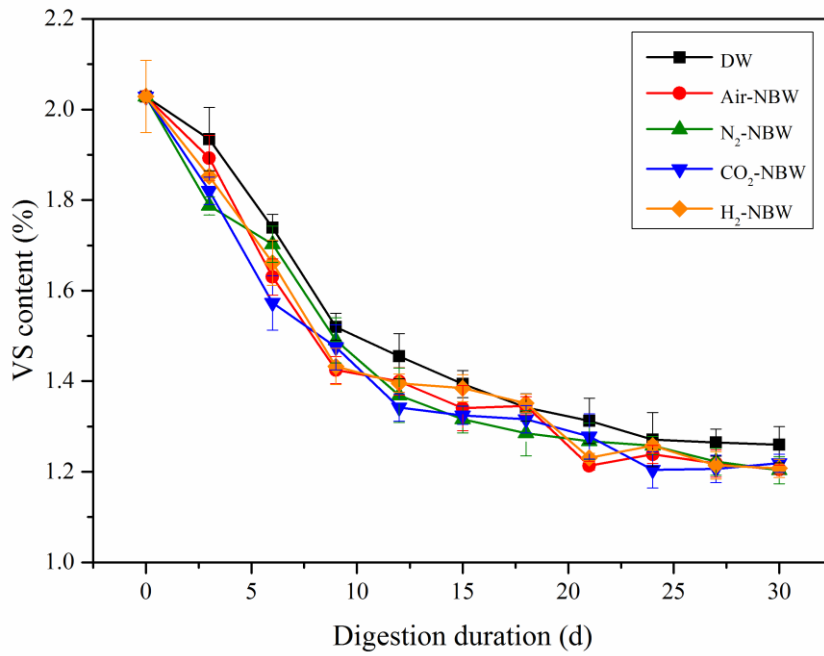


Figure 3-16. Reduction of VS after the whole AD process of WAS with and without UFBW addition.

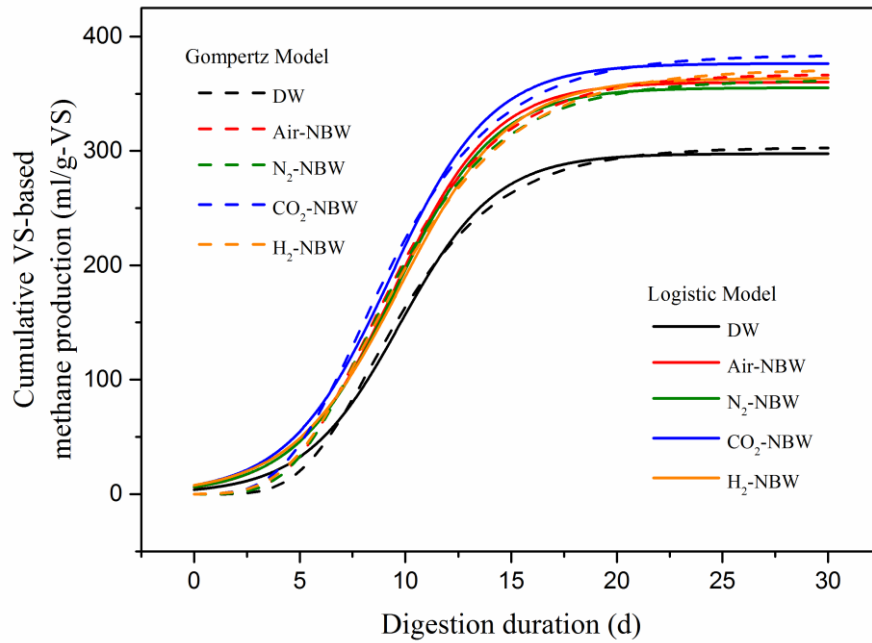


Figure 3-17. The modified Gompertz model and the Logistic model fitting curves for CH₄ production from WAS with and without UFBW addition.

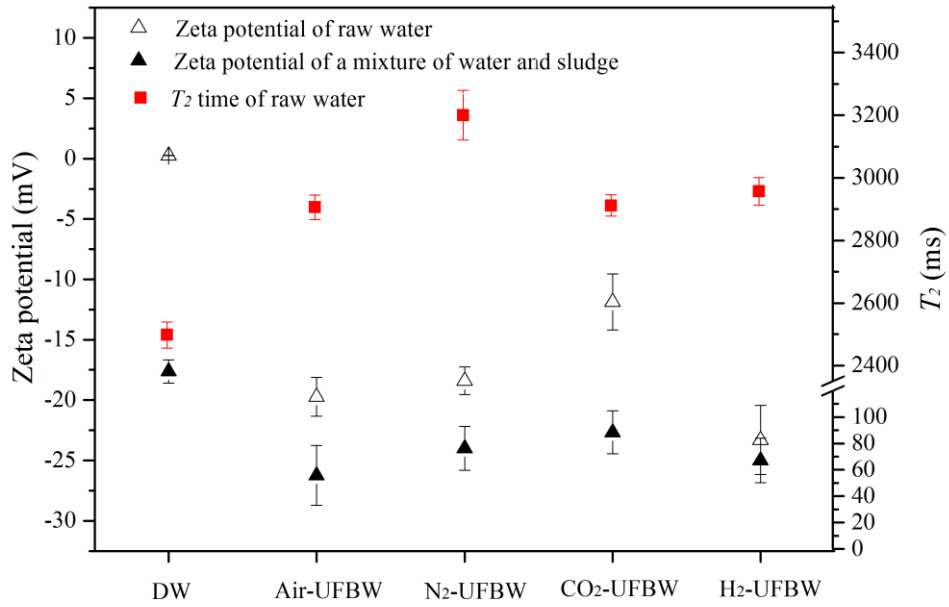


Figure 3-18. Zeta potential of the raw water (DW and different UFBW) and the mixture (sludge with DW and different UFBW), and T_2 time of the raw water (DW and different UFBW), and all without pH adjustment.

