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

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Abstract

In this study, CH_4 production from anaerobic digestion (AD) of refractory cellulose was investigated at a high loading of 3.5 ($VS_{cellulose}/VS_{inoculum}$) under nanobubble water (NBW) addition. A longer proton spin-spin relaxation time (2611–2906 ms) of NBW during 35 days' storage reflected its high mobility and diffusion of water molecules. Higher volatile fatty acids were yielded at the hydrolysis-acidification stage under NBW addition. Methanogenesis tests showed that Air-NBW and CO_2-NBW supplementation accelerated the utilization of crystalline cellulose, achieving methane yields of 264 and 246 mL CH_4/g-$VS_{reduced}$, increasing by 18% and 10% compared to deionized water addition (the control), respectively. In addition, under NBW addition the cellulose crystallinity reduction was enhanced by 14–20% with microbial community being enriched with hydrolytic and methanogenic bacteria. Results from this work suggest that NBW environment with no chemical addition and relatively low energy consumption is advantageous for enhanced AD process of cellulosic biomass.

Keywords: Nanobubble water; Proton spin-spin relaxation time; Anaerobic digestion; Cellulose crystallinity; Methane production

1. Introduction

Lignocellulosic biomass is likely to be the most abundant natural resource for CH_4 production (Li *et al.*, 2018a). As reported, cellulose is one of the most abundant, cheapest and easily available sources in nature, accounting for 40–50% of lignocellulosic biomass (Cocero *et al.*, 2018). Biogas from anaerobic digestion (AD) of cellulose is becoming more competitive due to the fact that the annual cellulose production is approximately 1.8×10^{11} tons (t) estimated from plants (Sundarraj and Ranganathan, 2018), thus cellulose is an important source of biomass energy. On the other hand, the huge amount of annual lignocellulosic biomass production may bring about environmental pollution and waste of resources if not being properly treated. In this context, AD is usually applied to treat lignocellulosic biomass for renewable energy (mainly CH_4) recovery.

To be specifically, cellulose, a type of linear polysaccharides, is composed of several glucose units linked by β-1,4 glycosidic bonds. The saccharide chains are connected by hydrogen bonds and aggregated to form a three-dimensional structure of fibrils, which can be characterized by toughness and water insolubility (Wang and Zhang, 2013). However, since its commercial success has been hampered, bioenergy is yet to compete with petroleum-based fuels due to the high cost of various processes, especially for the pretreatment and enzymatic digestion of lignocellulosic biomass (Nguyen *et al.*, 2016). Therefore, it is still necessary to explore new technologies to produce renewable energy from lignocellulosic biomass more efficiently and economically.

Various methods have been attempted on lignocellulose to improve its CH_4 production efficiency. Acids and alkalis are widely used as pretreatment to improve CH_4 production from AD of lignocellulosic biomass (Monlau *et al.*, 2012). Fungal pretreatment can also accelerate methane production from AD of rice straw due to its enhancement effect on hydrolysis (Kainthola *et al.*, 2019). However, these methods may bring about secondary pollution, a large quantity of chemicals consumption and high operation cost. These pretreatment methods to a great extent make them difficult to be compensated by the increased CH_4 yield. In addition, most researches on methane production from AD were conducted at low feed-to-microorganisms (F/M) ratios (≤ 2 (volatile solids (VS) basis)) (Xu *et al*., 2018; Díaz *et al*., 2011). The major reason is associated with the inhibition by fatty acids (propionic and valeric acid) accumulated during AD of excessive lignocellulosic biomass, which also makes properly mixing difficult to advance smoothly (Alzate *et al*., 2012). On the other hand, the oligosaccharides produced during high loading cellulose (F/M=3.5 (VS basis) in this study) degradation can create mass transfer resistance, and reduce the accessibility of water molecules to the surface of the cellulose particles (Vaquerizo *et al.*, 2018), thus hindering cellulose degradation to produce methane. Nanobubble water (NBW) has been reported to have high-diffusion water molecules, which may break down the oligosaccharide layer produced at high cellulose loadings. In this study we attempted to increase methane production from high loading cellulose through NBW addition, and at the same time explored both high loading cellulose

degradation mechanisms and the effect of NBW on methane production from cellulose via AD.

As reported, NBW technology has been widely applied in various fields, including water purification, biomedical engineering, agricultural and marine environments (Agarwal *et al.*, 2011). NBW contains bubbles with diameter smaller than 1000 nm and possesses unique properties such as long-term stability, high zeta potential value, and free radicals generation (Liu *et al*., 2017). One of the most amazing properties is its improvement effect on the physiological activity of living organisms (Ebina *et al.*, 2013). NBW supplemented for AD enhancement does not bring about additional pollutants due to no chemical addition and easy operation at relatively low energy consumption, which is consistent with the sustainable development goals (SDGs). NBW can promote the mass transfer of organics from liquid phase to microbial cells, which may enhance the bioactivity of microorganisms, promoting decomposition of substrates with improved biogas production from AD of waste activated sludge (Wang et al., 2019). In addition, Yang et al. (2019) found that NBW addition improved methane yield by enhancing not only the disintegration of high molecular weight compounds (proteins and polysaccharides) but also volatile fatty acids (VFAs) production during AD of waste activated sludge. The sludge usually contains a high cellulose content, accounting for 32–38% of the organic mass (Ruiken et al., 2013). NBW may have great potential for lignocellulosic biomass degradation with high adaptability due to its high mobility of water molecules. It thus is speculated that NBW could probably enhance cellulose conversion during AD at

high cellulose loading. Up to the present, however, little information is available regarding the impact of NBW addition on AD of refractory lignocellulosic biomass, especially under high cellulose loading conditions.

