Environmental Research 251 (2024) 118725

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

A novel microbial community restructuring strategy for enhanced hydrogen production using multiple pretreatments and CSTR operation

Jishan Jiang[a], Tielan Guo[b], Jingyuan Wang[b], Ao Sun[b], Xingping Chen[b], Xiaoxiao Xu[b], Shaojun Dai[b], Zhi Qin[b,*]

[a] School of Environmental and Geographical Sciences, Shanghai Normal University, Shanghai 200234, China

[b] Development Center of Plant Germplasm Resources, College of Life Sciences, Shanghai Normal University, Shanghai 200234, China

ARTICLE INFO

Handling Editor: Dr Aijie Wang

Keywords:
Multiple pretreatments
Microbial community
Activated sludge
Hydrogen production
CSTR
Anaerobic fermentation

ABSTRACT

To achieve rapid enrichment of the targeted hydrogen-producing bacterial population and reconstruction of the microbial community in the biological hydrogen-producing reactor, the activated sludge underwent multiple pretreatments using micro-aeration, alkaline treatment, and heat treatment. The activated sludge obtained from the multiple pretreatments was inoculated into the continuous stirred tank reactor (CSTR) for continuous operations. The community structure alteration and hydrogen-producing capability of the activated sludge were analyzed throughout the operation of the reactor. We found that the primary phyla in the activated sludge population shifted to Proteobacteria, Firmicutes, and Bacteroidetes, which collectively accounted for 96.69% after undergoing several pretreatments. This suggests that the multiple pretreatments facilitated in achieving the selective enrichment of the fermentation hydrogen-producing microorganisms in the activated sludge. The CSTR start-up and continuous operation of the biological hydrogen production reactor resulted in the reactor entering a highly efficient hydrogen production stage at influent COD concentrations of 4000 mg/L and 5000 mg/L, with the highest hydrogen production rate reaching 8.19 L/d and 9.33 L/d, respectively. The main genus present during the efficient hydrogen production stage in the reactor was *Ethanoligenens*, accounting for up to 33% of the total population. *Ethanoligenens* exhibited autoaggregation capabilities and a superior capacity for hydrogen production, leading to its prevalence in the reactor and contribution to efficient hydrogen production. During high-efficiency hydrogen production, flora associated with hydrogen production exhibited up to 46.95% total relative abundance. In addition, redundancy analysis (RDA) indicated that effluent pH and COD influenced the distribution of the primary hydrogen-producing bacteria, including *Ethanoligenens*, *Raoultella*, and *Pectinatus*, as well as other low abundant hydrogen-producing bacteria in the activated sludge. The data indicates that the multiple pretreatments and reactor's operation has successfully enriched the hydrogen-producing genera and changed the community structure of microbial hydrogen production.

1. Introduction

Hydrogen energy has the advantages of high energy density, cleanliness and non-pollution, and therefore has received extensive attention in the development of new renewable energy sources (Matamba et al., 2023). In recent years, anaerobic fermentation for hydrogen production using high concentration organic wastewater as a substrate has been favored due to its dual role of wastewater treatment and clean energy production. Anaerobic fermentation hydrogen production technology utilizing organic wastewater employs a continuous flow method to provide organic substrates. The fermentation of mixed anaerobic microorganisms in the hydrogen production reactor enables the continuous production of clean energy source hydrogen (Ren et al., 2011; Li et al., 2021).

Pretreatment of reactor-inoculated activated sludge at the beginning of the start-up operation of a biological hydrogen-producing reactor has become an important means of increasing the percentage of the target hydrogen-producing bacteria in the mixed sludge flora (Fu et al., 2021; Zhou et al., 2020). The researches on pretreatment of the activated sludge primarily focuses on heat treatment, acid and alkali treatment, and micro-aeration pretreatment (Alexandropoulou et al., 2023). The pretreatment method of heat treatment is currently more widely used.

* Corresponding author.
E-mail address: qinzhi@shnu.edu.cn (Z. Qin).

https://doi.org/10.1016/j.envres.2024.118725
Received 6 December 2023; Received in revised form 7 February 2024; Accepted 13 March 2024
Available online 20 March 2024

For example, the 100 °C pretreatment of activated sludge from soybean treatment plant and sewage treatment plant can significantly improve the hydrogen production capacity, the maximum was 1.9 mol H_2/mol glucose (Rossi et al., 2011; Han et al., 2015). Hydrogen production capacity from activated sludge was also significantly increased when alkali treatment and micro-aeration were used to pretreat activated sludge (Wang and Yin, 2017; Fu et al., 2020). Micro-aeration pretreatment contributed to the enrichment of hydrolytic and fermentative bacteria in activated sludge (Ruan et al., 2019) and was also beneficial for increasing the enzyme activity of hydrolytic bacteria and substrate efficient hydrolysis (Zhou et al., 2021).

In recent years, researchers have increasingly investigated the use of synergistic combinations of two or more pretreatment methods for activated sludge pretreatment, in addition to single pretreatment methods (Hassan et al., 2020; Hu et al., 2023). Pretreatment of wastewater activated sludge (WAS) with a combination of free nitrite and calcium hypochlorite showed that the combined pretreatment significantly increased the hydrogen production capacity of the activated sludge compared to pretreatment with either method alone, with the maximum hydrogen production rate increased by 76.2% compared to pretreatment with either method alone (Ye et al., 2023). A study of WAS pretreatment using a low temperature combined calcium hypochlorite (CH) method (−5 °C, 0.12 g/g VSS CH, CH 65% purity) found that the highest hydrogen production yield was up to 18.18 ± 0.43 mL/g VSS (Hu et al., 2023). Although research on combined pretreatment has been conducted, it has mainly focused on the combined pretreatment of anaerobic digested sludge, and the pretreatment of a small number of hydrogen-producing activated sludges is also based on batch fermentation experiments and lacks long-term monitoring (Hassan et al., 2020; Hu et al., 2021). Combined multiple pretreatments of anaerobic hydrogen-producing activated sludge and inoculation of the pretreated sludge into a continuous stirred tank reactor (CSTR) for long-term continuous cultivation, and long-term and systematic comprehensive tracking of the activated sludge flora during operation have rarely been reported.

In this study, three pretreatment methods, namely micro-aeration, alkaline treatment, and high-temperature heat treatment, were combined to reduce the number and activity of methanogenic bacteria and other hydrogen-consuming bacteria in activated sludge, and to effectively enrich the hydrogen-producing flora in the activated sludge. This process also improved the hydrogen-producing flora of activated sludge, ultimately resulting in a mixed bacterial flora of activated sludge with higher hydrogen-producing capacity and stability. The constructed high-efficiency hydrogen-producing flora were inoculated into the CSTR for continuous operation. A multi-stage approach was adopted to systematically track the change rule of microbial community in the reactor during the critical period of reactor operation. This laid an important research foundation for the realization of high-efficiency hydrogen production and stable operation of the biohydrogen production reactor. It also provides critical information for further optimizing the bacterial community structure and improving the hydrogen production capacity of the reactor.

2. Materials and methods

2.1. The pretreatment of seed sludge

The raw sludge, detoxified in the secondary sedimentation tank of the chemical pollutant treatment plant, is used as the activated sludge inoculant in the CSTR for hydrogen production. This original activated sludge is black and granular with a positive settling performance. The sludge settling velocity (SV30) is approximately 15%. After three stages of pretreatment, the activated sludge was inoculated as seeding sludge into the CSTR to start a continuous biological hydrogen production reaction. In the pretreatment stage I (Pre-I), the seeding sludge was diluted from 5 L to 20 L and then micro-aerated at 1 L/h aeration rate for 7 days (Nguyen and Khanal, 2018). In the pretreatment stage II (Pre-II), the sludge was treated with alkali (10% w/v NaOH, pH 10) for 24 h (Cai et al., 2004; Dessì et al., 2018; Kim et al., 2013; Zhou et al., 2020). And then the sludge was subjected to a heat treatment (115 °C) for 15 min (Wang and Yin, 2017). Which was the pretreatment stage III (Pre-III).

2.2. Experimental equipment

The reactor is designed as an integrated structure of the reaction area and the settlement area (Fig. 1). The reactor is equipped with an electric stirring device for complete mixing of organic waste water and activated sludge. The reactor also has a solid-liquid-gas three-phase separation device inside. the inlet water flow rate was automatically controlled by a metering pump, and the temperature was controlled at 35 ± 1 °C by a temperature probe installed in the inner cylinder of the reactor. The effective volume of the reaction area of the reactor is 6 L.

2.3. Acclimation of anaerobic activated sludge and reactor operation control

The substrate for the biohydrogen production reactor was sugarcane molasses. To keep the ratio of m(COD):m(N):m(P) at 1000:5:1, the NH_4Cl and K_2HPO_4 were added in sugarcane molasses.