Table 3-1. Parameters estimated from the Logistic and modified Gompertz models for CH₄ production from WAS with and without UFBW addition.

Conditions	DW	Air-UFBW	N ₂ -UFBW	CO ₂ -UFBW	H ₂ -UFBW
Gompertz model					
P_{max} (ml CH ₄ /g-VS)	303.09 ± 4.29 ^b	367.10 ± 5.51 ^a	361.73 ± 5.31 ^{ab}	383.80 ± 5.76 ^a	371.47 ± 6.79 ^a
R_{max} (ml CH ₄ /g-VS d)	32.99 ± 2.23	38.48 ± 2.69	37.83 ± 2.59	39.30 ± 2.72	35.68 ± 2.79
λ (d)	4.99 ± 0.32	4.57 ± 0.35	4.56 ± 0.34	4.20 ± 0.35	4.40 ± 0.42
R^2	0.9941	0.9933	0.9935	0.9931	0.9910
Logistic model					
P_{max} (ml CH ₄ /g-VS)	297.63 ± 5.61 ^b	360.16 ± 7.20 ^a	355.2 ± 6.85 ^{ab}	376.44 ± 7.65 ^a	363.52 ± 8.34 ^a
R_{max} (ml CH ₄ /g-VS d)	32.94 ± 3.36	38.42 ± 4.08	37.54 ± 3.82	39.25 ± 4.20	35.54 ± 4.00
λ (d)	5.23 ± 0.52	4.81 ± 0.56	4.78 ± 0.54	4.45 ± 0.57	4.66 ± 0.64
R^2	0.9867	0.9848	0.9858	0.9839	0.9813
P_{max} from the AD trials (ml CH ₄ /g-VS)	304.08 ± 10.78 ^b	369.47 ± 7.92 ^a	361.86 ± 11.52 ^{ab}	387.86 ± 2.92 ^a	372.77 ± 10.14 ^a

Data in the table are expressed as Means ± SD, and the different letters (a, b) indicate significant difference at 5% level ($p < 0.05$).

Chapter 4 Enhancement of trace metal elements uptake by microorganisms in AD system with UFBW addition

4.1 Introduction

The last part, the effects of UFBW on AD of WAS in both hydrolysis stage and the whole stage were investigated. As demonstrated in Chapter 3, with the addition of UFBW, the rate and production of hydrolysis and methane fermentation enhanced. The observation in this part may be attributable to the existing nanoscale UFBs in the aqueous solution, which plays an important role in the AD process. UFBs may affect the physical properties of aqueous environment because the negatively charged UFBs may contribute to the weakening or destruction of the hydrogen bonding network in the bulk water, which could reduce the water viscosity and accelerate the mobility of water molecules. The increased mobility of water molecules would accelerate the rate of mass transfer of nutrients from liquid to bacterial cells, which further has an indirectly stimulatory effect on improving the activity of microorganisms and enzymes, facilitating the decomposition of the substrates with the improvement of biogas production.

In this chapter, we attempted to carry out batch experiments to investigate whether the uptake of trace metals by microorganisms increases under the condition with UFBW.

4.2 Materials and methods

4.2.1 Raw materials

The acclimated inoculum was collected from the domesticated digestive sludge which has been well adapted for methanogens cultivation by using acetic acid as the only carbon source and medium [108] for one month. The water samples used in the experiment were DW (as the control) and different UFBW (including CO₂-UFBW, H₂-UFBW, Air-UFBW and N₂-UFBW). The preparation of nutritive salt solution (medium) was prepared by using different UFBW and DW (as control). Acetic acid was used as the only carbon source with the concentration of 2 g/L in each reactor.

4.2.2 Medium preparation

The medium with the same components as the medium for methanogens cultivation as mentioned in 4.2.1., was prepared by DW and different UFBW, respectively. For the medium with UFBW, the components contained in the medium were first dissolved in 1.5 L of distilled water. Then each solution was used to prepare UFBW with UFBW generator followed the method above by introducing different kinds of gases.

4.2.3 Experimental procedure

Figure 4-1 shows the experimental procedure. Fed-batch fermentation experiments (semi-continuous tests) were conducted under mesophilic conditions ($36 \pm 2^\circ\text{C}$) for about half a month. The 100 mL serum bottles were used in the anaerobic fermentation. For the whole process, three periods were conducted. Before the first period, 20 mL of inoculum and 60 mL of medium were mixed and added in each digester. Each serum bottle contained 0.378 g of TS or 0.150 g of VS. Then, all the reactors were carefully sealed and flushed with high purity nitrogen gas for 1 min to create an anaerobic environment. After that, at the beginning of each period a suitably additional carbon source of concentrated acetic acid was injected to make sure that the acetic acid concentration is close to 2g/L. In each reaction period, TS, VS, biogas and CH_4 production, VFAs and the bioavailability of trace metals were investigated.