In order to find out the cost-effective and environment-friendly process for the utilization of large amount of lignocellulosic biomass, in this study, the effects of Air-NBW and CO_2-NBW on the biological activity of anaerobic microorganisms were investigated with cellulose as the sole substrate. It is expected that Air-NBW or CO_2-NBW would enhance CH_4 yield from AD of cellulose, which may provide an effective means to sustainably manage the huge amount of lignocellulosic biomass and reduce the fossil fuel demand. In this study, much attention was paid to the hydrolysis-acidification stage and methanogenic stage of AD of cellulose, respectively. Namely, VFAs accumulation and pH changes in the hydrolysis-acidification stage, and cumulative CH_4 production and cellulose crystallinity reduction in the methanogenic stage were determined to evaluate the effect of NBW addition on AD of cellulose. In addition, bacterial and archaeal communities were analyzed to elucidate the possible mechanisms involved in the NBW-supplemented AD process. The insights gained from this work could lay the foundation for efficient bioconversion of lignocellulosic biomass into renewable energy.

2. Materials and methods

2.1 Materials

Anaerobically digested sludge was sampled from the Shimodate sewage treatment plant in Ibaraki, Japan, and stored in a refrigerator at 4°C before the experiments. The sludge was filtered through a 1-mm sieve before use and acclimated in an incubator at 35±1°C for 7 days to activate microbial activity and decompose the remaining biodegradable organics. Cellulose used in this study was purchased from Sigma-Aldrich (Avicel® PH-101). The preparation of NBW was described elsewhere (Wang *et al.*, 2019). The laboratory air was directly used for production of Air-NBW. CO_2 (purity ≥ 99.9995%) purchased from Taiyo Nippon Sanso Co., Ltd., Japan was used for CO_2-NBW production.

2.2 Batch AD tests

2.2.1 Hydrolysis-acidification stage of AD of cellulose

Batch experiments were performed using 120 mL closed Schott Duran serum bottles. Each bottle was loaded with 30 mL of digested sludge mixed with 30 mL of CO_2-NBW, Air-NBW or deionized water (DW). The addition of DW instead of NBW was deemed as the control. Previous researches on microbial lignocellulose degradation were conducted at F/M ratio ≤ 2 (VS based) (Xu *et al*., 2018; Díaz *et al*., 2011). In this study cellulose as the sole substrate was added into each AD reactor to achieve a higher F/M ratio of 3.5. To elucidate the effect of NBW on the hydrolysis-acidification stage of AD, 10 mM sodium 2-bromoethanesulfonate (BES) ($C_2H_4BrNaO_3S$, Tokyo Chemical Industry Co., Ltd, Japan), a coenzyme M analog, was added into each reactor to inhibit CH_4 production from methanogens. The

reactors without cellulose addition were also prepared as blanks in order to exclude the VFAs production from the residual organics in the digested sludge from the total VFAs production under NBW or DW addition condition. Before starting the AD process, the initial pHs in all the reactors were adjusted to 7.0 with 2.0 M NaOH or HCl. Then all the reactors were sealed, evacuated and flushed with N_2 gas for 5 min to create anaerobic condition. The experiments were conducted in an incubator at 35±1°C for 16 days. The bottles were shaken manually for 2 minutes right after the setup of the AD experiments and before each sampling time. All the experiments were performed in triplicate.

2.2.2 Methanogenesis of cellulose

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

2.3 Calculation

Net CH_4 production (mL/g-$VS_{reduced}$) from cellulose at all conditions was calculated according to Eq. (1) (He *et al.*, 2016).

CH_4(mL/g-$VS_{reduced}$) = {[CH_4(mL, cellulose+inoculum)]-[CH_4(mL, inoculum)]}/Reduced biomass(g-VS) (1)

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

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

$$TMP=22.4\times(\frac{1}{2}a+\frac{1}{8}b-\frac{1}{4}c)/(12a+b+16c) \quad (3)$$

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

Anaerobic biodegradability (BD) of cellulose can be estimated by comparing the experimental methane production (EMP, total CH_4 yield) with TMP (Raposo *et al.*, 2011) according to Eq. (4).

$$BD\ (\%) =100\times EMP/TMP \quad (4)$$

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

$$M(t) = P \times exp\{-exp[1 + R_{max}e(\lambda - t)/P]\} \quad (5)$$

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

Cellulose content was determined as follows: the samples were dried at 60±2°C to constant weight and milled to powders (0.5 mm), then boiled with 5 mL 72% w/w

H_2SO_4 solution for 4.5 h in order to hydrolyze the cellulose to glucose (Ververis *et al*., 2007). Glucose in liquid was determined with a dinitrosalicylic acid colorimetric method (Mendel *et al*., 1954). Cellulose content was calculated according to Eq. (6).

$$\text{Cellulose content (\%, w/w)} = 100 \times (0.9/0.96) \times (C_1 - C_0) \times V/M \quad (6)$$

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

2.4 Analytical methods for general properties of biomass and NBW

Total solids (TS) and volatile solids (VS) of the mixture were determined according to Standard Methods (APHA, 2012). pH value was measured with a semi-solid pH meter (Testo 206, Germany). VFAs including acetic (HAc), propionic (HPr), iso-butyric (iso-HBu), n-butyric (n-HBu), iso-valeric (iso-HVa) and n-valeric acid (n-HVa) were quantified using a gas chromatography (GC-8A, SHIMADZU CO., Japan) equipped with a flame ionization detector (FID) and a Unisole F-200 30/60 column. The pressure of carrier gas N_2 was maintained at 200 KPa. The temperatures of column and detector/injector were fixed at 150°C and 180°C, respectively. Biogas composition mainly including CH_4 and CO_2 was determined using a gas chromatography (GC) (GC-8A, SHIMADZU CO., Japan) packed with a thermal conductivity detector (TCD, 80°C) and a Porapak Q column (60°C). The method for