The activated sludge was inoculated into the CSTR after micro-aeration, alkali treatment, and heat pretreatment, and then operated continuously for 170 d. The hydraulic retention time (HRT) was controlled to be about 8 h. In the initial stage of the reactor start-up operation, the influent COD concentration of molasses was set at 3000 mg/L, which corresponds to an organic loading rate (OLR) of 9 kg/m^3·d (Stage I). The OLR of the reactor was subsequently controlled by adjusting the influent COD concentration of the reactor after the initial acclimation of the activated sludge. The influent COD concentration in the reactor was gradually increased to 4000 mg/L (Stage II), 5000 mg/L (Stage III), and 6000 mg/L (Stage IV). As a result, the OLR of the reactor also gradually increased to 12 kg/m^3·d, 15 kg/m^3·d, and 18 kg/m^3·d, respectively.

2.4. High-throughput sequencing

A total of 15 samples from the activated sludge in various periods of the seeding sludge (Origin), the pretreatment stages (Pre-I, Pre-II, and Pre-III), and the start-up and continuous operation stages (Stage-I, Stage-II, Stage-III, and Stage-IV; two sampling time points in Stage-I and three in Stage II, III, and IV, respectively) were used for the analysis of the microbial community structure (Supplemental table S1).

DNA extraction of 15 samples was carried out using a soil DNA kit. PCR amplification of the extracted DNA was performed using the universal primer 341F/805R for the conserved region V3–V4 of the samples. High-throughput sequencing was performed on the Illumina Miseq™/HiSeq™ platform (Zhang et al., 2014). Sequences are clustered into Operational Taxonomic Units (OTUs) as proxies for species (Edgar, 2018). Samples were analyzed and visualized for microbial community structure based on the R and RStudio platforms (R Core Team, 2023). Various indices such as the Abundance-based Coverage (ACE), the Chao1 estimator, the Good's coverage (Coverage), the Shannon index, the Simpson index, and the Pielous' evenness index (Pielou) were used to measure the microbial diversity of the activated sludge (Dou et al., 2023; Ren et al., 2022; Zhang et al., 2012). Redundancy analysis (RDA) was performed to identify the environmental variables that affect the microbial community at the genus level (Dou et al., 2023). These analyses were performed using the "Vegan" package (vegan 2.6–4) in the R environment (version 4.3.1) (Sun et al., 2024). The phyla with relative abundance greater than 1% in all samples were taken as the dominant flora (Shi et al., 2023).

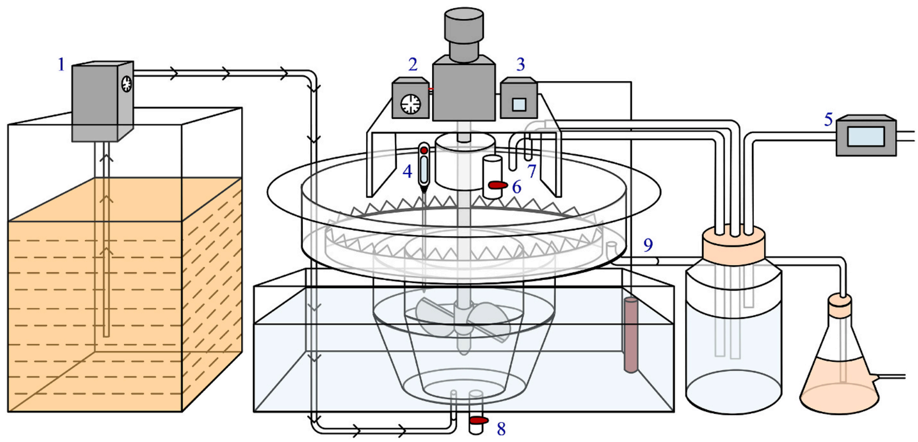

Fig. 1. Schematic of the CSTR. 1. Inflow pump. 2. Stirring paddle and speed control motor. 3. Heating rod thermostat. 4. Thermometer. 5. Gas meter. 6. Inlet port; 7. Outlet port. 8. Biological sampling port. 9. Drain port.

2.5. Analytical methods

The COD concentration was determined by potassium dichromate method (Walter, 1961). The pH was monitored by PHS-3C acidimeter (Li et al., 2004). The cumulative gas production was measured by wet gas flow meter (Yin et al., 2023). The hydrogen content was detected by GC1120 gas chromatograph with thermal conductivity cell detector (Simon and Arndt, 2002). Liquid-phase fermentation product compositions were analyzed using a 7890B-5977A GC-MS gas chromatograph and a Waters SQ Detector 2 liquid-liquid-mass spectrometer with a four-stage rod (Chen et al., 2014; Cordell et al., 2013).

3. Results and discussion

3.1. Changes in microbial community structure during the pretreatment stage

The microbial community structure and diversity were altered during the multiple pretreatment process (Table 1 and Fig. 2). The coverage of the samples in the Origin and three pretreatment stages is above 99%, indicating that the sequencing results can represent the real situation of the samples (Table 1). According to Shannon and Simpson's diversity index analysis, the microbial diversity was increased and reached the maximum value in stage Pre-I, which indicates that micro-aeration pretreatment is beneficial to the microbial diversity of activated sludge (Table 1). The microbial diversity showed a decrease during alkali treatment and heat treatment in stages Pre-II and Pre-III, with a significant decrease in Stage Pre-III (Table 1). The OTU index also reflected a significant decrease in stage Pre-III (Table 1). This indicates that heat treatment at stage Pre-III has a more significant effect on both the diversity of the microbial community and the number of microorganisms.

A total of 26 phyla were identified at the phylum level (Fig. 2a and b). The stages Origin and Pre-I shared the similar dominant phyla (relative abundance >1%) (Fig. 2a). Some of the dominant phyla exhibited significantly high abundance in stage Pre-I than that in stage Origin, such as Proteobacteria, Planctomycetes, Bacteroidetes, Acidobacteria, Actinobacteria, and Firmicutes (Fig. 2b). Micro-aeration pretreatment has been utilized to enhance the facultative hydrolytic and acid-producing bacteria in anaerobic digestion (Huiliñir et al., 2023; Zhang et al., 2021). Nevertheless, micro-aeration has not been employed as a pretreatment technique in anaerobic fermentation for hydrogen production. The current study reports the dominance of Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes, which are important phyla in the anaerobic degradation of complex substrates, hydrolysis, and acidification (Ruan et al., 2019). Interestingly, these phyla have also been reported in the anaerobic digestion system (Huiliñir et al., 2023). This suggests that these phyla were critical for the two-phase anaerobic fermentation. In stage Pre-I, the relative abundances of 22 phyla were increased when compared with the stage Origin (Fig. 2b). This indicates that micro-aeration in pretreatment stage favors the growth of aerobic bacteria and also indirectly favors the growth of facultative anaerobe and strict anaerobe bacteria in the activated sludge. The optimized community structure after micro-aeration contributed to further screening of hydrogen-producing bacteria.

To reduce the methanogenic activity of the seed sludge, alkaline pretreatments were used. The community composition and abundance of microorganisms were altered obviously after the treatments of alkali and thermal in stage Pre-II and Pre-III (Fig. 2). Significantly, the Firmicutes (59.58%) and Bacteroidetes (33.27%) were the most dominant phyla in stage Pre-II, which was quite different with other three stages (Fig. 2a). This indicates that the alkali treatment at stage Pre-II removed

Table 1
Sequence information and diversity analysis of samples in the multiple pretreatment stages.

Sample	Reader[a]	Coverage[b]	OTUs[c]	Chao1[d]	ACE[e]	Shannon[f]	Simpson[g]	Pielou[h]
Origin	26200	0.9957	736	838.0645	806.4766	5.1106	0.0171	0.7742
Pre-I	47774	0.9991	681	701.0233	700.2984	5.2000	0.0138	0.7971
Pre-II	52051	0.9977	769	838.3204	874.0187	5.0867	0.0190	0.7655
Pre-III	38670	0.9965	696	777.4865	801.6525	3.5077	0.1170	0.5359

[a] Reader is the sum of observed Operational Taxonomic Units (OTUs).
[b] Good's coverage (Coverage) refers to the coverage of the detection and sequencing of the species.
[c] The OTUs is the number of observed OTUs for an OTU definition.
[d] The Chao1 estimator and.
[e] the Abundance-based Coverage (ACE) estimator are indexes to measure the species richness of the community.
[f] the Shannon index and [g] the Simpson index are indexes to measure the diversity of the community.
[h] the Pielous' evenness index (Pielou) is index to measure species evenness for each community.