4.2.4 Analytical methods

The related parameters including VFAs, TS, VS and dry weight were performed in accordance with the procedures described in Chapter 3.2.4. In order to analyze the trace metal elements in methanogens, the method for samples digestion and analysis for trace metals analysis was based on the previous study [109]. After adding phosphate buffer, Samples were then centrifuged at 5000 rpm for 10 min and the sediments were obtained. This process was repeated for three times. After that, 10% HCl with twice the sample volume was added and then centrifuged at 300 rpm for 5 min, which could elute the extracellular metal [110]. After extraction, the extracellular metals were separated from the biomass into the digestate supernatant while the intracellular metals remained in the biomass. Then the treated biomass was placed in a 25 ml Teflon tube with 2 ml

concentrated HNO₃ added and then kept at 150°C for 15 min. Then the temperature was decreased and kept at 98°C for 6 h with 2 ml hydrogen peroxide addition until to the liquid becomes clarified. Trace metals of Fe, Co, Ni, Cu and Zn in samples were analyzed with ICP-MS spectrometer (PERKINELMER, ELAN DRC-e, JAPAN). Morphological characteristics of acclimated inoculum washed with phosphate buffer twice [109] were observed using scanning electron microscope (SEM) (JSM6330f, JAPAN).

4.2.5 Statistical analysis

The batch experiments in this part were performed in triplicate. Average value was taken for all the determinations and used for results and discussion. For data analysis, one-way analysis of variance (ANOVA) was used to analyze the statistical differences among the experimental scenarios by using Microsoft Office Excel 2013. Significant difference was assumed at $p < 0.05$.

4.3 Results and discussion

4.3.1 Biogas and CH₄ production

Figure 4-2 and Figure 4-3 show the daily and cumulative biogas production and CH₄ production from the AD of acetic-acid with and without UFBW addition, respectively. Total 14 days results were obtained with the first period lasted for 7 days, second and last period lasted for 4 and 3 days, respectively. From the daily results we can observe that in the 1st period the great difference of biogas production was observed between the experimental and control group. But in the 2nd and 3rd period, the difference is not obvious. Besides, the 1st period shows a longer duration time while less duration time was observed in the 2nd and 3rd period. The accumulated biogas production of N₂-UFBW group was the highest and 17.97% higher than that of control group, followed by the Air- (16.31%), CO₂- (15.42%) and H₂-UFBW (13.90%). For the accumulated CH₄ production, Air-UFBW group obtained the highest production and was 26.76% higher than control group, followed by the CO₂- (24.48%), N₂- (21.88%) and H₂-UFBW (19.09%). Figure 4-4 shows CH₄ content in different digesters each day. The greater difference of biogas production was observed between the UFBW and DW group in the 1st period than that in the 2nd and 3rd period. Results show that in the first 2 periods, the CH₄ content increased after adding

acetic acid each day, after reaching to the peak value (70%~80%), the CH₄ content decreased. This might be because after adding acetic acid there is sufficient organic carbon source available for methanogens. But at the 3rd period, when acetic acid was added into reactors, CH₄ content did not increase. This may be because although adequate carbon source exists, other nutrition elements such as N, P and trace elements closely related to the growth of microorganisms are insufficient that may inhibit the activity of CH₄ producing bacteria. However, there was no significant difference in CH₄ content between the experimental group and the control group.

4.3.2 VFAs variation

Figure 4-5 shows VFAs variation in each reactor from the AD of acetic-acid with and without UFBW addition at the beginning and end of the 1st, 2nd and 3rd period. At the beginning of each period, concentrated acetic acid was added into each reactor for timely replenishing the carbon source to make sure that the concentration of acetic acid at the beginning of each period is very high and approach to 2g/L. Some other VFAs can be detected in the initial samples at the beginning of the 1st period. While almost no VFAs could be observed at the end of each period. But at the beginning of the 2nd and 3rd period, no other VFAs except acetic acid can be detected. This is because all VFAs were consumed for the conversion of biogas in the previous stage, but only small amount of acetic acid was replenished in the new period.

4.3.3 Trace metals transformation analysis

According to previous researches [109, 111-115], trace elements of iron (Fe), cobalt (Co), nickel (Ni), copper (Cu) and zinc (Zn), which were closely related to the growth of methanogens, could be considered to be involved in many enzymatic processes and act in the methane formation during methanogenesis stage.

Figure 4-6 shows trace metals contents in the raw sample and final samples after 3 periods of AD with and without UFBW addition. After experiments finished, the intracellular trace metals concentrations increased in all groups but were relatively higher in UFBW groups. The uptake of different trace metals by microorganisms is different. Among these elements, Fe, Zn and Co increased greatly. The amounts of uptake follow the descending order of Fe > Zn > Co > Ni > Cu. Samples in UFBW groups took in more

trace metals than those in the control group. This might be because UFBs in the solution can affect the physical properties of the aqueous environment and improve the mobility and diffusion of the water molecules due to the longer proton spin relation time (showed in Figure 3-18) and further stimulate and promote mass transfer efficiency of nutrients into biological cells and improve the bioavailability of trace metals for methanogens. The negatively charged UFBs also might be as carriers that make a contribution for the nutrient to transport from extracellular to intracellular.

