

Metagenomic analysis reveals metabolic mechanism of enhancing lignocellulosic anaerobic digestion mediated by CO₂/O₂-nanobubble water

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Abstract

Nanobubble water (NW) has been reported to enhance anaerobic digestion (AD), but its influence on the metabolic pathways of microorganisms remains unclear. In this study, the specific methane yields of rice straw in the CO₂NW and O₂NW treatments increased by 6.9% and 18.3%, respectively. The electron transport system (ETS) and coenzyme F₄₂₀ activities were enhanced by the addition of NW. Metagenomic analysis showed that the abundances of most enzymes in the acidification were significantly increased by both CO₂NW and O₂NW. Regarding methanogenesis, CO₂NW promoted the expression of genes encoding enzymes of hydrogenotrophic methanogenesis, while O₂NW stimulated both the acetoclastic and hydrogenotrophic methanogenesis. With the addition of O₂NW, the expressions of modules related to the tricarboxylic acid (TCA) cycle and oxidative phosphorylation were enhanced, resulting in increased ATP production. This study provided fundamental evidence of the metabolic pathways of microorganisms mediated by NW at each stage of AD.

Keywords: Biogas production; Nanobubbles; Rice straw; Micro-oxygen; Microbial community

1. Introduction

Anaerobic digestion (AD) of crop straw to produce biogas is a representative technique that has been widely used in rural bioenergy systems (Sun et al., 2021). However, it faces challenges such as long fermentation periods and low specific biogas yields (Fu et al., 2023). Numerous studies related to straw pretreatment and AD accelerants have been carried out for higher biogas yield (Kainthola et al., 2019). In recent years, interest in nanobubble water (NW) technology has grown as an accelerant in the AD process due to its economic and environmentally friendly advantages.

NW is defined as water containing numerous nanobubbles with diameters smaller than 1000 nm, typically ranging from 50 to 200 nm (Chuenchart et al., 2021). Compared with large bubble, it exhibits longer residence time in water, the negative surface charge, high gas-liquid mass transfer efficiency, and the capability to produce hydroxyl radical (Lyu et al., 2019). X. Wang et al. (2020) reported that the highest cumulative methane yield was observed in the reactor with O₂NW addition, which was 30% higher than that of the DW treatment. Song et al. (2023) also reported that the addition of CO₂NW increased the cumulative methane yield by 17% compared with control group. Although adding NW (especially CO₂NBW and O₂NBW) to AD reactors has been proven to increase biogas yields, these studies primarily focus on enhancing AD performance and enzyme activity (Fan et al., 2020; X. Wang et al., 2020). The explanation of microorganisms remains focused on the microbial community characteristics interpreted through 16S rRNA high-throughput sequencing. However, 16S rRNA sequencing can only provide information about the relative abundance of microbial community composition, which limits the understanding of specific metabolic pathways during AD (Jünemann et al., 2017). Metagenomic analysis, a next-generation sequencing technology, has been widely utilized to reveal changes in key microbial genes encoding enzymes at different stages of the AD process (hydrolysis, acidification, and methanogenesis), while elucidating the detailed mechanisms of the metabolic interactions of different microbial populations (Kim et al., 2022). To the best of authors' knowledge, there have been no studies on the effects of NW addition on metabolic pathways using metagenomics analysis.

Therefore, it is necessary to elucidate the mechanisms underlying the effects of NW on microorganisms and metabolic pathways by combining 16S rRNA sequencing and metagenomics. First, substrate degradation and methane production from rice straw with the addition of CO₂NW and O₂NW were investigated. Then, the potential functional mechanisms of the effects of NW addition were elucidated by examining related enzyme activities and microbial community structure. Finally, the effects of NW addition on the metabolic pathways of lignocellulosic AD process were determined using metagenomic analysis. This study aimed to fill the knowledge gap regarding NW-promoted metabolism and identify the influence of NW on key enzymes at different stages of the AD process (hydrolysis, acidification, and methanogenesis).

2. Materials and methods

2.1 Substrate, inoculum, and nanobubble water

The rice straw used in this work was obtained from a farm in Hubei Province, China. The harvested straw was air-dried in the field and then transported to the laboratory. The anaerobic inoculum was obtained from stable operating digesters in a wastewater treatment plant in Beijing, China. After being transported to the laboratory, the inoculum was stored at 37 ± 2 °C for one week until no biogas production was observed before subsequent biogas production tests. The substrate and

inoculum characteristics are tested (See supplementary materials).

The CO₂NW and O₂NW were prepared by mixing deionized water (DW) with gases (CO₂ and O₂) using a commercial nanobubble generator (TL-MBG10-A, China) based on previous research (E. Wang et al., 2023). Briefly, the nanobubble generator was operated through hydraulic cavitation with a gas intake rate of 1 L/min and a DW volume of 20 L under ambient temperature conditions. The generation time for CO₂NW and O₂NW lasted 10 min and 15 min, respectively.

2.2 Batch anaerobic digestion experiment

This study was conducted to investigate the influence of NW on biogas production from rice straw. The experiments were performed in 250-mL serum bottles, which were placed in a water bath at 37 ± 2 °C to produce biogas. The rice straw and inoculum were loaded at a ratio of 1:1.5, based on volatile solids (VS). The total solids (TS) content of all bottles was adjusted to 5% by adding DW, CO₂NW, or O₂NW, labeled as DW, CO₂NW, or O₂NW treatment, respectively. Each treatment was tested in triplicate. Three bottles containing only the inoculum were labeled as blank. The serum bottles were purged with N₂ to remove air in the bottle before sealing. The bottles were manually shaken daily to ensure uniform mixing. The biogas volumes were periodically measured using syringes and normalized to standard temperature and pressure (273.1 K and 1 atm) condition. The batch AD experiment was performed for 45 days until biogas production ceased.