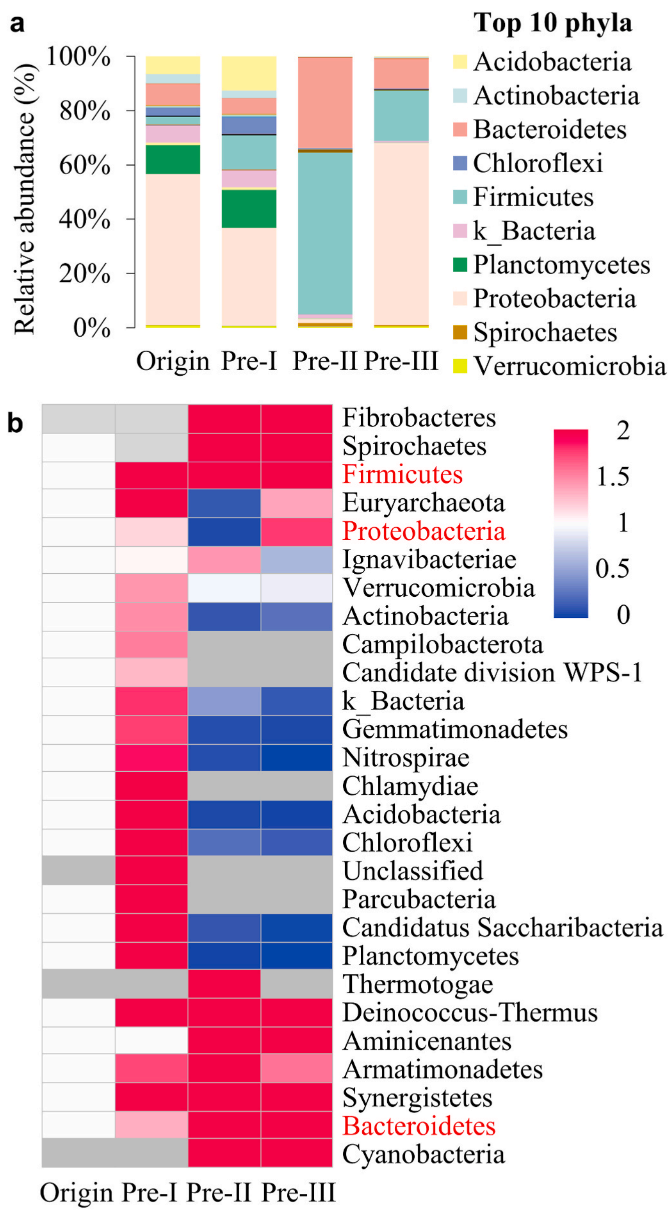

Fig. 2. Activated sludge microorganisms in the multiple pretreatment stages at the phylum classification level. (a) community structure analysis; (b) microbial community composition and relative abundance analyses. Undetermined phylum was named using a higher taxonomic level, such as k_Bacteria. Red and blue represent the increase and decrease of the phylum abundances, respectively. Gray represents phyla not identified.

most other bacteria in reactor. A recent study reported that the alkalization treatment was applied to the *in-situ* waste sludge in anaerobic bioreactors (ABRs). After 7 days of treatment and agitation at 400 rpm, the dominant phyla were enriched in the sludge such as Firmicutes, Bacteroidetes, and Proteobacteria under the optimized alkalinities of pH 9 and 10 (Zhou et al., 2020). The abundance of Proteobacteria showed a significant decrease from 36% in the stage Pre-I to 1.56% in the stage Pre-II, suggesting greater alkaline sensitivity of Proteobacteria in comparison to Bacteroidetes.

Following the alkali treatment, the heat treatment further contributed to the enrichment of hydrogen production bacteria and reduced the methanogenic activity (Wang and Yin, 2017). In the stage Pre-III, Proteobacteria (67.15%), Firmicutes (18.59%) and Bacteroidetes (10.95%) were selected as the dominant phyla (Fig. 2a). The Proteobacteria in stage Pre-III was increased 31.98 times when compared with that in stage Pre-II (Fig. 2a). These phyla have also been identified in the activated sludge from the batch reactor after the combined alkali and thermal pretreatment for anaerobic fermentation of hydrogen production (Kang et al., 2012). The Proteobacteria, Bacteroidetes, and Firmicutes are common fermentative acid-producing bacteria in mesophilic anaerobic reactors (Kang et al., 2012; Xin et al., 2021). They are microorganisms that have genes encoding hydrogenases (Ma et al., 2021). [FeFe]-hydrogenase homologs are mainly present in the Firmicutes, whereas [NiFe]-hydrogenase homologs are primarily found in the Proteobacteria (Peters et al., 2015). The significant enrichment of these flora in stage Pre-III implied that the combined pretreatments of alkali and heat facilitated the screening of hydrogen-producing bacteria in activated sludge.

The pretreatments have been reported to optimize the microbial community structure of activated sludge (Fu et al., 2023; Mohammadi et al., 2012; Rafieenia et al., 2018). The multiple pretreatments of micro-aeration, heat, and alkaline conditions was utilized in this study. Our findings indicate an increase in the abundance of anaerobic fermentation hydrogen-producing bacteria in the activated sludge, promoting hydrogen production in the reactor.

3.2. CSTR start-up and continuous operation

After multiple pretreatments, the activated sludge was inoculated into the biological hydrogen production reactor, and the reactor was then started up and operated. To complete the start-up of the reactor and the acclimation of the activated sludge, the influent COD concentration was gradually increased (Fig. 3a). In the initial two stages, the influent COD was regulated at 3000 mg/L and 4000 mg/L, respectively. The gradual increase of the influent COD substantially increased the COD removal efficiency under these circumstances (Fig. 3a). The average COD removal efficiencies at the two stages were 14.59% and 29.43%, respectively. This suggests that the activated sludge can be acclimated to attain a satisfactory COD removal efficacy when the influent COD concentration is at 4000 mg/L (Fig. 3a). In Stage-III, the reactor demonstrated the capacity to sustain proficient COD removal efficacy (30%) when handling an influent COD concentration of 5000 mg/L, the optimal operational condition for the reactor (Fig. 3a). Under the condition of the influent COD of 6000 mg/L in Stage-IV, the COD removal efficiency of the reactor was gradually decreased and the average COD removal efficiency was 26.99% (Fig. 3a). This indicates that the removal of organic material in the reactor is being hindered by an excessive substrate concentration. Therefore, in the stable operation stage (Stage III) of the biological hydrogen production reactor, the appropriate influent COD should be controlled at approximately 5000 mg/L to ensure higher COD removal efficiency.

The influent and effluent pHs changed during the start-up and operation of the CSTR (Fig. 3b). At the very beginning of Stage-I, the CSTR shifted from an aerobic to an anaerobic environment, and the effluent pH was decreased rapidly from 8.00 on day 1–5.49 on day 5. From Stage-I to Stage-IV, the anaerobic fermentation of the activated sludge produced organic acids, resulting in a gradual decrease of the pH value in the reactor. The average values of effluent pH in four stages were 5.17, 4.86, 4.61, and 4.38, respectively (Fig. 3b).

During the CSTR start-up and operation stages, the hydrogen production capacity is mediated by COD concentration and pH changes in various stages of the reactor (Fig. 3c). At the very beginning of Stage-I, the reactor had a low average hydrogen production rate and hydrogen production yield of 0.22 L/d and 0.07 mol H_2/mol glucose, respectively (Fig. 3c). The gas and hydrogen productions were gradually increased after the activated sludge entered the Stage-II. The hydrogen production rate was stabilized at 85 d–108 d, and the average gas and hydrogen production rate were 46.68 L/d and 6.58 L/d, respectively. During this period, the highest hydrogen production rate of 8.19 L/d was achieved at 91 d, along with the highest hydrogen production yield of 1.83 mol H_2/mol glucose (Fig. 3c). In Stage-III, the maximum hydrogen