Figure 4-7 shows scanning electron microscope (SEM) images ($\times 10,000$ and $\times 20,000$) of the diversity and the structure of the main microorganisms in sediment samples after washed with phosphate buffer and immersed by HCl. We can observe 2 main species in pictures in the microscope, where morphological structures are very similar to the species of *Methanosarcina* and *Methanothrix*, which are typical species producing methane by utilizing acetate as the carbon source. This will be confirmed in the future study.

4.4 Summary

In this chapter, we investigate the bioavailability of trace metals under the condition with UFBW addition. Acetate-enriched seed cultures were used to investigate the uptake and mass transfer of trace metals for methane-producing bacteria. Results show that:

All UFBW groups showed an enhancement effect on biogas production, especially Air-UFBW, achieving a 26.76% increase in CH₄ production in comparison to the control group. A relatively higher difference of biogas production was observed between the UFBW group and the DW group in the 1st period than that in the 2nd and 3rd periods.

As one of the most directly bioavailable VFAs, acetic acid can be rapidly consumed for biogas production. The addition of acetic acid effectively enhanced biogas production. But the lack of other nutrient elements becomes the limiting factor for CH₄ production even with sufficient carbon sources.

After 3 periods, the contents of trace metal elements in biomass increased. The order of the uptake amount follows the descending order of Fe>Co>Zn>Ni>Cu. Samples in the UFBW group took in more trace metals than those in the control group.

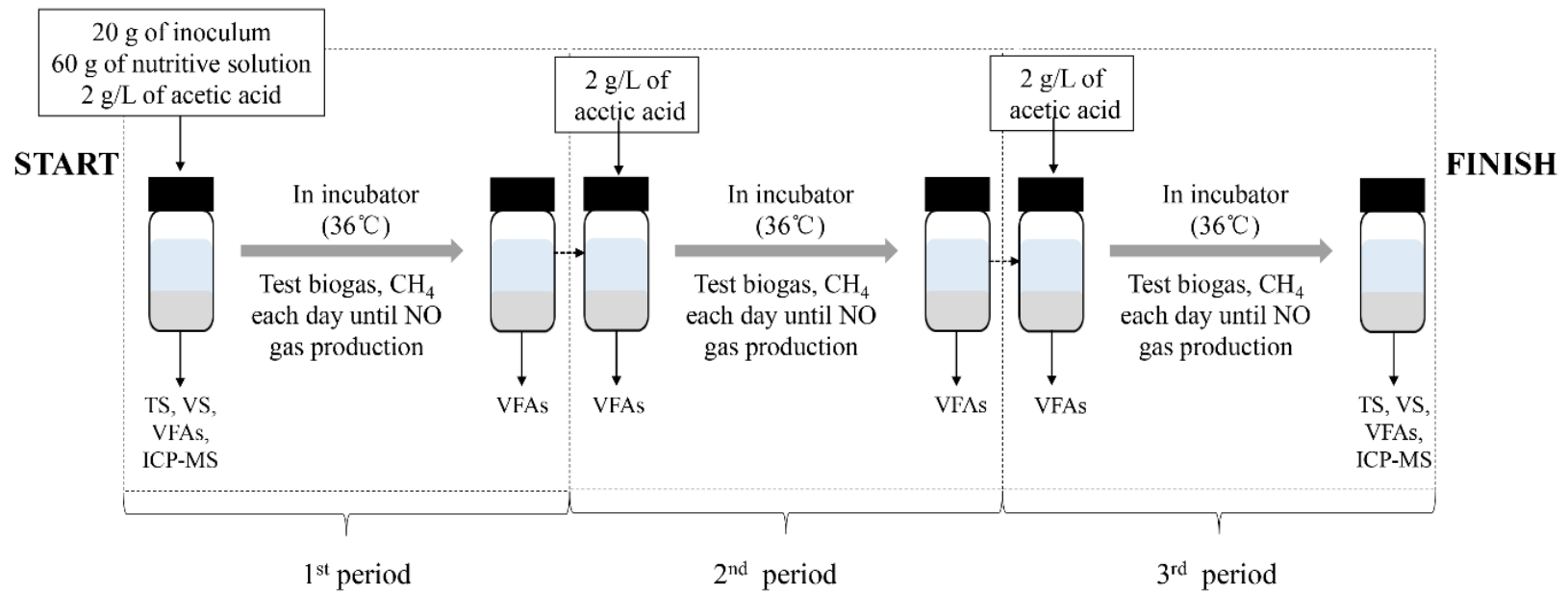


Figure 4-1. Experimental procedure of Chapter 4.