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

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

2.5 Microbial community analysis

To analyze the archaeal and eubacterial communities, the samples were collected from each bottle after 50 days' batch tests for methane production. The total DNA was extracted from the wet sludge sample using the E.Z.N.A.® soil DNA Kit (Omega

Bio-tek, Norcross, GA, US) according to manufacturer's instructions. DNA quality and quantity were checked by 2% agarose gel electrophoresis and NanoDrop 2000 UV-vis spectrophotometry (260 nm/280 nm ratio) (Thermo Scientific, Wilmington, USA). The V3-V4 region of bacterial 16S rDNA was amplified using the universal primer pair of 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The archaeal 16S rDNA was amplified with the primer pair of 524F10extF (5'-TGYCAGCCGCCGCGGTAA-3') and Arch958RmodR (5'-YCCGGCGTTGAVTCCAATT-3'). According to the standard protocol of Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China), the purified amplicons were combined on an Illumina MiSeq platform (Illumina, San Diego, USA) with equimolar and paired end sequencing (2×300 bp).

2.6 Statistical analysis

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

3. Results and discussion

3.1 Change in T_2 during 35 days' storage of NBW

Proton-nuclear magnetic resonance relaxation time can be used to detect interactions between weak molecules such as spatial effects and molecular mobility (Liu *et al.*, 2013). It has been widely used to explore the mobility and diffusion of the water molecules in the biological and chemical fields. Fig. 1 shows the changes of T_2 of Air-NBW, CO_2-NBW and DW at 35±1°C during 35 days' storage. The longest T_2 of NBW was detected right after NBW generation for 20 minutes. As shown, NBW formed from pressurized mechanical circulation, rotating flow and cavitation is a kind of liquid with high water molecule activity. After one day storage, the T_2 of Air-NBW decreased from 2906.0 ms to 2711.8 ms, while the T_2 of CO_2-NBW decreased from 2768.0 ms to 2657.7 ms. After that, their T_2 values kept relatively stable till day 21. From day 21 to day 28, the T_2 values decreased again to 2631.2 ms for Air-NBW and to 2611.0 ms for CO_2-NBW, respectively. From day 28 onwards, the concentrations of NBs in Air-NBW and CO_2-NBW were also detected to decrease slightly. This phenomenon was positively correlated with the changes of NBs concentration in the corresponding NBW: the correlation coefficients between the NBs concentration and T_2 value were found to be 0.74 (p = 0.030) for Air-NBW and 0.90 (p = 0.002) for CO_2-NBW, respectively. Besides, the absolute values of zeta potentials of Air-NBW (-19.4±1.8 ~ -25.0±1.2 mV) and CO_2-NBW (-18.2±1.9 ~ 21.9±1.8 mV) were always much higher than those of DW (-1.6±0.23 mV) during the 35 days' storage. As stated by Takahashi (2005), a higher zeta potential (absolute value) may bring about a high moving speed, which might be the reason for the higher mobility of water molecules in NBW after being stabilized. In general, a longer T_2 time indicates a higher mobility

and diffusion of water molecules (Liu *et al.*, 2013), which has been demonstrated to improve the penetration of water molecules into the cellulose matrix (Vaquerizo *et al.*, 2018). Thus, in this work, the longer T_2 of NBW (Fig. 1) is expected to have some enhancement effect on hydrolysis during the high cellulose loading AD process.

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

These batch tests were to identify whether NBW addition could contribute to methane production from AD at a high cellulose loading (F/M) of 3.5. Firstly, the effects of CO_2-NBW and Air-NBW on the hydrolysis-acidification stage of cellulose during AD were investigated by adding BES. VFAs yield and pH value were monitored during the 16 days' AD of cellulose and the results are shown in Fig. 2. As it can be seen, the VFAs yields (39. 7– 40.4 mg/g-cellulose$_{reduced}$) in all the reactors were detected to be significantly high during the initial 3 days. The reactors with Air-NBW addition achieved the highest VFAs yields, increasing by 30.3%, 17.8% and 17.3% compared to the control reactors with DW addition on day 6 (35.6 mg/g-cellulose$_{reduced}$), day 9 (33.6 mg/g-cellulose$_{reduced}$) and day 16 (16.9 mg/g-cellulose$_{reduced}$), respectively, followed by the reactors with CO_2-NBW addition, increasing about 11.4%, 13.0% and 16.2%. When compared to the reactors with CO_2-NBW addition, the reactors with Air-NBW addition increased by 17.0%, 17.8% and 0.9% on days 6, 9 and 16, respectively. Relatively low VFAs yields were detected in all the reactors on day 16, most probably due to the following two reasons: (1) after

16 days' AD test, the pH values decreased to 4.6–4.7 in all the reactors, which are not within the optimum range (5.5–6.5) for acidogenesis (Mao *et al.*, 2015); and (2) probably almost no hydrolyzable acidified cellulose was available. Generally, a higher VFAs production can yield a higher CH_4 production during the methanogenesis stage, thus the produced VFAs during the hydrolysis-acidification stage may function as a good indicator for the subsequent methanogenesis process. This observation might be the major reason for the Air-NBW reactors to achieve the highest methane yield.