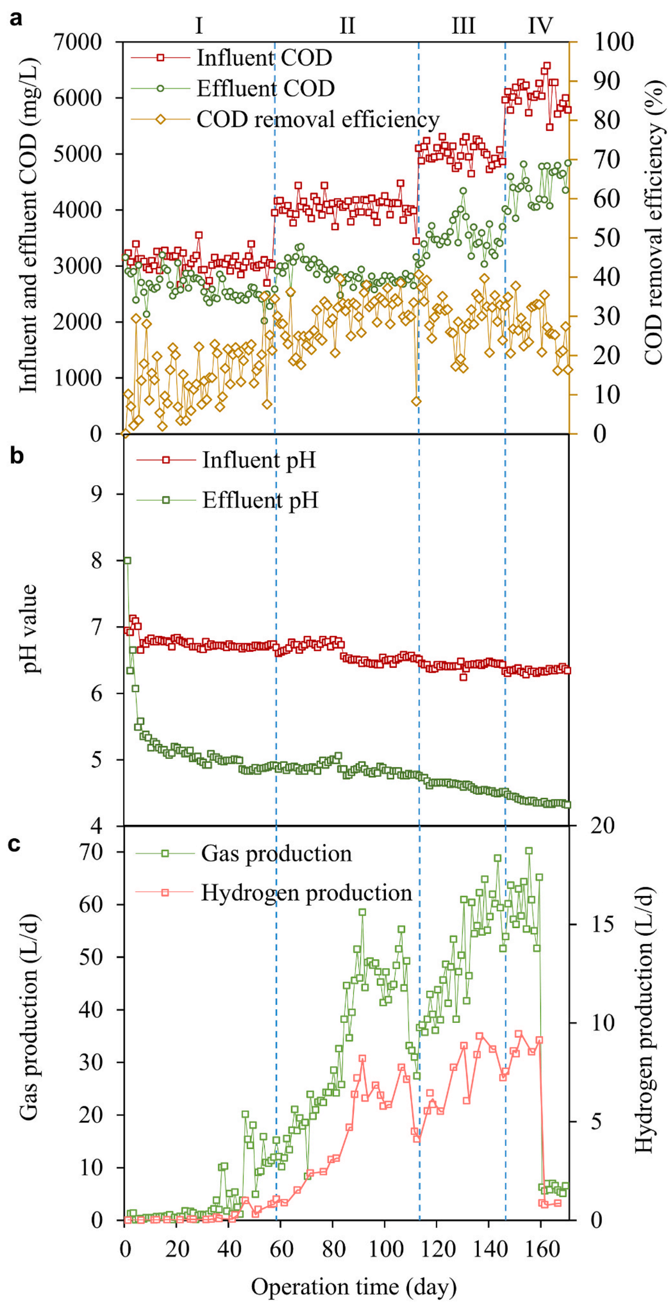

Fig. 3. Changes of operational parameters during the CSTR start-up and continuous operation. (a) influent COD, effluent COD, and COD removal efficiency. (b) influent pH and effluent pH. (c) gas and hydrogen production yields.

production rate and hydrogen production yield were 9.33 L/d and 1.67 mol H_2/mol glucose, respectively. At the beginning of Stage-IV, the hydrogen production rate and hydrogen production yield were 9.44 L/d and 1.40 mol H_2/mol glucose, respectively. However, at the end of Stage-IV (after 160 d), the production of gas and hydrogen was significantly reduced. This suggests that the excessive concentration of substrate is detrimental to the hydrogen production process in the reactor.

Various anaerobic fermentation products from activated sludge at different stages of CSTR operation were detected using LC-MS method (Table 2). At the outset of Stage-I, acetic acid and butyric acid were the predominant fermentation metabolites of the activated sludge. This indicates that the CSTR fermentation type at this stage was butyric acid-type (Table 2). By the end of Stage-I (Stage-I-2), the concentration of butyric acid was comparatively lower, whereas the yields of valeric and propionic acids were higher than those from Stage-I-1. This suggests that the butyric acid-type fermentation began to decline. (Table 2). Furthermore, it is noteworthy that the middle of Stage–II–2 indicated a significantly higher concentration of ethanol. This observation implies that the reactor had successfully transitioned from butyric acid-type fermentation to ethanol-type fermentation during Stage-II. (Table 2). Additionally, there was an increase in the total amount of fermentation products during Stage-II and Stage-III, primarily attributed to the rise in influent COD concentration in the reactor. Conversely, a decrease in the total amount of fermentation products occurred during Stage-IV, indicating that the activated sludge curbed the degradation of organic substrate to generate organic acids under extreme COD conditions (Table 2).

In summary, during reactor operation, the collected data indicates that at the beginning of the CSTR start-up stage, there was a predominant fermentation of the butyric acid type under conditions of 3000 mg/L influent COD and an average effluent pH value of 5.17. In Stage-II, as the influent COD was kept under control at 4000 mg/L and pH at 4.86, the fermentation transitioned into the ethanol type. Higher pH environments benefit the achievement of butyric acid type fermentation, whereas lower pH environments promote the conversion of ethanol type fermentation (Li et al., 2007). For instance, in the study of anaerobic fermentation for hydrogen production in the CSTR, butyric acid fermentation was observed at pH levels 5.0, 5.5, and 6.0, while ethanol fermentation was observed at pH 4.0 (Wu et al., 2017). The pH range of the effluent between 4.35 and 4.92 is optimal for improving hydrogen production in the reactor. The maximum hydrogen production rate and hydrogen production yield recorded were 8.13 L/d and 1.83 mol H_2/mol glucose in Stage-II. Stage III was marked by high-efficiency hydrogen production, yielding a maximum of 9.33 L/d and 1.67 mol H_2/mol glucose with a 5000 mg COD/L and an effluent pH value of 4.61.

Compared to activated sludge with micro-aeration pretreatment, the efficiencies of hydrogen production and COD removal were significantly improved (Table 3). The average hydrogen production of activated sludge with micro-aeration pretreatment after acclimation of different substrate concentrations was highest at 5000 mg COD/L, 189.45 mL/d. At 3000 mg COD/L, the dominant bacteria were *Spartobacteria*

Table 2
Changes in fermentation production mass concentration during CSTR start-up operation.

Sample	Ethanol (mg·kg^{-1})	Butanol (mg·kg^{-1})	Valerate (mg·kg^{-1})	Lactate (mg·kg^{-1})	Propionate (mg·kg^{-1})	Butyrate (mg·kg^{-1})	Acetate (mg·kg^{-1})
Stage-I-1	(<10)	(<5)	220	(<20)	620	4230	6500
Stage-I-2	(<10)	(<5)	350	(<20)	900	2580	4890
Stage–II–1	(<10)	(<5)	480	(<20)	1290	2060	5230
Stage–II–2	5200	(<20)	1000	(<5)	1000	2000	5000
Stage–II–3	(<20)	(<20)	1000	(<5)	1000	2000	7000
Stgae–III–1	(<20)	(<20)	1000	(<5)	1000	3000	8000
Stgae–III–2	(<20)	(<20)	1000	(<5)	1000	2000	1000
Stage–III–3	(<20)	(<20)	1000	(<5)	2000	2000	8000
Stage-IV-1	(<10)	(<5)	130	(<2.5)	570	240	1400
Stage-IV-2	(<10)	(<5)	97	(<2.5)	600	170	1600
Stage-IV-3	(<10)	(<5)	89	(<2.5)	510	170	1700

Table 3
Parameters of activated sludge after micro-aeration pretreatment inoculated into a CSTR reactor.

Parameters	3000 mg/L COD	4000 mg/L COD	5000 mg/L COD
pH	From 4.7 to 4.9		
Average COD removal efficiencies	10.62%	20.3%	22.4%
Gas production rate	1216 mL/d	1488 mL/d	1506 mL/d
Hydrogen production rate	84 mL/d	159 mL/d	189.45 mL/d
Main metabolites	Acetate, butyrate, and valerate	–	Acetate, butyrate, and propionate
Dominant genera (relative abundance)	*Spartobacteria* (26.10%), *Megasphaera* (13.69%), and *Clostridium* IV (4.69%)	*Novosphingobium* (55.95%), *Ethanoligenens* (3.45%), and *Clostridium sensu stricto* (2.13%)	*Ethanoligenens* (24.16%), *Megasphaera* (21.44%), and *Clostridium* IV (15.52%)

(26.10%), *Megasphaera* (13.69%), *Clostridium* IV (4.69%). At 4000 mg COD/L, the dominant organisms were *Novosphingobium* (55.95%), *Ethanoligenens* (3.45%), and *Clostridium sensu stricto* (2.13%). At 5000 mg COD/L, the dominant species were *Ethanoligenens* (21.16%), *Megasphaera* (21.44%), and *Clostridium* IV (15.52%). Under the micro-aeration pretreatment condition, the activated sludge microbial community was poorly stable, which could have resulted in the low hydrogen production during this CSTR operation (Wang et al., 2018).

Compared to other studies, our multiple pretreatment strategy for activated sludge can achieve a higher hydrogen production rate or hydrogen production yield. For instance, the activated sludges acclimated in the CSTR resulted in the maximum hydrogen production yield of 1.40 mol H_2/mol glucose at a condition of volumetric loading rate of 4400 mg COD/L (Chang et al., 2011) and a maximum hydrogen production rate of 6.6 L H_2/d under the condition of 8000 mg COD/L (Wang et al., 2013). Additionally, in the expanded granular sludge bed (EGSB) reactor, the granular sludge after mild pretreatment at 60 °C for 15 days resulted in the maximum hydrogen.

production yield of 1.64 mol H_2/mol glucose (Yin et al., 2023). Their maximum hydrogen production rate or hydrogen production yield were all lower than our values of 9.33 L/d or 1.83 mol H_2/mol glucose, respectively (Fig. 3c). Although the specific parameters of the reactors are different in these studies, we can still speculate that employing a multiple pretreatment strategy for activated sludge could improve the capacity for hydrogen production.