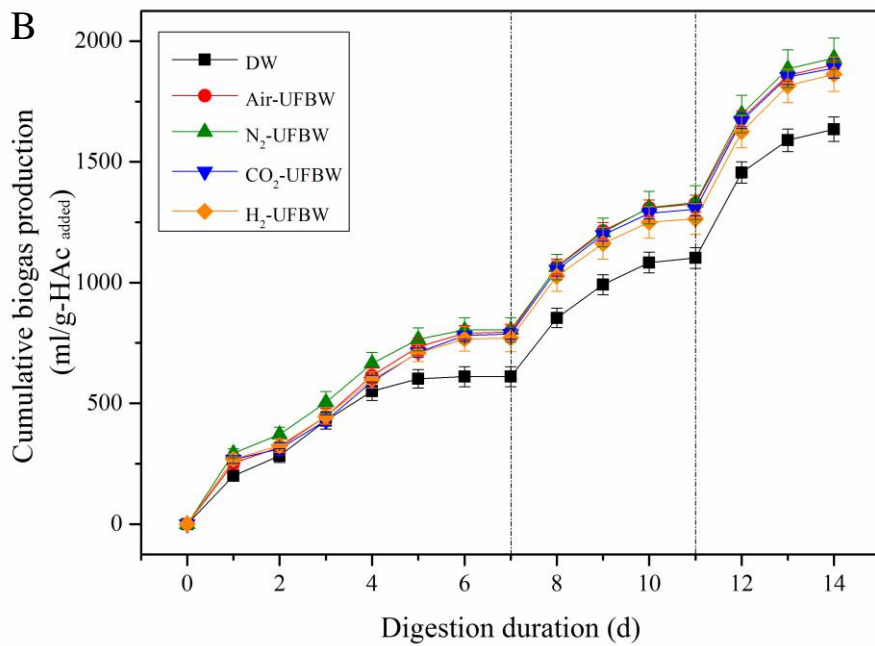
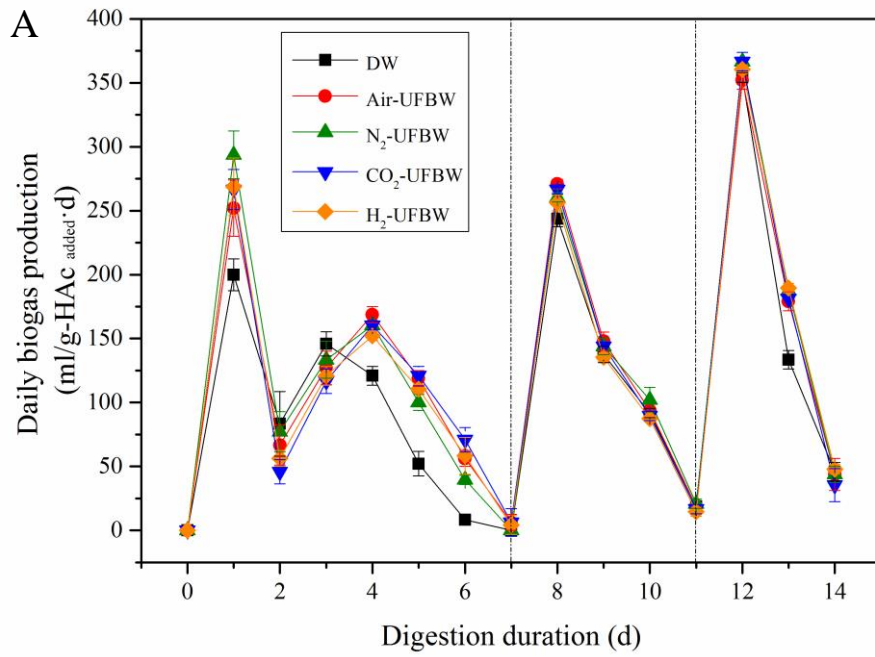


Figure 4-2. Daily (A) and cumulative (B) biogas production from the AD of HAC with and without UFBW addition.