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

(Fig. 2). Interestingly, Air-NBW addition achieved lower inhibition factors during the hydrolysis-acidification stage of cellulose (Fig. 2). Restated, the promotion effect of NBW on the metabolism of microorganisms during the hydrolysis-acidification stage of high cellulose loading AD is most probably resulted from its high mobility of water molecules. Therefore, NBW addition can favor the microbial decomposition of refractory cellulose to VFAs, which are then available to methanogens for more methane production.

3.3 Methane production

3.3.1 Effect of NBW on CH_4 production from cellulose

The effects of NBW addition on cumulative CH_4 production during AD are illustrated in Fig. 3a. The CH_4 production increased rapidly before day 40; after day 40, it reached a plateau. The CH_4 yields from the DW reactors (Control) were 223.3±0.1 mL/g-$VS_{reduced}$ after 50 days' AD of cellulose. As expected, the highest CH_4 yields, 263.6±10.9 mL/g-$VS_{reduced}$ ($p < 0.05$) were achieved in the Air-NBW reactors during the 50 days' AD, followed by the CO_2-NBW reactors (246.0±6.0 mL/g-$VS_{reduced}$), increasing by 18% and 10% when compared to the control. This observation was probably and mainly contributed by the enhanced hydrolysis-acidification of cellulose under NBW addition. As for the two kinds of NBWs, Air-NBW showed better enhancement effect on CH_4 production than CO_2-NBW, most probably owing to the microaerobic environment created by Air-NBW addition, favoring the growth of facultative anaerobes which may have synergetic utilization of

cellulose (Wushke *et al*., 2015) and then contribute to the enhanced hydrolysis-acidification process. As recently reported, microaerobic pretreatment by a pure bacterial strain achieved 17% increase in methane yield from corn straw (Xu *et al.*, 2018). Microaeration has also been claimed by Fu *et al.* (2016) to be responsible for the enhanced methanogenesis of corn straw. During the hydrolysis-acidification stage, the produced HPr and HVa might be consumed by the facultative anaerobes under Air-NBW addition, thus enhancing CH_4 production from the subsequent methanogenesis stage of high cellulose loading AD.

3.3.2 Kinetic analysis by the modified Gompertz model

The modified Gompertz model was applied to simulate the kinetic patterns of CH_4 production during the high cellulose loading AD with NBW addition. Table 1 lists the results of TMP, EMP, biodegradability, the maximum CH_4 production rate (R_{max}) and lag phase time (λ) for all the reactors, which are helpful to better understand the methanogenesis process of cellulose. According to Eq. (2), the TMP of cellulose used in this work was calculated as 414.8 mL/g-$VS_{reduced}$. The experimental CH_4 productions from the reactors with CO_2-NBW and Air-NBW addition were 59% and 64% of TMP, respectively according to Eq. (3) (Table 1) in comparison to 54% of TMP in the DW-reactors. This observation also suggests that NBW addition can enhance the CH_4 production from high cellulose loading AD. According to the results simulated from the modified Gompertz model, the λ values under NBW addition conditions were shortened to 0.3–0.8 day from 1.0 day with DW addition, implying

that the high cellulose loading AD process took a shorter start-up period when NBW was supplemented to the AD system. A shorter lag phase duration is critical to improve the economic benefits of the process since the resultant shorter AD duration can bring about a smaller AD reactor system and thus lower operation cost. Moreover, the R_{max} was determined as 13.3±0.9 mL/g-$VS_{reduced}$/day with the addition of Air-NBW, followed by 10.2±0.6 mL/g-$VS_{reduced}$/day and 10.1±0.6 mL/g-$VS_{reduced}$/day with the addition of CO_2-NBW and DW (Control), respectively. These results indicate that NBW addition could shorten the lag phase period and accelerate CH_4 production from the refractory cellulose. In addition, the simulated maximum cumulative CH_4 production (P) was quite similar with the experimental CH_4 yield, indicating the good fitness of the modified Gompertz equation to the experimental data (Table 1). This observation might be attributable to no or few inhibitory factors involved in the high cellulose loading AD process in this study, which is further evidenced by the high R^2 values (0.990–0.994) under the test conditions.

3.3.3 Changes in cellulose content and crystallinity degree

The results of refractory cellulose reduction during the methanogenesis stage are shown in Fig. 3b. Clearly, the highest cellulose reduction was detected in the Air-NBW reactors ($p < 0.05$). The cellulose contents in the Air-NBW and CO_2-NBW reactors were found to decrease sharply to 25.2±1.1% and 28.1±0.9% after 50 days' AD, resulting in 65% and 60% of cellulose reduction, respectively, about 29% and 21% higher than those in the DW reactors. It is also observed that a higher cellulose

reduction corresponded to a higher CH_4 production under the test conditions. Since cellulose was the sole substrate in these experiments, TOC removal was also correlated with the cellulose reduction as shown in Table 1. The TOC removals by the Air-NBW and CO_2-NBW reactors were 43.1±6.4% and 39.6±4.3%, about 12% and 3% higher than those by the DW reactors, respectively. In addition, VS reductions as shown in Table 1 also reflect the similar trend: A higher VS reduction (54–58%) was achieved in the NBW supplemented reactors in comparison to those with DW addition (45%). The above results imply that NBW addition enhanced cellulose reduction or degradation for methane production.