3.3. Microbial community structure during the CSTR start-up and continuous operation stages

Microbial community structure of the activated sludge during the start-up operation of the CSTR hydrogen-producing reactor was analyzed using high-throughput sequencing method (Table 4). The analysis of Shannon's and Simpson's index showed that the diversity of the microflora did not change significantly from Stage-I to Stage-III when compared to those at the end of the multiple pretreatments (Table 4). In the Stage-IV, the microbial diversity and evenness were significantly reduced, which could be the main reason for the decrease in COD removal efficiency and gas production (Table 4).

The high-throughput sequencing analysis showed that the relative abundance patterns of microorganisms in the activated sludge changed during the different stages of reactor operation (Fig. 4). Among the 27 phyla detected in the activated sludge, Firmicutes (49.00% ~ 79.20%), Bacteroidetes (8.45–34.72%), and Proteobacteria (4.10% ~ 25.68%) were taken as the stable core anaerobic fermentation flora at different stages of reactor operation (Fig. 4a).

Species annotation revealed that 352 bacterial genera were identified in the activated sludge, with significant changes observed during reactor operation, leading to a clear stage specificity of the microbial community structure (Fig. 4b). In Stage I, the predominant genera were *f_Ruminococcaceae* (15.37%), *Ethanoligenens* (12.99%), and *o_Bacteroidales* (12.79%). Throughout Stage II, there was a shift in the dominant community of microorganisms, with *Ethanoligenens* (21.84%), *f_Ruminococcaceae* (16.73%), and *Prevotella* (9.28%) becoming the most prevalent. In Stage III, the primary types of microbial genera were *Ethanoligenens* (18.71%), *Propionispira* (14.36%), and *Enterobacter* (11.68%). During Stage IV, the reactor was dominated by a microbial community consisting of *Ethanoligenens* (30.38%), *Prevotella* (25.51%), and *f_Ruminococcaceae* (12.05%) (Fig. 4b). Importantly, the hydrogen-producing *Ethanoligenens* was the stable dominant genus in all these stages. The relative abundance of *Ethanoligenens* were increased significantly to 33% in stage-II, and remained at a high level (~22%) in stage-III (Fig. 5b). This indicates that *Ethanoligenens* is the main hydrogen-producing genus in the efficient hydrogen production stage in current study (Castro et al., 2013; Li et al., 2019). It has been reported that

Table 4
Sequence information and diversity analysis of samples during CSTR start-up and continuous operation.

Sample	Reader[a]	Coverage[b]	OTUs[c]	Chao1[d]	ACE[e]	Shannon[f]	Simpson[g]	Pielou[h]
Stage-I-1	41171	0.9978	379	470.0000	467.9091	3.8156	0.0460	0.6426
Stage-I-2	54231	0.9982	390	479.3000	485.4139	3.2760	0.0820	0.5491
Stage–II–1	51838	0.9981	375	485.2500	475.6629	2.8289	0.1746	0.4773
Stage–II–2	21519	0.9958	360	462.3750	448.8079	3.8020	0.0523	0.6459
Stage–II–3	28660	0.9967	369	489.0000	462.8075	3.4248	0.0953	0.5794
Stage–III–1	25697	0.9972	349	414.7000	404.8980	3.7044	0.0528	0.6327
Stage–III–2	29821	0.9970	373	479.3333	449.8733	3.5869	0.0731	0.6057
Stage–III–3	34986	0.9972	391	513.5263	480.7764	3.7041	0.0613	0.6206
Stage-IV-1	86784	0.9984	550	644.4660	677.8373	2.8374	0.1141	0.4497
Stage-IV-2	91029	0.9982	567	726.0000	723.6618	2.5695	0.1621	0.4053
Stage-IV-3	77298	0.9984	508	591.8022	612.1239	2.5961	0.2010	0.4167

[a] Reader is the sum of observed Operational Taxonomic Units (OTUs).
[b] Good's coverage (Coverage) refers to the coverage of the detection and sequencing of the species.
[c] The OTUs is the number of observed OTUs for an OTU definition.
[d] The Chao1 estimator and.
[e] the Abundance-based Coverage (ACE) estimator are indexes to measure the species richness of the community.
[f] the Shannon index and [g] the Simpson index are indexes to measure the diversity of the community.
[h] the Pielous' evenness index (Pielou) is index to measure species evenness for each community.

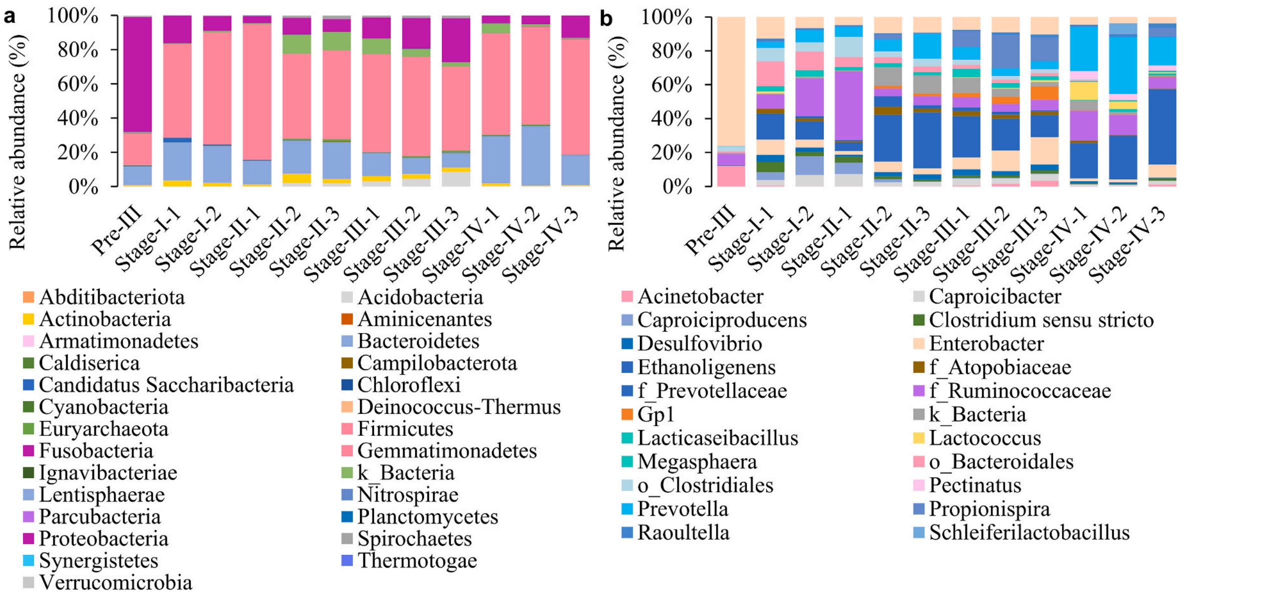

Fig. 4. Changes in relative abundance of activated sludge microorganisms during the CSTR start-up and continuous operation stages. (a) analysis at the phylum level. (b) analysis at the genus level of the "top 10″ samples. Unidentified genera are named using higher taxonomic levels such as family (f_), order (o_), and kingdom (k_).

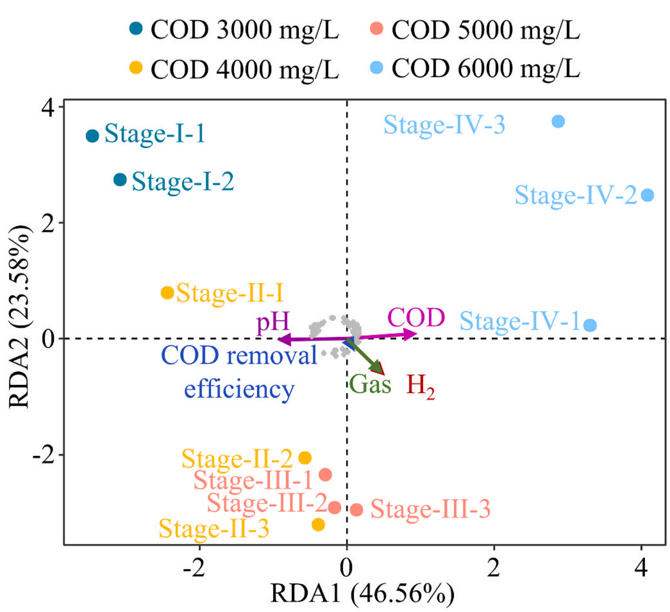

Fig. 5. Redundancy analysis (RDA) biplot depicting the relationship between environment factors and microbial community (at the genus level) during CSTR start-up and continuous operation. Colored dots represent the stage with the influent COD concentration. Colored arrows indicate environmental factors, and the length of the arrows indicates the effect of biochemical parameters on the on the genera. The cosine value between the arrow links indicates the correlation between them. The "envfit" function from the "vegan" R package was used to testing of the correlation between genera and environmental factors. r^2 represents the proportion of the total variance in the response variable that can be explained by the explanatory variable. the *p*-value is used to assess the significance of the model. There are 108 genera ($r^2 > 0.6$, $p < 0.05$) from the activated sludge that are marked with gray dots. The detailed information is shown in table S1.