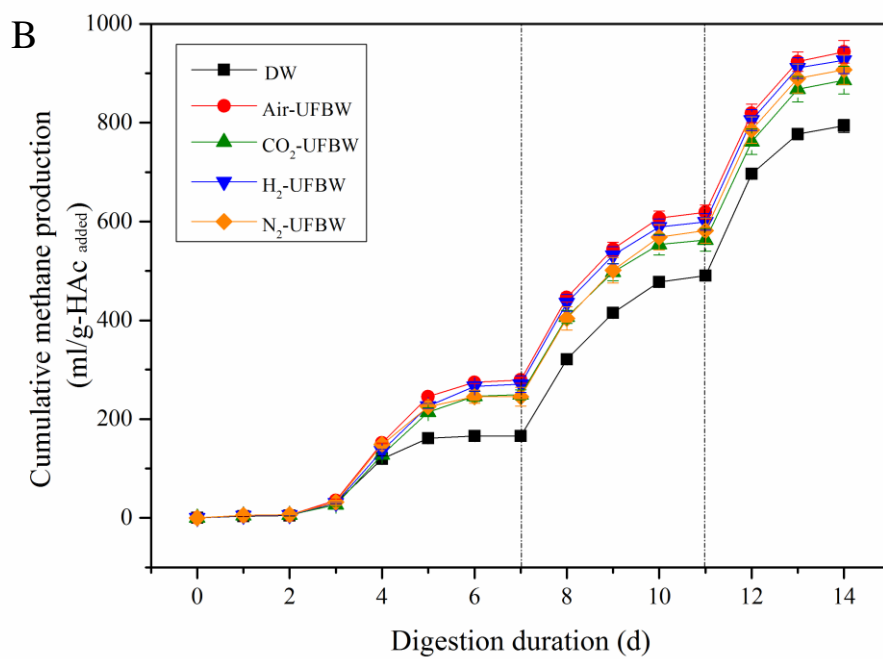
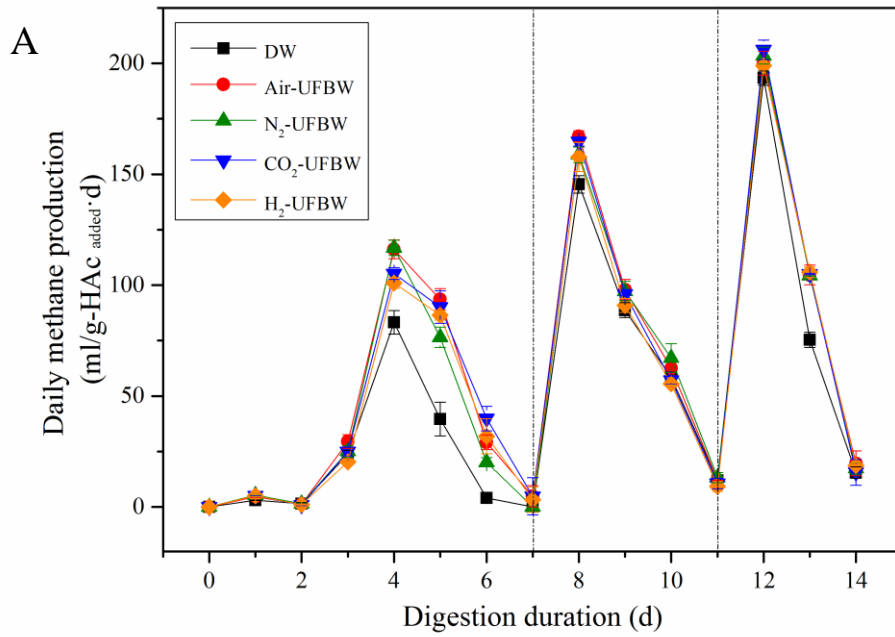


Figure 4-3. Daily (A) and cumulative (B) CH₄ production from the AD of HAc with and without UFBW addition.

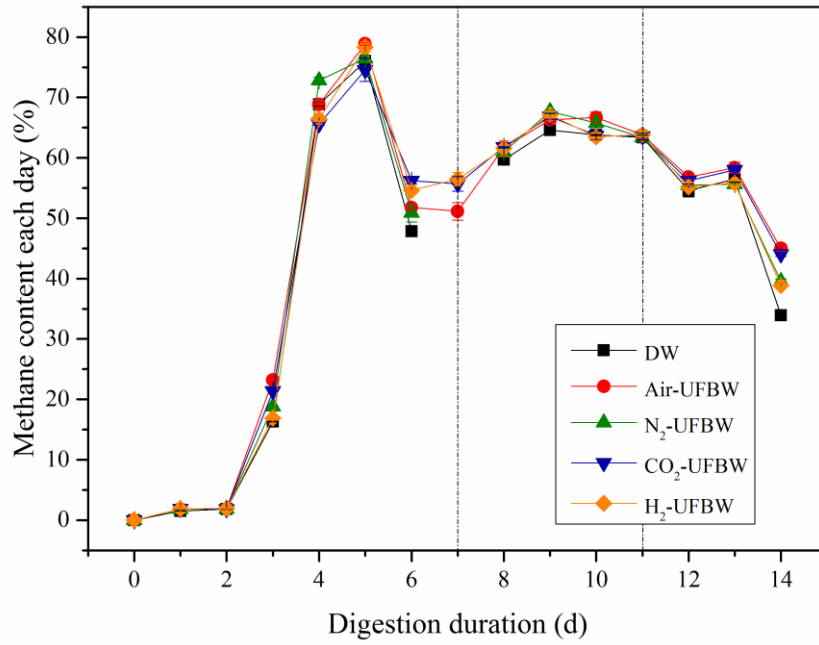


Figure 4-4. CH₄ content in biogas from each AD reaction fed with HAc with and without UFBW addition.