On the other hand, cellulose structure can be divided into two regions, namely the amorphous region that is easily digested by microorganisms and enzymes, and the crystalline region that is difficult to be digested (Gupta *et al.*, 2016). Table 1 also shows the crystallinity reduction of the samples after 50 days' AD. The XRD diffraction data indicate that the crystallinity reductions were 80.7±2.1% and 76.9±0.2% in the Air-NBW and CO_2-NBW reactors, increasing by 20% and 14% compared to the DW reactors, respectively. This observation implies that NBW addition could enhance the reduction of cellulose crystallinity, probably through improving the metabolic activity of hydrolytic bacteria or facultative anaerobes in the AD process. Crystallinity of cellulose is one of the limiting factors affecting its hydrolysis (Chávez-Guerrero *et al.*, 2019). Thus, the reduction of crystallinity might be the major reason for the promoted hydrolysis-acidification of cellulose under NBW addition and then increased VFAs production for methanogenesis.

3.4 Changes in microbial communities under NBW addition

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

3.4.1 Diversity of microbial communities

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

reactors with CO_2-NBW addition, implying that different gas NBW also has some effect on the species richness of both bacteria and archaea.

3.4.2 Bacterial phyla

To better understand the correlation between CH_4 production and microbial community structure in the high loading cellulose AD process with NBW addition, bacterial and archaeal communities were analyzed through Illumina sequencing. Fig. 4a shows the bacterial community at phylum level under the test conditions. The results indicate that *Aminicenantes*, *Spirochaetae*, *Parcubacteria*, *Bacteroidetes*, and *Chloroflexi* were the most dominant phyla in all the reactors, which are considered to be actively involved in AD process. *Aminicenantes* was found to be the dominant species in all the reactors, accounting for 8.3%, 20.5% and 21.9% in the DW, CO_2-NBW, Air-NBW reactors, respectively. *Aminicenantes* is claimed to be related to the production of VFAs (Robbins *et al*., 2016), which has been proven in this study that the reactors with NBW addition yielded higher VFAs during the hydrolysis-acidification stage of cellulose. *Spirochaetae* was found in all the samples, while the relative abundance of this phylum was rather high in the NBW supplemented reactors (7.4%, 11.0% and 5.7% for the CO_2-NBW, Air-NBW, and DW reactors, respectively). This phylum participates in the decomposition of lignocellulose to simple compounds (Zhao *et al.*, 2018). *Parcubacteria* is also considered to contribute to CH_4 yield from cellulose (Dai *et al.*, 2016). Besides, *Bacteroidetes* can play an important role in the degradation of complex polymers, residual stubborn substances

and high molecular weight organic compounds (Liu *et al.*, 2016). The metabolic products are beneficial for enhanced biogas production from high cellulose loading AD. The enrichment of all these bacteria implies their important contributions to AD of cellulose, especially under Air-NBW addition condition which may be partially contributed by its microaerobic environment. A higher relative abundance (RA) of *Chloroflexi* was noticed in the NBW reactors, which can primarily utilize various carbohydrates during the initial stage of AD, i.e. hydrolysis and acidification (Ruan *et al.*, 2019).

3.4.3 Archaeal genera

As it is known, archaea play a critical role in AD process. The major archaea at genus level in all the reactors are shown in Fig. 4b. The RA of *Methanosaeta* in the Air-NBW (73.4%) and CO_2-NBW (52.7%) reactors were 63.8% and 17.6% higher than that in the DW reactors (44.8%), respectively. *Methanosaeta*, belonging to the order *Methanosarcinales*, is an obligate aceticlastic methanogen which can produce CH_4 through direct electron transfer (DIET) (Rotaru *et al.*, 2014). *Methanosaeta* can efficiently utilize HAc to produce CH_4 (Chen *et al.*, 2016), which might explain why little accumulation of HAc was detected in the methanogenesis at high cellulose loading in this study (data not shown). In the DW reactors, no *Methanospirillum* was observed; however, it appeared in the Air-NBW and CO_2-NBW reactors with RA of 0.04% and 0.09%, respectively. *Methanospirillum*, a genus of strictly mesophilic hydrogenotrophic methanogens, can utilize H_2 or formate to produce CH_4 (Jiang *et*

al., 2018). This genus belongs to the family of hydrogenotrophic methanogen and is involved in the interspecies hydrogen transfer (Lin *et al.*, 2019). The enrichment of *Methanomassiliicoccus* in the NBW reactors may also contribute to enhanced CH_4 production by using hydrogen as the substrate (Li *et al.*, 2018b). These observations may explain why almost no H_2 was detected throughout the whole methanogenesis of cellulose. It was found that the Air-NBW reactors had the highest RA (1.01%) of *Methanobacterium* among the test reactors. Fu *et al.* (2016) reported that the RA of *Methanobacterium* was doubled under microaerobic condition. Thus the microaerobic environment created by Air-NBW addition might contribute to the shift of microbial community and promote the CH_4 formation. In addition, *Methanolinea* can utilize CO_2 to produce CH_4 (Cayetano *et al.*, 2019), which offers a reasonable explanation for the reactors with CO_2-NBW addition resulting in the highest RA of *Methanolinea*, most probably due to CO_2 could be collapsed and generated from CO_2-NBW and then used for CH_4 production. Taking all the results together, it can be denoted that the above-mentioned bacteria and archaea are fundamentally important for the high cellulose loading AD process in this study. Meanwhile, it can also be inferred that high mobility of water molecule in NBW may promote the functional metabolism of microorganisms.