Ethanoligenens has autoaggregation ability in activated sludge, which helps to improve the settling performance of activated sludge and reduce the loss of activated sludge (Ren et al., 2009). *Ethanoligenens* also has excellent hydrogen production potential, with a maximum hydrogen production yield of up to 2.14 mol H_2/mol glucose (Zhao et al., 2017). In our study, *Ethanoligenens* became the dominant hydrogen-producing genus in the activated sludge, ensuring continuous operation of the reactor with high hydrogen production.

In addition to *Ethanoligenens*, a large number of hydrogen production-associated microorganisms were enriched by activated sludge acclimation in our reactor operation, such as *Enterobacter* (Zhang et al., 2011), *Caproiciproducens* (Flaiz et al., 2020), *Raoultella* (Wang et al., 2019), *Pectinatus* (Castelló et al., 2009), *Megasphaera* (Kalia, 2015; Ohnishi et al., 2012; Prabhu et al., 2012), *Clostridium sensu stricto* (Chi et al., 2018) and *Caproicibacter* (Flaiz et al., 2020). The sum of the relative abundance of all these dominant hydrogen-producing communities were 46.06% in Stage-II and 46.95% in Stage-III, respectively. This suggests that the enrichment of various hydrogen-producing bacteria increased the diversity and ecological stability of the hydrogen-producing activated sludge in the reactor.

By comparing the dominant hydrogen-producing community in reactor operation with that at the stage Pre-III, we find that the hydrogen-producing associated flora showed an obvious increase in abundance during reactor operation (Fig. 4b). The total abundance of hydrogen-producing genera was induced to 43.19% in the Stage-I-1, which was much higher than that (0.05%) in the stage Pre-III of multiple pretreatments (Fig. 4b). For example, the abundance of *Clostridium sensu stricto* was significantly increased to 5.95% at the beginning of Stage-I-1 (Fig. 4b). *Clostridium sensu stricto* mainly contributes to butyric acid-type fermentation (Zhao et al., 2022). This was consistent with our findings that the higher butyric acid was detected in the beginning of Stage-I-1 (Table 2). Therefore, our pretreatment and reactor operation have successfully enriched the hydrogen-producing bacteria, and then switched from butyric acid-type fermentation to ethanol-type fermentation.

Additionally, the hydrogen-producing community analysis indicated that propionic acid-type fermentation related *Prevotella* and *Propionispira* were enriched in Stage-IV with excessive influent COD concentration (Fig. 4b). *Prevotella* and *Propionispira* have been found in the conditions with higher influent COD concentration, which competes with hydrogen-producing bacteria for substrates and consumes hydrogen (Lim et al., 2014). Thus, we suggest that the enrichment of propionic acid-type fermentation bacteria causes the decrease in the hydrogen-producing ability of the reactor.

3.4. Relationship between the bacterial community and the environment factors in CSTR

Throughout the activated sludge acclimation period, a total of 352 genera were identified. Out of these, 180 genera exhibited a positive correlation with the hydrogen production rate, while 172 genera showed a negative correlation (Supplemental table S2). Notably, during the period of increased hydrogen production capacity, from Stage-I to Stage-III, 109 genera showed a positive correlation while 165 genera exhibited a negative correlation with the hydrogen production rate (Supplemental table S2). Among them, 100 genera showed a positive correlation during the acclimation period (Stage-I to Stage-IV) and the period of hydrogen production increase (Stage-I to Stage-III) (Supplemental table S2). These included the representative hydrogen-producing bacteria, such as *Pectinatus* (Castelló et al., 2009), *Enterococcus* (Zhang et al., 2011), *Syntrophomonas* (Lozano et al., 2023), *Caloramator* (Ciranna et al., 2014), *Odoribacter* (Göker et al., 2011), *Phocaeicola* (Miebach et al., 2023), *Clostridium XlVa* (Gong et al., 2021), *Victivallis* (Yi et al., 2017), and *Bacteroides* (Ichikawa et al., 2023). These bacteria may contribute significantly to the variation in hydrogen production capacity. For example, *Enterococcus faecium* INET2 has been reported to have the ability to adjust its fermentation type under different pH conditions (Yin and Wang, 2019). Additionally, the 100 genera included a significant number of bacteria related to hydrogen production, such as *Propionispira* for reversible hydrogen production (Thompson et al., 1984), *Acetanaerobacterium* for carbohydrate degradation (Su et al., 2018), *Akkermansia* for cysteine production (Ottman et al., 2017), as well as *Turicibacter* and *Acetatifactor* for organic acid production (Zhong et al., 2015; Pfeiffer et al., 2012). Interestingly, 78 genera were exclusively identified at Stage-IV (Supplemental table S2). These included low abundance *Sporacetigenium* (Chen et al., 2006), *Opitutus* (Göker et al., 2011), and *Acetivibrio* (Mai et al., 2023) for hydrogen-producing bacteria, and *Dialister* for inhibiting hydrogen production (Kim et al., 2023), as well as high abundance *Schleiferilactobacillus* for protein degradation (Zheng et al., 2021). There were 27 genera of species were negatively correlated with hydrogen production rate at Stage-IV (hydrogen production decrease period), such as *Prevotella* and *Leuconostoc* competing with hydrogen producing bacteria (Lim et al., 2014; Stiles, 1994), as well as hydrogen-consuming *Streptomyces* (Collins and Gaines, 1964), *Hydrogenophaga* (Suzuki et al., 2014), and *Rhizobium* (O'Brian and Maier, 1988). These Stage-IV specific or negatively correlated bacteria may be one of the reasons for the decline in hydrogen production.

To better depict the relationship between the activated sludge community and environmental factors in CSTR, the RDA was performed (Fig. 5). The explanatory variables accounted for 70.14 %, which suggest that CSTR operation parameters could explain 70.14 % of the variation in the microbial community structure (Fig. 5).

RDA showed that most sample points in each stage were closely spaced (Fig. 5). For example, Stage-I-1 and Stage-I-2, Stage–II–2 and Stage–II–3, as well as three points in Stage III and Stage IV have close distribution, respectively. This indicated that each stage has relatively stable microbial community. However, Stage–II–1 located between Stage-I-2 and Stage–II–2, implying that Stage–II–1 is the transition period from Stage-I to Stage-II (Fig. 5). In addition, most of the sample points of Stage-II and Stage-III were close to each other, indicating that the two stages have similar and stable microbial communities for hydrogen production (Fig. 5).

RDA revealed that effluent COD concentration ($r^2 = 0.9; p = 0.002$), effluent pH ($r^2 = 0.8; p = 0.002$), and H_2 production ($r^2 = 0.6; p = 0.039$) significantly affected the microbial community (Fig. 5). The concentration of effluent COD and pH displayed slight angles with the RDA1 axis, indicating their crucial involvement in the alteration of the microbial population.

The RDA demonstrated significant correlation between environmental factors and a total of 134 genera containing dominant hydrogen-producing bacteria *Ethanoligenens*, *Pectinatus*, and *Raoultella* (Supplemental table S3). In addition to the three bacteria, several hydrogen-producing bacteria with low abundances were also included, such as *Aeromonas* (Cho et al., 2018), *Clostridium XlVa* (Lin et al., 2018), and *Fermentimonas* (Wang et al., 2023). The increased functional flora diversity capable of producing hydrogen could enhance stability of continuous hydrogen production systems. Therefore, the RDA further indicates that, after undergoing multiple pretreatments and acclimation in a CSTR, the activated sludge enriched a diverse range of hydrogen-producing microorganisms and restructured the microbial community involved in hydrogen production.

4. Conclusion

The pretreatment of activated sludge and the control of operating parameters are critical for the operation of hydrogen-producing reactors. In the current study, the multiple pretreatments of microaeration, alkaline and thermal conditions were applied to the activated sludge for hydrogen-producing reactor operation. Proteobacteria, Firmicutes and Bacteroidetes were successfully enriched in the activated sludge after the multiple pretreatments, contributing in the formation of a novel microbial community structure.