2.3 Chemical and physical analysis

TS and VS were determined following standard methods from American Public Health Association (APHA, 2005). Volatile fatty acids (VFAs) were quantified by gas chromatography (GC-2010 Plus, Shimadzu, Japan) after centrifuging the digestate at 8000 rpm for 20 min and filtering it through 0.22 µm filter membrane. The lignocellulose contents of the rice straw before and after AD was tested by the Ankom2000 fiber analyzer. The methane content was measured by gas chromatography (GC8A, Shimadzu, Japan). The pH of the samples was tested by a pH meter (FE28, Mettler-Toledo, Switzerland). Coenzyme F₄₂₀ was detected as described by Tian et al. (2017). The electron transfer system (ETS) activities of the samples were determined using the INT (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride)-ETS method.

The modified Gompertz model was used to analyze the methane yield kinetics for each treatment. The model is expressed as in Eq. (1).

$$P(t) = P_m \times \exp \left\{ -\exp \left[\frac{R_m e}{P_m} (\lambda - t) + 1 \right] \right\} \quad (1)$$

where $P(t)$ is the cumulative methane yield (mL/gVS); t is the digestion day (d); P_m is the methane yield potential (mL/gVS); R_m is the maximum methane yield rate (mL/gVS/d); and λ is the delay phase (d).

2.4 Microbial community and metagenomic analyses

The digestate of DW, CO₂NW, and O₂NW treatments was collected after 30 d for microbial analysis. DNA was extracted from centrifuged samples using an E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) following the manufacturer's instructions. The DNA quality and concentration were tested by 1% agarose gel electrophoresis and then stored at -80 °C for further testing. The 16S rRNA gene primer pairs were amplified as follows: for bacteria with 338F and 806R

primers, and for archaea with 524F10extF and Arch958RmodR primers. The samples were paired-end sequenced on an Illumina MiSeq platform (Illumina, San Diego, USA) following the standard protocols of Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

To construct paired-end libraries, the extracted DNA was fragmented to an average size of approximately 400 bp using the NEXTFLEX Rapid DNA-Seq kit. Sequencing was conducted on an Illumina NovaSeq (Illumina, San Diego, USA) following the manufacturer's instructions. Adaptors were trimmed from Illumina reads and low-quality reads (length < 50 bp, quality value < 20, or having N bases) were removed using Fastp (version 0.20.0). The annotation was conducted using Diamond (version 0.8.35) against the KEGG database (<http://www.genome.jp/kegg/>) with an e-value cutoff of $1e^{-5}$. The raw sequencing data in this study has been submitted to National Center for Biotechnology Information (NCBI) with project number PRJNA1128434.

2.5 Data analysis

All data were presented as the mean values $\pm$ standard deviations ($n=3$). The kinetics and Pearson correlation analysis were performed using the Origin 2021 software. The significance of differences (Duncan's test) was determined using the SPSS 25 software.

3. Results and discussion

3.1 Effect of nanobubble water on methane production

The cumulative and daily methane yields from the DW, CO₂NW, and O₂NW treatments are shown in Fig.1. After 45 days of AD, the specific methane yields for the CO₂NW and O₂NW treatments were 257.4 ± 2.5 and 284.8 ± 2.0 NmL/gVS, respectively, which were 6.9% and 18.3% higher than that of the DW treatment ($p < 0.05$). Additionally, the daily methane yields for the DW, CO₂NW, and O₂NW treatments peaked on the 6th and 7th days, reaching 18.9, 22.0, and 27.6 NmL/gVS/d, respectively. The significant increase of the peak of daily methane yield in CO₂NW and O₂NW, which also demonstrated that adding NW in the AD process could benefit to accelerate biogas production. Previous studies have reported similar findings, in which a two-stage food waste AD system based on NW showed a higher peak daily yield compared with the DW treatment (Hou et al., 2021). In addition, the highest yield being obtained under the O₂NW treatment could be attributed to the increased facultative bacteria activity due to the presence of a small amount of exogenous oxygen, which facilitated more efficient organic material decomposition and increased biogas production (Nguyen et al., 2019).

The kinetic parameters are presented in Table 1. All treatments achieved $R^2 > 0.99$, indicating good fitting. The lag phases (λ) for the DW, CO₂NW, and O₂NW treatments were 3.6, 2.7, and 3.1, respectively, indicating that NW could decrease the fermentation time and accelerate biogas production. The O₂NW treatment exhibited the highest maximum methane potential yield (P_m) and maximum methane yield rate (R_m) values at 263.5 ± 5.5 mL/gVS and 20.1 ± 0.5 mL/gVS/d, respectively. This result demonstrated a significant increase compared with the DW and CO₂NW treatments, which was consistent with the experimental results.

3.2 Hydrolysis and acidification performance affected by nanobubble water

3.2.1 Substrate degradation

The TS, VS, and lignocellulose degradation rates in rice straw after AD are shown in Fig. 2a.

The highest degradation rates of TS and VS were obtained for the O₂NW treatment of $31.8\% \pm 0.3\%$ and $54.0\% \pm 0.1\%$, respectively. Meanwhile, The O₂NW treatment also achieved the highest cellulose and hemicellulose degradation rates of $77.6\% \pm 0.8\%$ and $59.8\% \pm 8.5\%$, respectively. Compared with the DW treatment, the degradation rate of cellulose and hemicellulose were significantly increased by 8.5% and 18.3% ($p < 0.05$). But there was no significant difference in the degradation rates of cellulose and hemicellulose in the CO₂NW treatment ($p > 0.05$). This indicated that O₂NW could promote the breakdown of complex and refractory lignocellulose structure into smaller molecular substances (VFAs and H₂). The presence of O₂ nanobubbles in O₂NW created a micro-oxygen environment which enhanced the facultative bacteria activity, resulting in better rice straw degradation. According to the report by Nirmalkar et al. (2018), the collapse of O₂ nanobubbles could generate very high local temperatures and pressures, stimulating the formation of “hotspots” and producing highly reactive hydroxyl radicals. The strong oxidizing ability of these radicals might have caused the higher lignocellulose degradation rate in the O₂NW treatment.