3.5 Mechanisms analysis

Hydrolysis of cellulose particles can generate an oligosaccharide layer under high cellulose loading condition, which could create a mass transfer resistance

(Vaquerizo *et al.*, 2018), thus increasing the resistance to the surrounding environment (Bouchard *et al.*, 2016). The high-mobility water molecules can diffuse from the bulk phase to the surface of the cellulose particles where the oligosaccharide layer is formed (Vaquerizo *et al.*, 2018), which is then attacked by the high-mobility water molecules in NBW. NBW has a positive effect on overcoming the obstacles of cellulose hydrolysis to produce the oligosaccharide layer. On the other hand, the hydrophobic and negatively charged surface of NBs is capable to adsorb trace metals that are essential for anaerobic microorganisms (Yang *et al.*, 2007). Small molecules of organic matters and trace elements can be adsorbed on the surface of NBs and act on the oligosaccharide layer together with the microorganisms. The hydrolysis-acidification of high loading cellulose can then be enhanced with the increased production of hydrolysate (VFAs) for more CH_4 production by the methanogens. The enhanced hydrolysis of high loading cellulose can also provide abundant carbon and nutrient sources for the growth of microorganisms, further enriching microbial communities. Results from the current research provide a new alternative to acquire more methane as renewable energy from high cellulose loading AD process with NBW supplementation, which can help to establish and maintain the sustainable society by utilizing the huge amount of lignocellulosic biowaste in the world.

4. Conclusions

This study demonstrated the positive effects of high-mobility NBW on the AD of refractory cellulose. VFAs yields were increased by 11–30% during the hydrolysis-

acidification stage of cellulose under NBW addition. When Air-NBW was supplemented, 18% increase in CH_4 yield (263.6 mL/g-$VS_{reduced}$) and 20% enhancement in cellulose crystallinity reduction (81%) were achieved in comparison to the control. These enhancements are attributable to the greatly enhanced hydrolysis-acidification stage of high cellulose loading AD, most probably due to the microaerobic environment created by Air-NBW addition. These findings can provide valuable insights into the mechanisms involved in the NBW-supplemented AD process.

Supplementary materials

Supplementary data of this work can be found in online version of the paper.

Declarations of Interest

None

Acknowledgments

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Table

Table 1

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

Water	TMP (mL/g-$VS_{reduced}$)	EMP (mL/g $VS_{reduced}$)	BD (%)	Modified Gompertz model				Crystallinity reduction (%)	TOC reduction (%)	VS reduction (%)
				P	R_{max}	λ	R^2			
DW	414.8	223.3±0.1	53.8	221.9±3.2	10.1±0.6	1.0	0.994	67.4±3.8	38.6±0.2	44.7±0.3
CO_2-NBW		246.0±6.0	59.3	244.6±4.1	10.2±0.6	0.8	0.994	76.9±0.2	39.6±4.3	53.6±1.3
Air-NBW		263.6±10.9	63.5	259.5±4.3	13.3±0.9	0.3	0.990	80.7±2.1	43.1±6.4	58.0±3.4

BD, biodegradability; DW, deionized water; EMP, experimental methane production; NBW, Nano-bubble water; P, simulated maximum methane yield (mL/g-$VS_{reduced}$); TMP, theoretical methane production; TOC, total organic carbon; R^2, correlation coefficient; R_{max}, maximum methane production rate (mL/g-$VS_{reduced}$/day); λ, lag phase time (day).

Figures

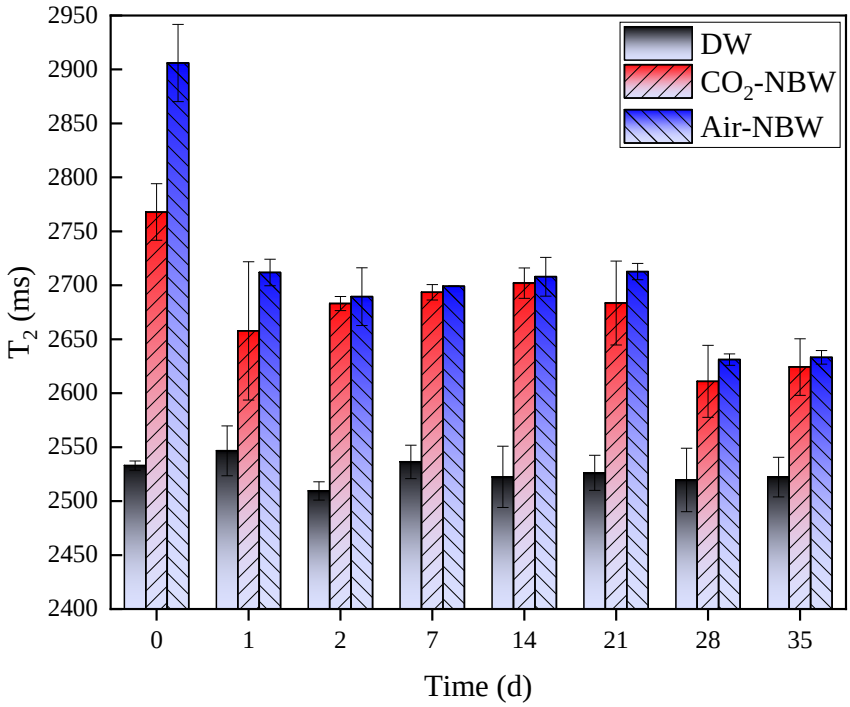

Fig. 1. Variations of spin-spin relaxation times (T_2) of Air-NBW, CO_2-NBW and DW at 35±1°C during 35 days' storage. DW, deionized water; NBW, nano-bubble water.