The CSTR, which was inoculated with pretreated activated sludge, continuously produced hydrogen at rates of 8.19 L/d and 9.33 L/d under controlled influent COD levels of 4000 mg/L and 5000 mg/L, respectively. The effluent pH during these high-efficiency hydrogen production stages of the CSTR operation ranged between 4.35 and 4.92. The primary bacterial genus responsible for hydrogen production was *Ethanoligenens*, and hydrogen-producing related flora accounted for approximately 46.95% of the total relative abundance during these stages. From Stage-I to Stage-III, 100 genera showed a positive correlation with the hydrogen production, which included the representative hydrogen-producing bacteria *Pectinatus*, *Enterococcus*, *Syntrophomonas*, *Caloramator*, *Odoribacter*, *Phocaeicola*, *Clostridium XlVa*, *Victivallis*, and *Bacteroides*. The RDA also pointed that the multiple pretreatments and reactor operation facilitated to the enrichment and reconstruction of the hydrogen-producing flora in the activated sludge in the hydrogen-producing reactor.

CRediT authorship contribution statement

Jishan Jiang: Investigation, Formal analysis, Data curation. **Tielan Guo:** Investigation. **Jingyuan Wang:** Investigation. **Ao Sun:** Investigation. **Xingping Chen:** Investigation. **Xiaoxiao Xu:** Formal analysis, Data curation. **Shaojun Dai:** Writing – review & editing, Supervision, Resources, Investigation. **Zhi Qin:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgements

This study was supported by the Fund of Shanghai Engineering Research Center of Plant Germplasm Resources, China (No. 17DZ2252700) to Zhi Qin.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

org/10.1016/j.envres.2024.118725.

References

Alexandropoulou, M., Antonopoulou, G., Ntaikou, I., Lyberatos, G., 2023. The impact of alkaline/hydrogen peroxide pretreatment on hydrogen and methane production from biomasses of different origin: the case of willow sawdust and date palm fibers. Sustain. Chem. Pharm. 32, 100971 https://doi.org/10.1016/j.scp.2023.100971.

Cai, M., Liu, J., Wei, Y., 2004. Enhanced biohydrogen production from sewage sludge with alkaline pretreatment. Environ. Sci. Technol. 38, 3195–3202. https://doi.org/10.1021/es0349204.

Castelló, E., García Y Santos, C., Iglesias, T., Paolino, G., Wenzel, J., Borzacconi, L., Etchebehere, C., 2009. Feasibility of biohydrogen production from cheese whey using a UASB reactor: links between microbial community and reactor performance. Int. J. Hydrogen Energy 34, 5674–5682. https://doi.org/10.1016/j.ijhydene.2009.05.060.

Castro, J.F., Razmilic, V., Gerdtzen, Z.P., 2013. Genome based metabolic flux analysis of *Ethanoligenens harbinense* for enhanced hydrogen production. Int. J. Hydrogen Energy 38, 1297–1306. https://doi.org/10.1016/j.ijhydene.2012.11.007.

Chang, S., Li, J., Liu, F., 2011. Continuous biohydrogen production from diluted molasses in an anaerobic contact reactor. Front. Environ. Sci. Eng. China 5, 140–148. https://doi.org/10.1007/s11783-010-0258-2.

Chen, L., Xie, B., Li, L., Jiang, W., Zhang, Y., Fu, J., Guan, G., Qiu, Y., 2014. Rapid and sensitive LC–MS/MS analysis of fatty acids in Clinical samples. Chromatographia 77, 1241–1247. https://doi.org/10.1007/s10337-014-2708-7.

Chen, S., Song, L., Dong, X., 2006. *Sporacetigenium mesophilum* gen. nov., sp. nov., isolated from an anaerobic digester treating municipal solid waste and sewage. Int. J. Syst. Evol. Microbiol. 56, 721–725. https://doi.org/10.1099/ijs.0.63686-0.

Chi, X., Li, J., Wang, X., Zhang, Y., Leu, S.-Y., Wang, Y., 2018. Bioaugmentation with *Clostridium tyrobutyricum* to improve butyric acid production through direct rice straw bioconversion. Bioresour. Technol. 263, 562–568. https://doi.org/10.1016/j.biortech.2018.04.120.

Cho, S.-K., Jeong, M.-W., Choi, Y.-K., Shin, J., Shin, S.G., 2018. Effects of low-strength ultrasonication on dark fermentative hydrogen production: start-up performance and microbial community analysis. Appl. Energy 219, 34–41. https://doi.org/10.1016/j.apenergy.2018.03.047.

Ciranna, A., Pawar, S.S., Santala, V., Karp, M., Van Niel, E.W., 2014. Assessment of metabolic flux distribution in the thermophilic hydrogen producer *Caloramator celer* as affected by external pH and hydrogen partial pressure. Microb. Cell Factories 13, 48. https://doi.org/10.1186/1475-2859-13-48.

Collins, R.P., Gaines, H.D., 1964. Production of hydrogen Sulfide by *Streptomyces odorifer*. Appl. Microbiol. 12, 335–336. https://doi.org/10.1128/am.12.4.335-336.1964.

Cordell, R.L., Pandya, H., Hubbard, M., Turner, M.A., Monks, P.S., 2013. GC-MS analysis of ethanol and other volatile compounds in micro-volume blood samples—quantifying neonatal exposure. Anal. Bioanal. Chem. 405, 4139–4147. https://doi.org/10.1007/s00216-013-6809-1.

Dessì, P., Porca, E., Frunzo, L., Lakaniemi, A., Collins, G., Esposito, G., Lens, P.N.L., 2018. Inoculum pretreatment differentially affects the active microbial community performing mesophilic and thermophilic dark fermentation of xylose. Int. J. Hydrogen Energy 43, 9233–9245. https://doi.org/10.1016/j.ijhydene.2018.03.117.

Dou, Y., Liao, J., An, S., 2023. Importance of soil labile organic carbon fractions in shaping microbial community after vegetation restoration. Catena 220, 106707. https://doi.org/10.1016/j.catena.2022.106707.

Edgar, R.C., 2018. Updating the 97% identity threshold for 16S ribosomal RNA OTUs. Bioinformatics 34, 2371–2375. https://doi.org/10.1093/bioinformatics/bty113.

Flaiz, M., Baur, T., Brahner, S., Poehlein, A., Daniel, R., Bengelsdorf, F.R., 2020. *Caproicibacter fermentans* gen. nov., sp. nov., a new caproate-producing bacterium and emended description of the genus *Caproiciproducens*. Int. J. Syst. Evol. Microbiol. 70, 4269–4279. https://doi.org/10.1099/ijsem.0.004283.

Fu, Q., Wang, D., Li, X., Yang, Q., Xu, Q., Ni, B.-J., Wang, Q., Liu, X., 2021. Towards hydrogen production from waste activated sludge: principles, challenges and perspectives. Renew. Sustain. Energy Rev. 135, 110283 https://doi.org/10.1016/j.rser.2020.110283.

Fu, S., Lian, S., Angelidaki, I., Guo, R., 2023. Micro-aeration: an attractive strategy to facilitate anaerobic digestion. Trends Biotechnol. 41, 714–726. https://doi.org/10.1016/j.tibtech.2022.09.008.

Fu, S.-F., Liu, R., Sun, W.-X., Zhu, R., Zou, H., Zheng, Y., Wang, Z.-Y., 2020. Enhancing energy recovery from corn straw via two-stage anaerobic digestion with stepwise microaerobic hydrogen fermentation and methanogenesis. J. Clean. Prod. 247, 119651 https://doi.org/10.1016/j.jclepro.2019.119651.

Göker, M., Gronow, S., Zeytun, A., Nolan, M., Lucas, S., Lapidus, A., Hammon, N., Deshpande, S., Cheng, J.-F., Pitluck, S., Liolios, K., Pagani, I., Ivanova, N., Mavromatis, K., Ovchinikova, G., Pati, A., Tapia, R., Han, C., Goodwin, L., Chen, A., Palaniappan, K., Land, M., Hauser, L., Jeffries, C.D., Brambilla, E.-M., Rohde, M., Detter, J.C., Woyke, T., Bristow, J., Markowitz, V., Hugenholtz, P., Eisen, J.A., Kyrpides, N.C., Klenk, H.-P., 2011. Complete genome sequence of *Odoribacter splanchnicus* type strain (1651/6T). Stand. Genomic Sci. 4, 200–209. https://doi.org/10.4056/sigs.1714269.

Gong, X., Wu, M., Jiang, Y., Wang, H., 2021. Effects of different temperatures and pH values on volatile fatty acids production during codigestion of food waste and thermal-hydrolysed sewage sludge and subsequent volatile fatty acids for polyhydroxyalkanoates production. Bioresour. Technol. 333, 125149 https://doi.org/10.1016/j.biortech.2021.125149.