3.2.2 VFAs production

VFAs were main intermediate metabolites utilized by methanogenic archaea in the AD process. The VFAs concentrations for each treatment are shown in Fig. 2b. The VFAs concentrations in all treatments rapidly increased and peaked within the first 6 days. On the 6th day, the total VFAs concentration in the DW and CO₂NW treatments reached 421.0 ± 58.5 mg/L and 545.1 ± 5.7 mg/L, respectively. And the O₂NW treatment obtained the highest total VFAs concentration at 898.5 ± 55.4 mg/L, which was 112.7% and 64.3% higher than that of the DW and CO₂NW treatments, respectively. This result indicated that adding O₂NW could promote VFAs production and the organic matter degradation. The VFAs concentration exhibited an initial increase, followed by a decrease, eventually reaching a dynamic balance between production and consumption until the end of AD. This trend was consistent with the daily methane production (as discussed in Section 3.1).

Among the VFAs compositions, acetate was the main component in the early stages, whereas propionate became predominant after 6 days. Generally, acetate could be directly utilized by methanogens, while propionate needed to be converted into acetate before being further utilized. The Gibbs free energy (ΔG) of the propionate degradation process was positive, indicating thermodynamic disadvantage, which resulted in propionate accumulation during the initial stages (Wu et al., 2022). Additionally, the O₂NW treatment exhibited higher propionate concentrations compared with DW and CO₂NW treatments. Some researchers have reported that higher oxidation-reduction potential (ORP) levels in a micro-oxygen environment might promote propionic acid production (Cohen et al., 1984; Zhuge et al., 2015). However, its concentration did not reach the threshold level. The previous study reported that propionic acid concentration above 900 mg/L adversely affected the growth of methanogens (Wang et al., 2009). Furthermore, the propionate concentration fell below the detection limit after 30 days of fermentation. This indicated that, despite the dominance of propionic acid, it did not reach a level sufficient to hinder biogas production or cause acidification.

3.2.3 Electron transport system and coenzyme F₄₂₀ activities

The ETS activity represented the rate of electron transfer to the microbial respiratory chain in the digestate, which indirectly indicated the microbial metabolic activity (Lizama et al., 2019; Tian

et al., 2017). The changes in ETS of each treatment are shown in Fig. 3a. The highest ETS activity was exhibited in O₂NW treatment on the 6th day, increasing by 41.6% and 14.3% compared with the DW and CO₂NW treatments, respectively. This indicated that O₂NW could promote microbial activity to accelerate substrate hydrolysis and acidification. Oxygen in O₂NW could function as an electron acceptor in the oxidation of NADH, releasing significant energy to drive ATP synthesis and the intermediates transfer (X. Wang et al., 2020). On the other hand, negatively charged nanobubbles had a stronger attraction to cations, which might accelerate the mass transfer of intermediates such as NAD⁺ (Kyzas et al., 2019). Therefore, the increase in ETS activity might be the reason why the O₂NW treatment achieved the highest methane production. Besides, the ETS activity showed an increasing trend during the early stages of AD, followed by a gradual decreased over time. Pearson correlation analysis also showed a positive correlation between ETS activity and VFA (See supplementary materials).

Coenzyme F₄₂₀ played an important role in the methanogenic activity (Zhao et al., 2018). At the end of AD process, the coenzyme F₄₂₀ activity were 6.9 ± 0.5 , 8.6 ± 0.3 , and 9.7 ± 0.8 $\mu\text{mol/L}$ in the DW CO₂NW and O₂NW treatments, respectively (Fig. 3b). The significantly highest activity of coenzyme F₄₂₀ obtained in O₂NW treatment might be the reason for the high specific methane production. These results agreed with those of a previous study that a 34% increase in coenzyme F₄₂₀ activity by adding AirNW to food waste AD compared with the DW treatment (Hou et al., 2021). Additionally, Nguyen et al. (2019) reported that O₂ had the potential to oxidize VFAs into CO₂, consequently increasing the production of CH₄. Coenzyme F₄₂₀ was an essential enzyme for hydrogenotrophic methanogenesis (M. Wang et al., 2023). Thus, O₂NW potentially stimulated the growth of hydrogenotrophic methanogens by oxidizing VFAs into CO₂ and promoting the secretion of coenzyme F₄₂₀.

3.3 Effect of nanobubble water on microbial community

The microbial community was analyzed based on 16S rRNA sequencing to elucidate the impact of NW during the AD process. The coverage of all samples exceeded 0.99, suggesting that the results accurately represented the actual state of the microbial community (See supplementary materials). Compared with the DW treatment, the CO₂NW and O₂NW treatments showed higher Shannon and lower Simpson values of bacteria. It indicated that CO₂NW and O₂NW were increase in species diversity of acid-producing bacteria. However, the indices of archaea showed the opposite trend, which indicated NW resulted in lower archaeal diversity due to their heightened sensitivity to the environment. Species richness was often measured using the ACE and Chao 1 indices (Zhu et al., 2022), and the results showed that NW did not significantly affect the richness of bacteria and archaea.

The *Firmicutes*, *Caldatribacteriota*, *Synergistota*, *Bacteroidota*, *Chloroflexi*, and *Actinobacteriota* were the predominant bacterial phyla in each treatment (Fig. 4a). These bacteria were typically associated with cellulose degradation and organic acid production. The CO₂NW and O₂NW treatments significantly decreased the relative abundance of *Firmicutes* compared with the DW treatment, from 78.3% to 46.3% and 62.5%, respectively. However, in the CO₂NW and O₂NW treatments, *Caldatribacteriota*, *Synergistota*, *Bacteroidota*, *Chloroflexi*, and *Actinobacteriota* exhibited higher relative abundance compared with the DW treatment.