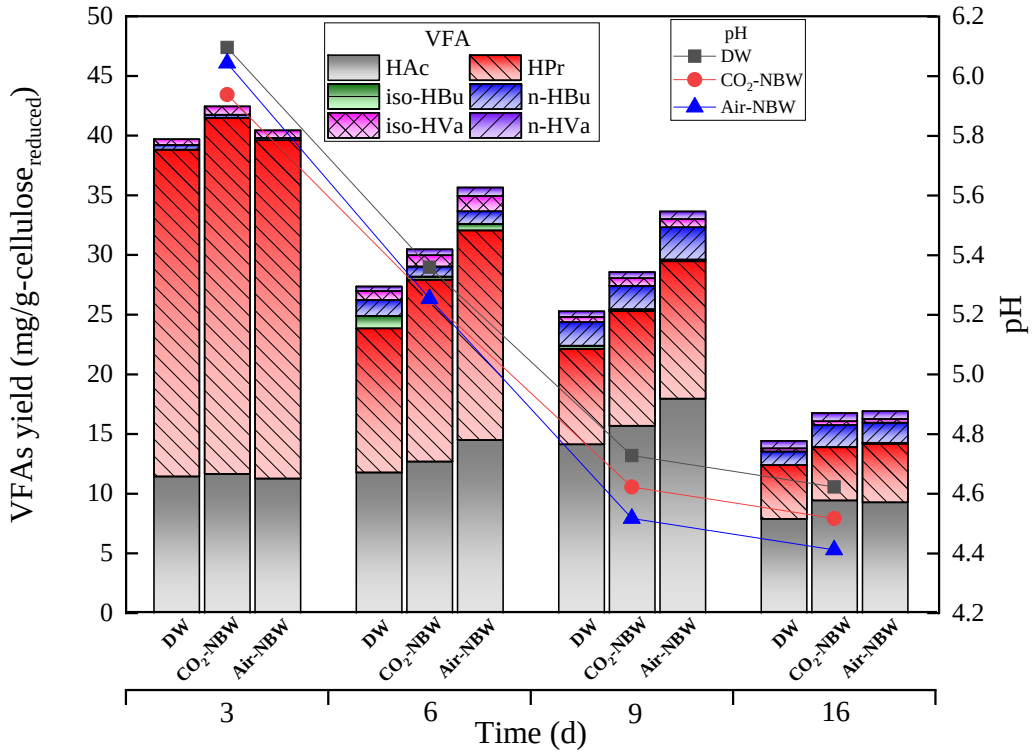

Fig. 2. Variations of VFAs yield and pH value during the hydrolysis-acidification stage of AD of cellulose after DW or NBW addition. AD, anaerobic digestion; DW, deionized water; HAc, acetic acid; HPr, propionic acid; iso-HBu, iso-butyric acid; n-HBu, n-butyric acid; iso-HVa, iso-valeric acid; n-HVa,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