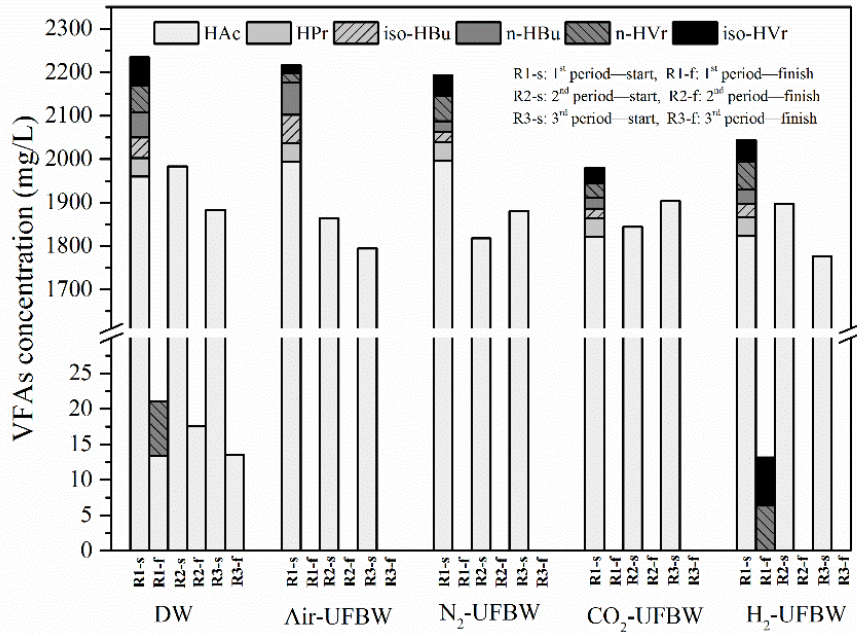


Figure 4-5. VFAs variation in each AD reactor fed with HAc with and without UFBW addition before and after the 1st, 2nd and 3rd period.

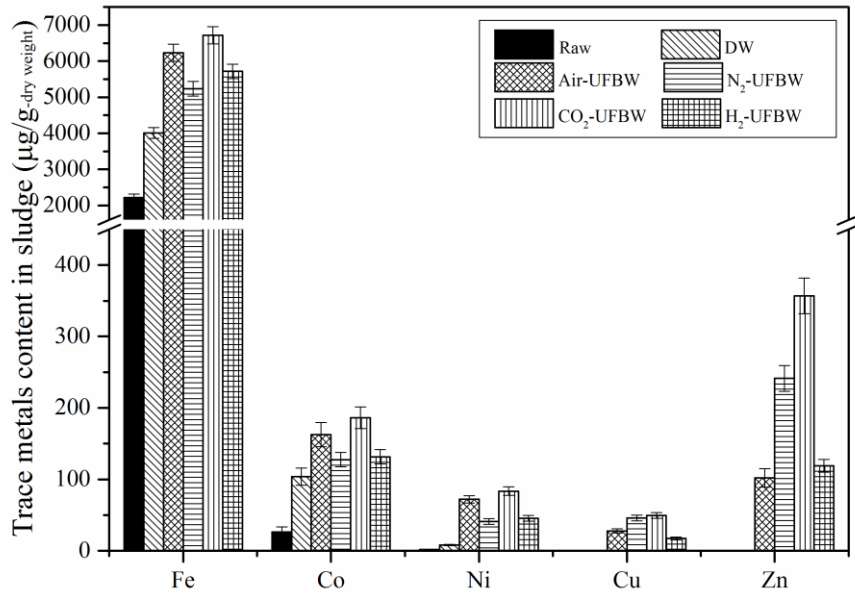


Figure 4-6. Trace metals contents in raw sample (inoculum) and final samples after 3 period of AD with and without UFBW addition.

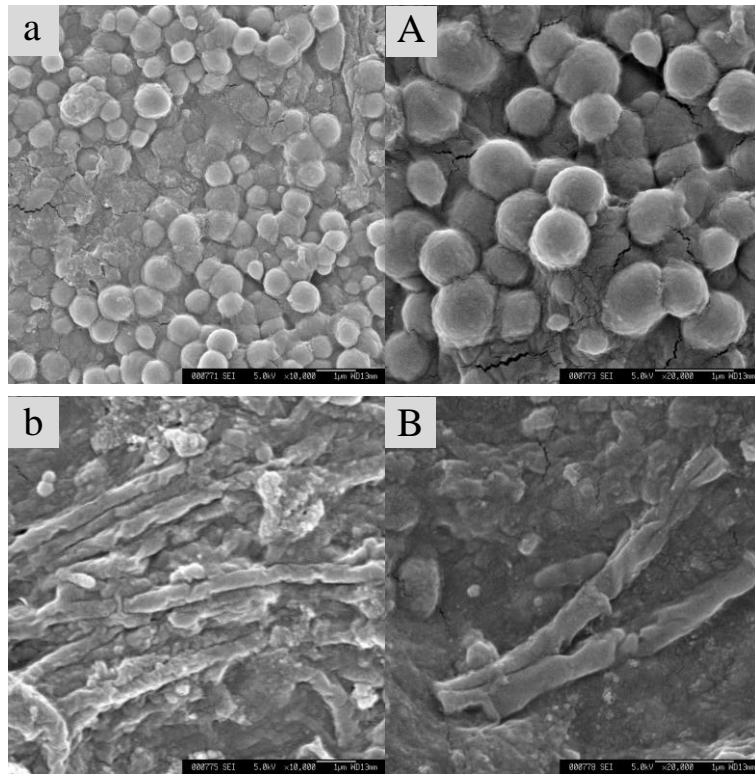


Figure 4-7. SEM photos of the diversity and the structure of the microorganisms ($\times 10,000$ (a, b) and $\times 20,000$ (A, B)).

Chapter 5 Conclusions and future research

5.1 Conclusions

In this research, four kinds of UFBW (Air-, N₂-, CO₂- and H₂-) were prepared in lab followed by the analysis of their physicochemical characteristics, and then they were first introduced into AD system to investigate their effects on both hydrolysis/acidification stage and methanogenesis stage.