At the bacterial genus level, *norank_c_D8A-2*, *Candidatus_Caldatribacterium*, and *Acetomicrobium* were the predominant bacteria in all treatments (Fig. 4b). The genus *norank_c_D8A-*

2, belonging to the *Firmicutes*, had the capability to degrade organic materials, such as polysaccharides and proteins (Yao et al., 2022). *Candidatus_Caldatribacterium* was primarily involved in the degradation of lignocellulose to produce hydrogen, acetate, and ethanol (Dodsworth et al., 2013). *Acetomicrobium* could convert propionate and butyrate into acetate, thus playing a crucial role in maintaining the balance of intermediary metabolism (Zhu et al., 2024). In the CO₂NW and O₂NW treatments, the relative abundances of *norank_c_D8A-2* decreased from 47.0% in the DW treatment to 28.0% and 35.7%, respectively. However, the relative abundances of *Candidatus_Caldatribacterium* and *Acetomicrobium* significantly increased by adding NW, particularly in the CO₂NW treatment, reaching 22.1% and 18.5%, respectively. This indicated the positive effects of adding NW on the enrichment of these two genera.

Syntrophomonas and *Petrimonas* were all mainly involved in the carbohydrate degradation for acetate production (Zhu et al., 2024). The relative abundances of *Syntrophomonas* and *Petrimonas* in the O₂NW treatment increased by 88.2% and 27.3%, respectively, compared with the DW treatment. Interestingly, some studies reported an association between *Syntrophomonas* and *Methanosarcina* via DIET (Jiang et al., 2022; Zhang et al., 2020). G. Wang et al. (2020) also reported that DIET between *Petrimonas* and *Methanosarcina* was likely stimulated to accelerate the VFAs oxidation and methanogenesis. Therefore, the enrichment of *Syntrophomonas* and *Petrimonas* in the O₂NW treatment was beneficial for VFAs oxidation by promoting DIET-based syntrophic metabolism. However, owing to the relatively low total abundance of electroactive bacteria, such as *Syntrophomonas* and *Petrimonas*, which were found to be 2.8%, 3.7%, and 4.6% in the DW, CO₂NW, and O₂NW treatments, respectively. Further validation of DIET involving electroactive bacteria and methanogens is still required in future research.

Among the archaeal, at the genus level, *Methanosarcina* and *Methanobacterium* were predominant in all treatments (Fig. 4c). The relative abundances of *Methanosarcina* in the CO₂NW and O₂NW treatments were 84.7% and 82.0%, respectively, representing a significant increase compared with the DW treatment (59.2%). However, the relative abundances of *Methanobacterium* decreased from 40.7% (in the DW treatment) to 14.5% and 17.5%, respectively. Some studies have reported that *Methanosarcina* is resistant to micro-oxygen conditions (Angel et al., 2012) and high ammonia nitrogen environments (De Vrieze et al., 2012). In contrast, other archaea were typically damaged by these adverse environments, allowing *Methanosarcina* to occupy a larger ecological niche (Ma et al., 2015). The behavior may explain the observed enrichment of *Methanosarcina* in the NW treatment. *Methanobacterium*, an obligate hydrogenotrophic methanogen (Wu et al., 2021), was another important archaeon identified in this study. Although the relative abundance of *Methanobacterium* significantly decreased in the NW treatments, *Methanosarcina*, a typical mixotrophic methanogen, exhibited an increasing trend. *Methanosarcina* has the capability to produce methane through acetoclastic, hydrogenotrophic, and methylotrophic pathways (Ma et al., 2015).

In this study, the addition of CO₂NW might have provided more CO₂ for the fermentation system to participate in hydrogenotrophic methanogenesis. Moreover, CO₂NW might alter the partial pressure of CO₂ and equilibrium of the carbonate system in AD reactors (Antonio Cerrón-Calle et al., 2022). Thermodynamically, the increased partial pressure of CO₂ favors hydrogenotrophic methanogenesis (HM) over acetoclastic methanogenesis (AM). However, in the CO₂NW treatment, the relative abundance of hydrogenotrophic methanogens did not observably increase, whereas that

of mixotrophic methanogens increased. The O₂NW treatment exhibited the same trend as the CO₂NW treatment, showing *Methanosarcina* enrichment. On one hand, O₂NW accelerates substrate degradation and increases acetate concentration, thereby promoting the AM pathway for methane production. On the other hands, Oxygen injection provided an opportunity to bypassing the thermodynamically limited reaction of syntrophic acetate oxidation (Wu et al., 2023). This might result in the partial oxidation of VFAs to CO₂ in lignocellulose AD reactors, thereby promoting the HM pathway (Wu et al., 2021). Importantly, *Methanosarcina*, a typical mixotrophic methanogen, dominated in this study, making it difficult to accurately determine methanogenesis pathways by 16S rRNA sequencing.

3.4 Metabolic pathway analysis

To further elucidate the mechanism by which NW promoted AD process, metagenomic analysis was performed. The abundance of AD-related pathways in both the CO₂NW and O₂NW treatment was significantly higher than that of the DW treatment (See supplementary materials). The addition of NW promoted substrate metabolism (carbohydrates and amino acids) and energy metabolism (oxidative phosphorylation and methane metabolism), which might have been the reason for the higher methane production. We also observed that O₂NW increased the abundance of ABC transports pathway. This indicates that O₂NW could accelerate the transmembrane transport of organic material, which might stimulate the growth of acidogenic and methanogenic microorganisms.