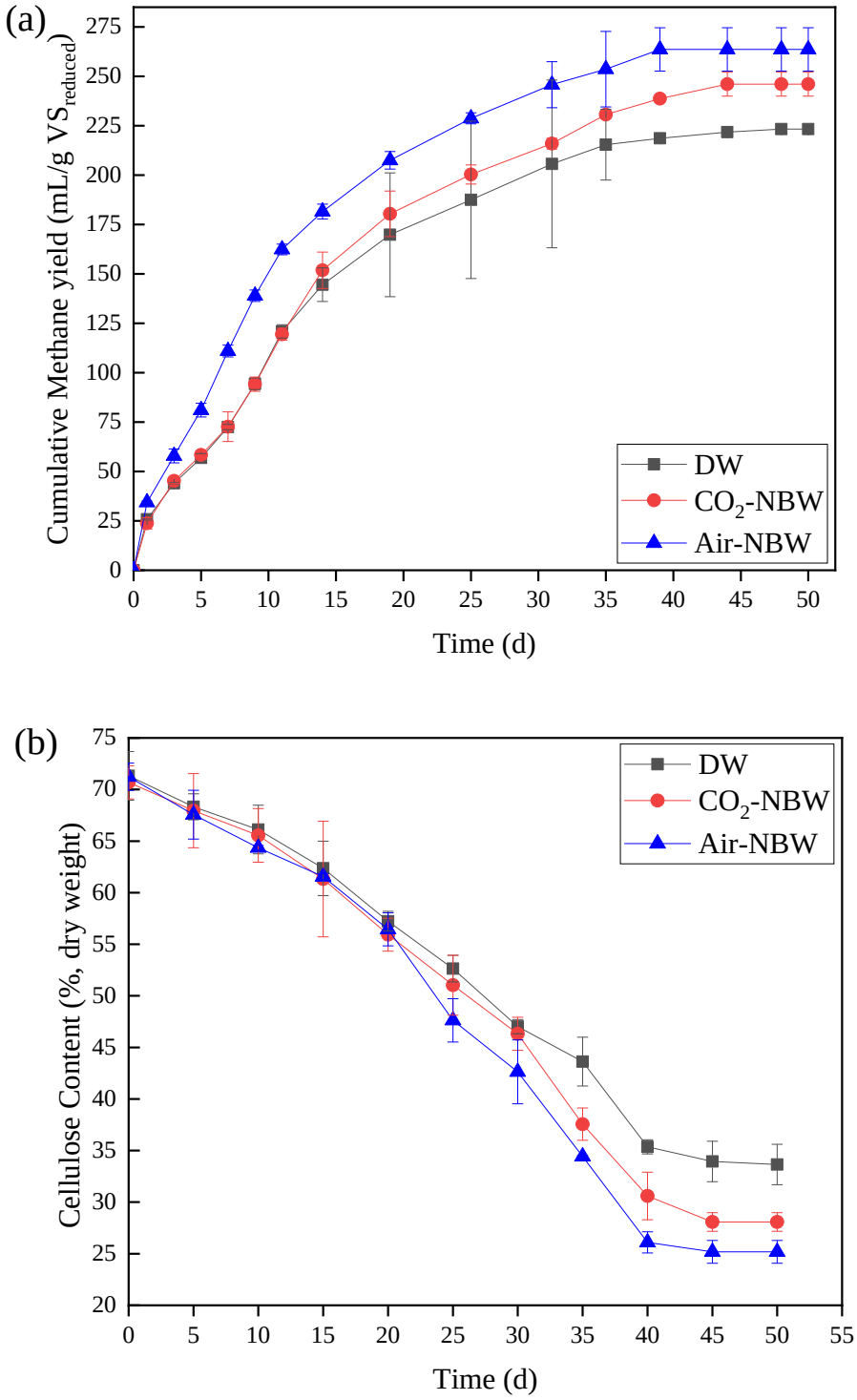

Fig. 3. Changes in cumulative methane production (a) and cellulose content (b) during AD of cellulose after NBW addition. DW, deionized water; NBW, nano-bubble water.

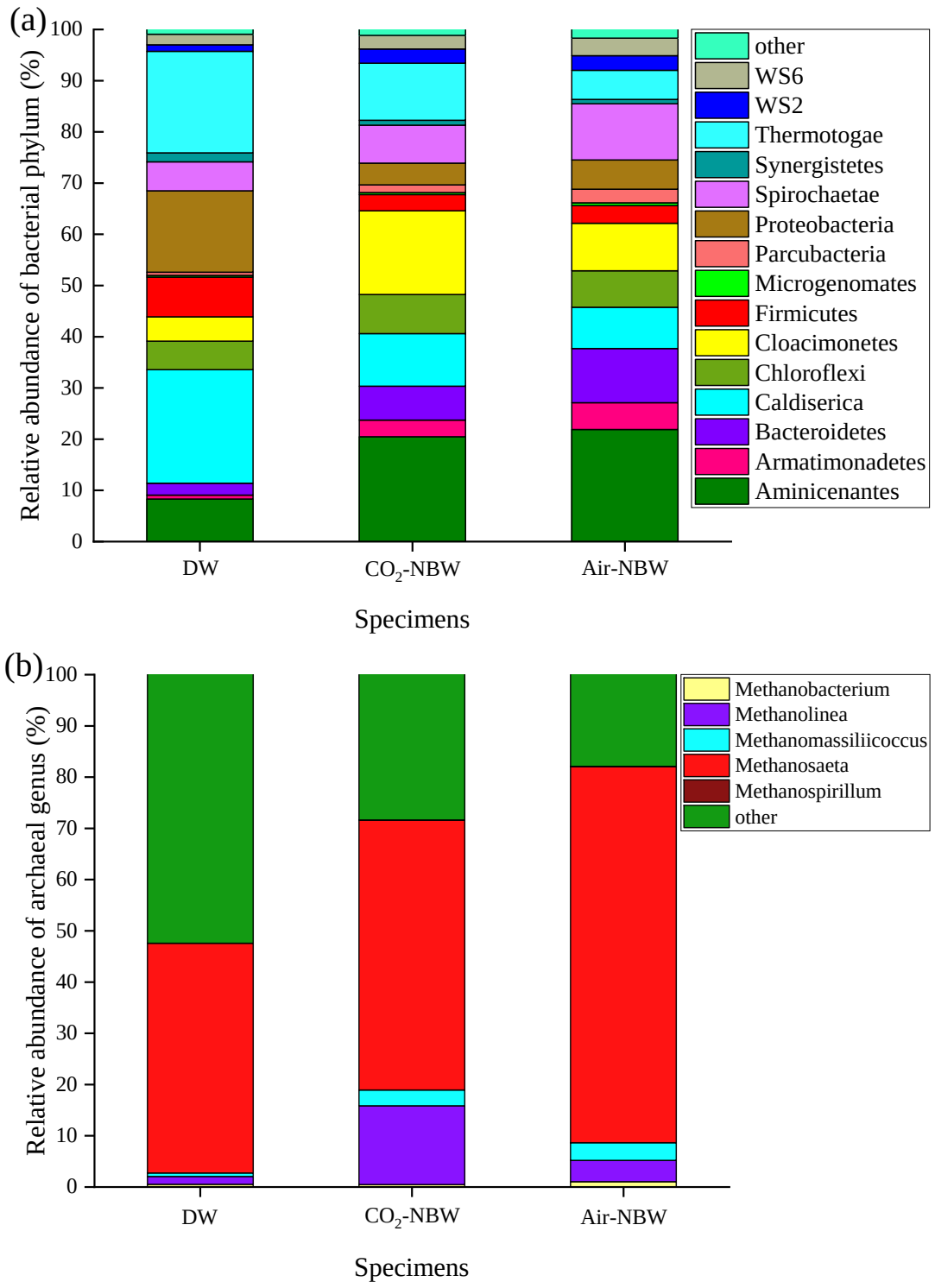

Fig. 4. Bacterial (a) and archaeal (b) community changes in the samples taken at the end of AD of cellulose with NBW addition using high-throughput 16S rDNA pyrosequencing. AD, anaerobic digestion; DW, deionized water; NBW, nano-bubble water.