5.1.1 Basic properties of UFBs and UFBW

The UFBs concentration increased with prolonging preparation time. The UFBs concentration in the UFBW was much higher than that in TW or DW, and the latter two have almost no UFBs detected. The UFBs number could maintain above 1.00×10^8 particles/ml in UFBW even after 2 weeks storage.

Zeta potential of UFBs was affected by pH value. All zeta potential values of UFBW were negative at pH 3.0 - 12.0. The zeta potential values were positively correlated with the number and concentration of UFBs, while no significant relationship was found between zeta potential and bubble diameter.

5.1.2 Effect of UFBW on AD of WAS

In the hydrolysis-acidification process, the biogas production from all the experimental groups, including H₂ and CO₂ production, were higher than that from the DW group. The VFAs concentration increased sharply during the first 3 days, and then increased gradually from day 3 to day 7 with the acetate accounting for almost half of the TVFAs in the UFBW groups. sTOC in the UFBW group increased higher compared with those in the raw material and control group. The reductions of soluble proteins and polysaccharides in all the experimental groups were greater than that in the control group. The addition of UFBW may promote the reduction of TB-EPS to LB- and soluble-EPS, especially during proteins reduction. Furthermore, the average VS reduction with UFBW addition was greater than that in control group, which is consistent with the changes of biogas production, VFAs, and sTOC.

During hydrolysis stage, the addition of UFBW may promote the degradation of EPS,

increasing the bioavailability of VS, resulting in the decrease of VS and the increase of sTOC and VFAs, thus the increase of biogas production.

During the whole AD process, compared with the control group, the production rate, the peak of daily production and total production of biogas and CH₄ were higher and the lag phase was shorter with no significant difference detected in the different gas-UFBW groups. The rate of accumulation and reduction of VFAs in UFBW groups, especially acetate, was faster and higher than that in the control group, leading to the higher production of CH₄. During day 7 to day 9, the maximum daily CH₄ yield was observed which corresponds to the rapid reduction of butyrate. While no acid inhibition and no big pH fluctuation occurred in this reactor which reflects the high stability of the reactor. All the digesters including the UFBW groups and the DW group kept a stable system or only a little unstable for most of digestion duration. Moreover, the groups with UFBW addition could shorten the unstable start-up stage (3 days) compared with the DW group (6 days).

During the whole AD process, compared to the DW group, the UFBW addition may improve the enzyme contents of dehydrogenase and CoF₄₂₀ then accelerate the conversion rate of macro organics to soluble organics resulting the higher sTOC peak accumulation rate and sTOC reduction rate. And faster VS reduction rate than that with the addition of DW, which means relatively faster hydrolysis and reduction rate of sludge was caused by UFBW addition, finally resulting in higher production of biogas and CH₄.

5.1.3 Enhancement of UFBW on trace metal elements uptake by microorganisms.

Compared with the control group, the addition of UFBW improved the biogas production, especially, Air-UFBW group achieved a 26.76% increase in CH₄ production. After 3 periods, the contents of trace metal elements in biomass were increased following a descending order of Fe > Zn > Co > Ni > Cu. Furthermore, more trace metals were uptaken by microbes in the UFBW groups than those in the control group.

Overall, in this study, the UFBW, with its unique physicochemical properties was introduced into AD system to investigate the effects on the AD process. The addition of UFBW was beneficial to the hydrolysis-acidification process and the whole AD process in view of the rapid reduction of VS and accumulation of sTOC and VFAs, the increase of biogas in the hydrolysis-acidification process, as well as, the higher enzymes contents, the stable pH and ALK condition, the higher biogas and CH₄ production, the larger trace

metal uptake during the whole AD process compared with the control group. This study provides opportunities for the application of UFBW in the AD system based on the unique physicochemical characteristics of UFBW and promoting CH₄ production.

5.2 Future research

To make a full understanding of the effect and potential application of UFBW on AD, future research should be followed up at least in the following two aspects.

5.2.1 Substrate categories

In future research, complex substrates, such as manure, straw, kitchen waste, yard waste, etc. can be selected to degrade and to study the effect of UFBW on their degradation rate.

5.2.2 Other meaningful products from AD

The hydrolysis-acidification stage can be further focused on studying degradation rate, VFAs recovery and H₂ production of complex substrate.

5.2.3 Mechanisms involved

The following hypotheses could be taken into account to be further confirmed:

(1) The negatively charged UFBs in the liquid may contribute to the weakening of the hydrogen bonding network in the bulk water and then accelerate the mobility of water molecules. The increased mobility of water molecules would accelerate the rate of mass transfer of nutrients from liquid to bacterial cells, which further has an indirectly stimulatory effect on improving the activity of microorganisms and enzymes, facilitating the decomposition of the substrates with improved biogas production.

(2) The moderately level exogenous ROS produced by UFBW plays an important role in cell growth, which also provides another reasonable explanation for physiological promotion and oxidation effects of UFBs.

(3) The nanoscale-UFBs in liquid might be considered as carriers for nutrient transport from the extracellular to the intercellular due to its negatively charged surface, hydrophobic nature, high viscous and irregular Brownian motion.

(4) The negatively charged UFBs also might be considered as an electron transfer

mediator to promote the direct interspecies electron transfer, which will facilitate creating electrical connections among syntrophic microorganisms and therefore achieve faster recovery of soured reactors and stimulate methane production.

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Appendix

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