Fig. 5 illustrates the key metabolism pathways and functional enzymes from the KEGG database during the hydrolysis, acidogenesis, and methanogenesis stages. Considering that crop straw mainly consisted of cellulose and hemicellulose, its hydrolysis products mainly included hexoses (like glucose) and pentoses (like xylose) (Wu et al., 2021). The cellulase (EC: 3.2.1.4), glucosidase (EC: 3.2.1.21), xylanase (EC: 3.2.1.8), and xylosidase (EC: 3.2.1.37) were key enzymes related to cellulose and hemicellulose hydrolysis. For the acidogenesis stage, glucose, the cellulose hydrolysis product, was converted into glucose-6P by hexokinase (EC: 2.7.1.1) and glucokinase (EC: 2.7.1.2), and then decomposed into pyruvate through glycolysis (Embden-Meyerhof pathway, EMP) or the pentose phosphate pathway (PPP). Hemicellulose, another major component of crop straw, was hydrolyzed into xylose and glucose. Xylose was converted into xylulose-5P through the xylose metabolism and then entered the PPP to form pyruvate. Pyruvate was an important intermediate metabolite in VFAs and methane production (Zhu et al., 2022) and was converted into acetyl-CoA by oxidative decarboxylation. Subsequently, acetyl-CoA could be further converted into acetic acid via either a two-step reaction catalyzed by phosphate acetyltransferase (EC: 2.3.1.8) and acetate kinase (EC: 2.7.2.1) or a one-step reaction catalyzed by an acetate—CoA ligase (EC: 6.2.1.1). It showed that the addition of CO₂NW and O₂NW significantly increased the abundance of most enzymes in the acidification stage, rather than the hydrolysis enzymes. This suggests that the addition of NW was more effective in promoting acidification (intracellular metabolism) compared with hydrolysis (extracellular metabolism). This discrepancy might result from changes in the extracellular environment induced by the NW, resulting in differences the expression of genes encoding hydrolytic and acidifying enzyme. The previous studies only utilizing 16S rRNA sequencing, which also suggested that O₂NW promoted acidification (He et al., 2022; E. Wang et al., 2023; X. Wang et al., 2020). However, it was not appropriate to attribute the strengthening acidification solely to an increase in bacterial abundance, since bacteria were involved in both hydrolysis and acidification

processes. In contrast, this work utilized metagenomic analysis to separately examine the changes in enzyme abundance across the hydrolysis, acidification, and methanogenesis stages. Future research should explore the impact of oxygen on microbial damage and investigate the reasons for the observed lack of significant improvement in enzyme gene expression during the hydrolysis stage with the addition of O₂NW.

In the traditional AD process (without oxygen), glucose could only be degraded via the EMP to produce small amounts of NADH and ATP by substrate-level phosphorylation. However, the addition of O₂NW alters the strictly anaerobic environment, which could create micro-oxygen environment (X. Wang et al., 2020). During the substrate metabolism, exogenous O₂ was the preferred electron acceptor because of its thermodynamics (Nguyen and Khanal, 2018). Thus, the energy metabolism exhibited differences compared with that in the strictly anaerobic environment (DW treatment); particularly significant increases in the expression of oxidative phosphorylation module in the O₂NW treatment were observed. In addition, the expression of most functional enzymes involved in the tricarboxylic acid (TCA) cycle was significantly upregulated. As the TCA cycle was activated under the microaerobic conditions, partial acetyl-CoA molecules could be completely oxidized to CO₂, generating NADH in the process (Xiao et al., 2024). The produced NADH was then completely oxidized to synthesize a large amount of ATP by oxidative phosphorylation system (Yang et al., 2024). The upregulation of key enzymes involved in oxidative phosphorylation, such as NADH dehydrogenase (EC: 7.1.1.2), succinate dehydrogenase (EC: 1.3.5.1), cytochrome c oxidase (EC: 7.1.1.9), and ATP synthase (EC: 7.1.2.2), was also a manifestation of enhanced oxidative phosphorylation by O₂NW (Fig 5). The increased abundance of NADH dehydrogenase helps to prevent the accumulation of NADH/NAD⁺, which promotes substrate degradation and organic acid formation. The expression of cytochrome c oxidase also reflected a similar upregulation trend in the O₂NW environment. Those results suggested that O₂NW (micro-oxygen environment) could promote ATP synthesis and stimulates higher energy metabolism, which was beneficial for providing energy for substrate degradation and cellular processes such as metabolism, growth, and signal transduction.

In general, the predominantly expression of bacteria is F-type ATPase (M00157), whereas archaea employ V/A-type ATPase (M00159) to catalyze the conversion of ADP to ATP (Zhu et al., 2022). The abundance of ATPase in the O₂NW treatment was higher than that in both the DW and CO₂NW treatments, particularly a significant increase in F-type ATPase was obtained (See supplementary materials). This suggests that the enrichment of F-type ATPase associated with the acidification stage by adding O₂NW was an important factor in improving biogas production.

To explore the impact of NW on methanogenic archaea, the methane metabolism pathway was analyzed, as shown in Fig.5, based on map00680. In the AM pathway, acetate—CoA ligase (EC: 6.2.1.1) could catalyze the conversion of acetate to acetyl-CoA. Alternatively, another predominant route involves the conversion of acetate to acetyl-CoA by the acetate kinase (EC: 2.7.2.1) and phosphate acetyltransferase (EC: 2.3.1.8). This conversion is strongly related to the higher abundance of *Methanosarcina* (Welte and Deppenmeier, 2014). Under the O₂NW treatment, the abundance of these key enzymes significantly increased compared with the DW treatment, while their abundance decreased in the CO₂NW treatment. On the other hand, formylmethanofuran dehydrogenase (EC: 1.2.7.12), which is the key enzyme of HM pathway, was enriched in the presence of CO₂NW and O₂NW additions. The key enzymes of the shared nodes of acetoclastic and hydrogenotrophic methanogenesis are shown in Fig. 5. Most of their abundances increased with the addition of NW,

suggesting that NW could synthesize and activate many enzymes related to methane metabolism. Overall, compared with the DW treatment, O₂NW could increase the abundance of the genes encoding enzymes of the AM pathway, while CO₂NW had a negative impact. For the HM pathway, both the CO₂NW and O₂NW could promote the expression of gene encoding enzymes.

3.5 Implications and future perspective of nanobubble water mediated anaerobic digestion process

This study revealed the succession of microbial communities and metabolic pathways with the addition of NW in the AD process, which may provide fundamental evidence for the mediation of nanobubbles at each stage of AD. The gene expressions of glycolysis, pyruvate metabolism, and fatty acid metabolism were obviously upregulated due to the exist of CO₂NW or O₂NW. Meanwhile, the enhancement of the oxidative phosphorylation pathway and the TCA cycle in the O₂NW treatment could provide more energy for substrate conversion, mass transfer, and microbial metabolism activity. Therefore, nanobubble technology combined with AD can serve as a low-cost and environmentally friendly biogas-upgrading strategy.

However, there are still some problems in this work that need further investigation. For instance, O₂NW increases the ORP of the AD system and promotes propionic acid formation, potentially resulting in acidification or even failure of continuously operating reactors. Secondly, although the addition of NW increased the specific methane yield, it downregulated the abundance of extracellular enzymes involved in the hydrolysis process. Further research is needed to explore the interactions between nanobubbles and cells, and to reveal how nanobubbles mediate extracellular and intracellular metabolic mechanism. Finally, the O₂NW treatment increased the abundance of electroactive bacteria (*Syntrophomonas* and *Petrimonas*) in this study, potentially enhancing DIET for increased biogas production. However, due to only a microbial community test was performed and other evidence (such as electron transfer capacity) to support the DIET enhancement is lacking, further validation is required.

4. Conclusion

This study revealed mechanism by which CO₂-nanobubble water (CO₂NW) and O₂NW mediated the anaerobic digestion process. Metagenomic analysis revealed that CO₂NW specifically enhanced the expression of enzymes for the hydrogenotrophic methanogenesis pathway, whereas O₂NW stimulated both the acetoclastic and hydrogenotrophic methanogenesis. The micro-oxygen environment created by O₂NW upregulated the energy metabolism module, including tricarboxylic acid cycle and oxidative phosphorylation, thereby promoting ATP production. Furthermore, O₂NW increased substrate degradation rate and enhanced the activities of electron transport system and coenzyme F₄₂₀. The above results explained that the cumulative methane yields were increased by 6.9% and 18.3% for CO₂NBW and O₂NBW, respectively.

E-supplementary data for this work can be found in e-version of this paper online.

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Table 1 Kinetic parameters for methane yield according to the modified Gompertz model.

Treatment	P_m (mL/gVS)	R_m (mL/gVS/d)	λ (d)	R^2
DW	203.4 ± 4.8	16.4 ± 0.6	3.6 ± 0.1	0.993
CO ₂ NW	230.3 ± 4.8	16.4 ± 0.4	2.7 ± 0.1	0.996
O ₂ NW	263.5 ± 5.5	20.1 ± 0.5	3.1 ± 0.1	0.997

Notes: P_m : the methane potential yield; R_m : the maximum methane yield rate; λ : delay phase; The values were the mean $\pm$ standard deviation (n=3).

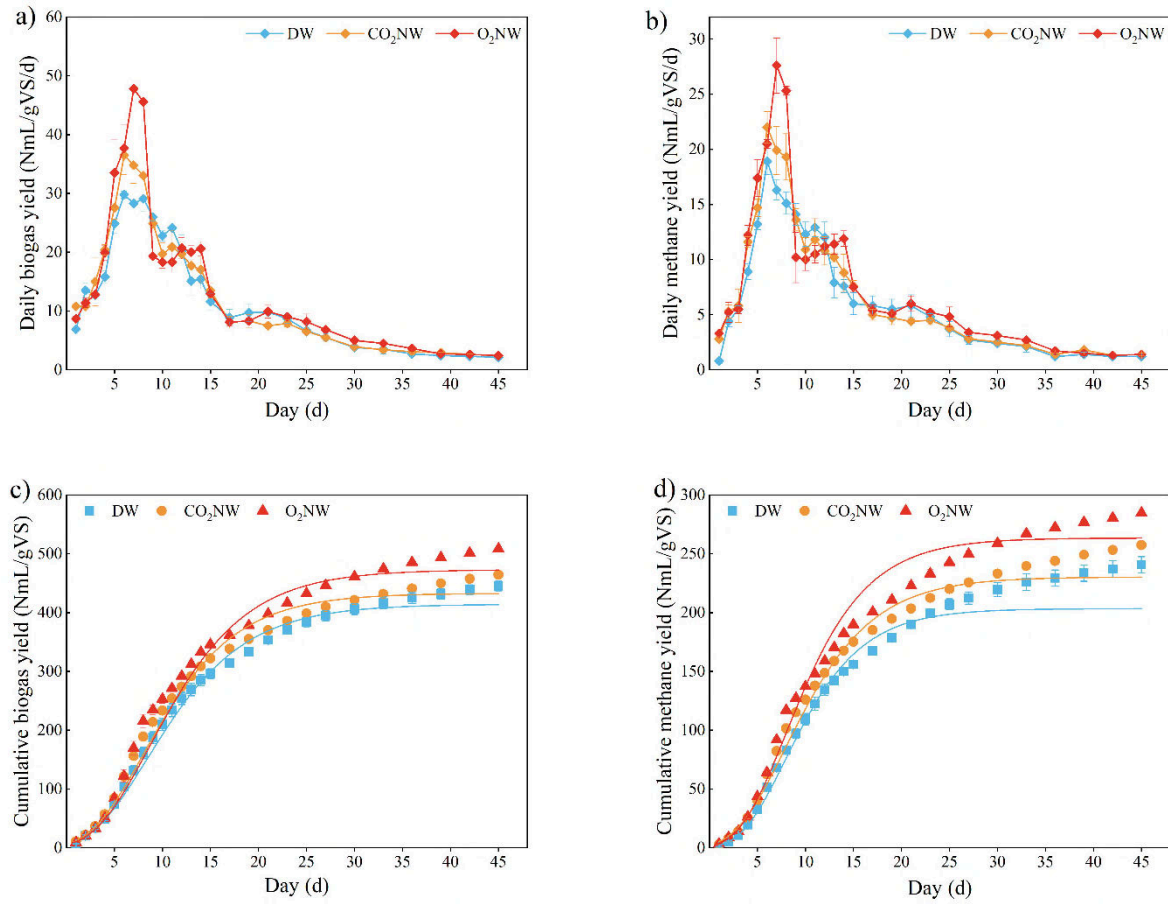


Fig. 1. The change of daily biogas yield (a), daily methane yield (b), cumulative biogas yield (c), and cumulative methane yield (d). The dots indicate batch experimental data and lines indicated Gompertz model prediction.

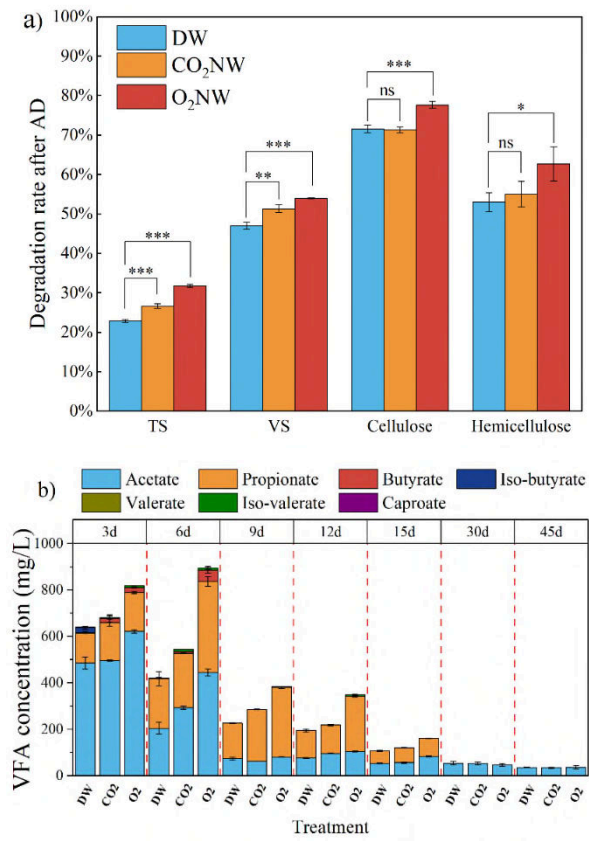


Fig. 2. The effects on substrate degradation rate (a) and volatile fatty acids (VFA) concentration during AD (b). (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, $p > 0.05$)

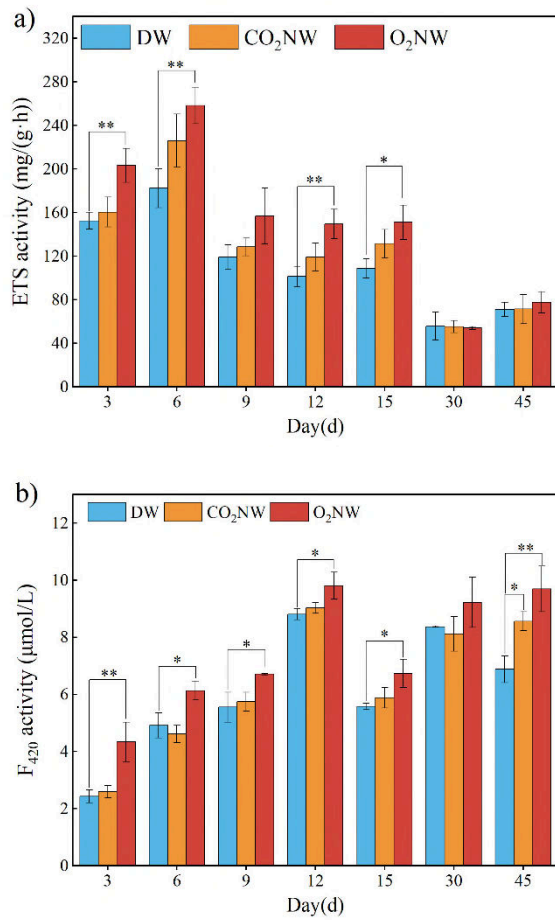


Fig. 3. The effects on the electron transport system (ETS) activity (a) and the coenzyme F₄₂₀ activity (b). (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

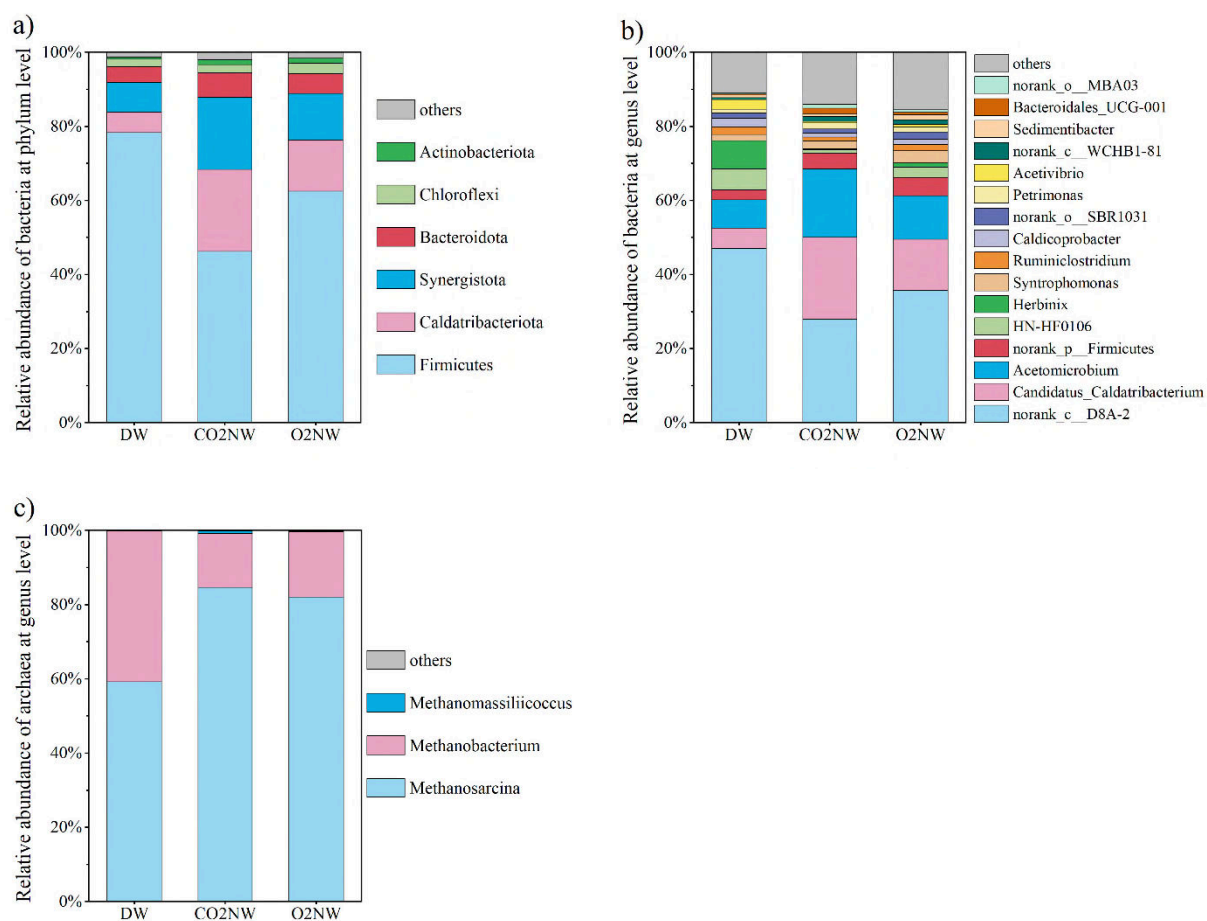


Fig. 4. Relative abundance of the dominant bacteria at phylum level (a) and genus level (b), and archaea at genus level (c).

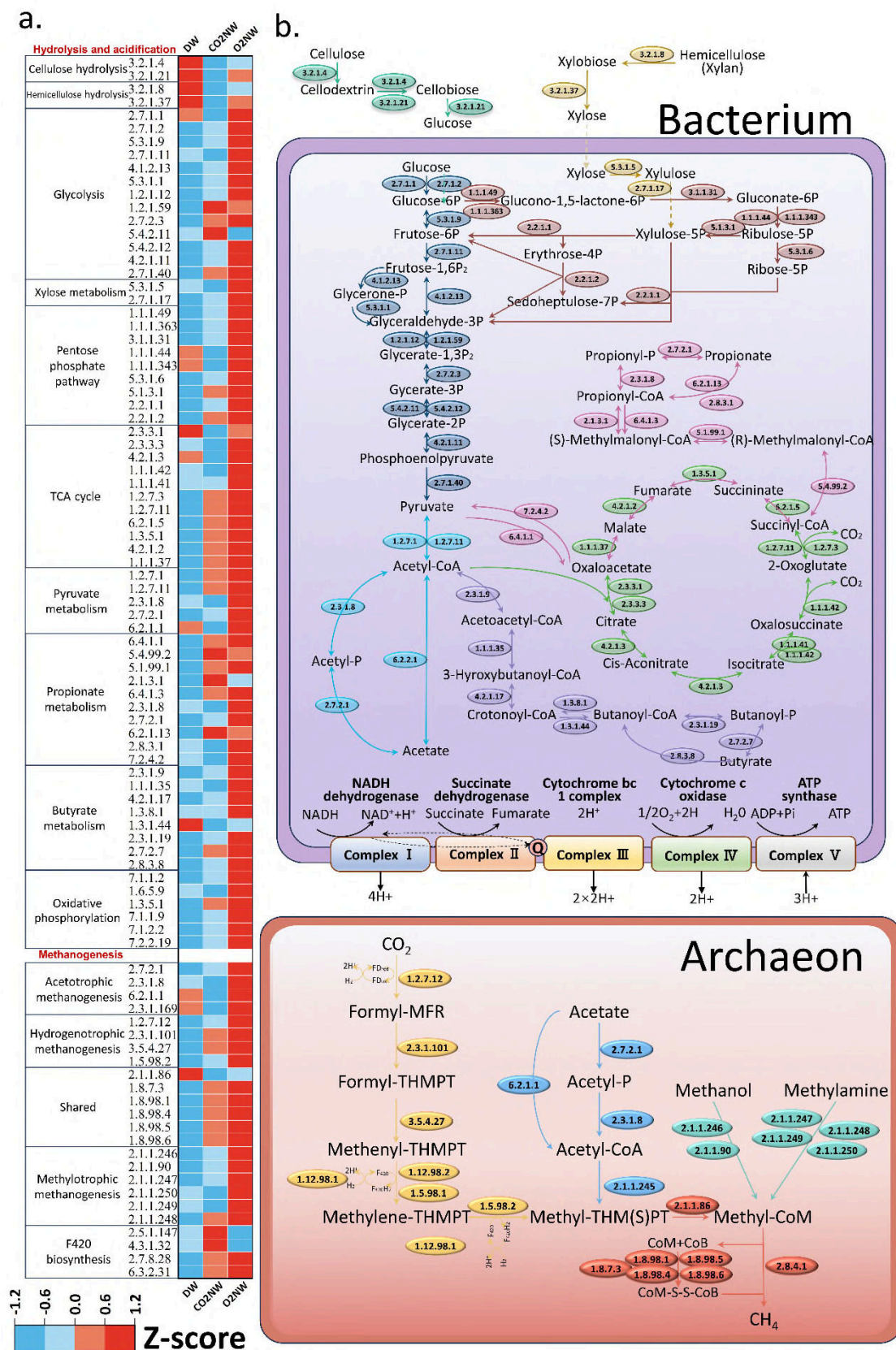


Fig. 5. Heatmap for the relative abundances of functional enzymes (a) and metabolic pathway map of lignocellulose degradation and methane production (b).



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