Han, W., Wang, X., Ye, L., Huang, J., Tang, J., Li, Y., Ren, N., 2015. Fermentative hydrogen production using wheat flour hydrolysate by mixed culture. Int. J. Hydrogen Energy 40, 4474–4480. https://doi.org/10.1016/j.ijhydene.2015.02.016.

Hassan, M., Zhao, C., Ding, W., 2020. Enhanced methane generation and biodegradation efficiencies of goose manure by thermal-sonication pretreatment and organic loading management in CSTR. Energy 198, 117370. https://doi.org/10.1016/j.energy.2020.117370.

Hu, J., Guo, B., Li, Z., Wu, Z., Tao, W., 2021. Freezing pretreatment assists potassium ferrate to promote hydrogen production from anaerobic fermentation of waste activated sludge. Sci. Total Environ. 781, 146685 https://doi.org/10.1016/j.scitotenv.2021.146685.

Hu, J., Zuo, Y., Guo, B., Shi, H., 2023. Enhanced hydrogen production from sludge anaerobic fermentation by combined freezing and calcium hypochlorite pretreatment. Sci. Total Environ. 858, 160134 https://doi.org/10.1016/j.scitotenv.2022.160134.

Huiliñir, C., Pagés-Díaz, J., Vargas, G., Vega, S., Lauzurique, Y., Palominos, N., 2023. Microaerobic condition as pretreatment for improving anaerobic digestion: a review. Bioresour. Technol. 384, 129249 https://doi.org/10.1016/j.biortech.2023.129249.

Ichikawa, Y., Yamamoto, H., Hirano, S., Sato, B., Takefuji, Y., Satoh, F., 2023. The overlooked benefits of hydrogen-producing bacteria. Med. Gas Res. 13, 108. https://doi.org/10.4103/2045-9912.344977.

Kalia, V.C. (Ed.), 2015. Microbial Factories. Volume 1: Biofuels, Waste Treatment. Springer, New Delhi.

Kang, J., Kim, D., Lee, T., 2012. Hydrogen production and microbial diversity in sewage sludge fermentation preceded by heat and alkaline treatment. Bioresour. Technol. 109, 239–243. https://doi.org/10.1016/j.biortech.2012.01.048.

Kim, D.-H., Lee, M.-K., Jung, K.-W., Kim, M.-S., 2013. Alkali-treated sewage sludge as a seeding source for hydrogen fermentation of food waste leachate. Int. J. Hydrogen Energy 38, 15751–15756. https://doi.org/10.1016/j.ijhydene.2013.05.120.

Kim, S.M., Sim, Y.-B., Baik, J.-H., Yang, J., Pandey, A.K., Joo, H.-H., Jung, J.-H., Kim, S.-H., 2023. Formation and characterization of H_2-producing granule in a pilot-scale dynamic membrane bioreactor. Chem. Eng. J. 452, 139384 https://doi.org/10.1016/j.cej.2022.139384.

Li, D., Zuo, Y., Meng, S., 2004. Separation of thorium(IV) and extracting rare earths from sulfuric and phosphoric acid solutions by solvent extraction method. J. Alloys Compd. 374, 431–433. https://doi.org/10.1016/j.jallcom.2003.11.055.

Li, Y., Qiren, N., Chen, Y., Xiangzheng, G., 2007. Ecological mechanism of fermentative hydrogen production by bacteria. Int. J. Hydrogen Energy 32, 755–760. https://doi.org/10.1016/j.ijhydene.2006.08.004.

Li, Z., Gu, J., Ding, J., Ren, N., Xing, D., 2021. Molecular mechanism of ethanol-H_2 co-production fermentation in anaerobic acidogenesis: challenges and perspectives. Biotechnol. Adv. 46, 107679 https://doi.org/10.1016/j.biotechadv.2020.107679.

Li, Z., Liu, B., Cui, H., Ding, J., Li, H., Xie, G., Ren, N., Xing, D., 2019. The complete genome sequence of *Ethanoligenens harbinense* reveals the metabolic pathway of acetate-ethanol fermentation: a novel understanding of the principles of anaerobic biotechnology. Environ. Int. 131, 105053 https://doi.org/10.1016/j.envint.2019.105053.

Lim, J.W., Chiam, J.A., Wang, J.-Y., 2014. Microbial community structure reveals how microaeration improves fermentation during anaerobic co-digestion of brown water and food waste. Bioresour. Technol. 171, 132–138. https://doi.org/10.1016/j.biortech.2014.08.050.

Lin, R., Cheng, J., Ding, L., Murphy, J.D., 2018. Improved efficiency of anaerobic digestion through direct interspecies electron transfer at mesophilic and thermophilic temperature ranges. Chem. Eng. J. 350, 681–691. https://doi.org/10.1016/j.cej.2018.05.173.

Lozano, D.A., Niño-Navarro, C., Chairez, I., Salgado-Manjarrez, E., García-Peña, E.I., 2023. Intensification of hydrogen production by a Co-culture of *Syntrophomonas wolfei* and *rhodopseudomonas* palustris employing high concentrations of butyrate as a substrate. Appl. Biochem. Biotechnol. 195, 1800–1822. https://doi.org/10.1007/s12010-022-04220-z.

Ma, L., Wu, G., Yang, J., Huang, L., Phurbu, D., Li, W.-J., Jiang, H., 2021. Distribution of hydrogen-producing bacteria in Tibetan hot springs, China. Front. Microbiol. 12, 569020 https://doi.org/10.3389/fmicb.2021.569020.

Mai, J., Hu, B.-B., Zhu, M.-J., 2023. Metabolic division of labor between *Acetivibrio thermocellus* DSM 1313 and *Thermoanaerobacterium thermosaccharolyticum* MJ1 enhanced hydrogen production from lignocellulose. Bioresour. Technol. 390, 129871 https://doi.org/10.1016/j.biortech.2023.129871.

Matamba, T., Tahmasebi, A., Yu, J., Keshavarz, A., Abid, H.R., Iglauer, S., 2023. A review on biomass as a substitute energy source: *polygeneration* influence and hydrogen rich gas formation via pyrolysis. J. Anal. Appl. Pyrolysis 175, 106221. https://doi.org/10.1016/j.jaap.2023.106221.

Miebach, K., Finger, M., Scherer, A.M.K., Maaß, C.A., Büchs, J., 2023. Hydrogen online monitoring based on thermal conductivity for anaerobic microorganisms. Biotechnol. Bioeng. 120, 2199–2213. https://doi.org/10.1002/bit.28502.

Mohammadi, P., Ibrahim, S., Annuar, M.S.M., 2012. Comparative study on the effect of various pretreatment methods on the enrichment of hydrogen producing bacteria in anaerobic granulated sludge from brewery wastewater. Korean J. Chem. Eng. 29, 1347–1351. https://doi.org/10.1007/s11814-012-0018-z.

Nguyen, D., Khanal, S.K., 2018. A little breath of fresh air into an anaerobic system: how microaeration facilitates anaerobic digestion process. Biotechnol. Adv. 36, 1971–1983. https://doi.org/10.1016/j.biotechadv.2018.08.007.

O'Brian, M.R., Maier, R.J., 1988. Hydrogen metabolism in *Rhizobium*: energetics, regulation, enzymology and genetics. In: Advances in Microbial Physiology. Elsevier, pp. 1–52. https://doi.org/10.1016/S0065-2911(08)60345-8.

Ohnishi, A., Abe, S., Bando, Y., Fujimoto, N., Suzuki, M., 2012. Rapid detection and quantification methodology for genus *Megasphaera* as a hydrogen producer in a

hydrogen fermentation system. Int. J. Hydrogen Energy 37, 2239–2247. https://doi.org/10.1016/j.ijhydene.2011.10.094.
Ottman, N., Geerlings, S.Y., Aalvink, S., De Vos, W.M., Belzer, C., 2017. Action and function of *Akkermansia muciniphila* in microbiome ecology, health and disease. Best Pract. Res. Clin. Gastroenterol. 31, 637–642. https://doi.org/10.1016/j.bpg.2017.10.001.
Peters, J.W., Schut, G.J., Boyd, E.S., Mulder, D.W., Shepard, E.M., Broderick, J.B., King, P.W., Adams, M.W.W., 2015. [FeFe]- and [NiFe]-hydrogenase diversity, mechanism, and maturation. Biochim. Biophys. Acta BBA - Mol. Cell Res. 1853, 1350–1369. https://doi.org/10.1016/j.bbamcr.2014.11.021.
Pfeiffer, N., Desmarchelier, C., Blaut, M., Daniel, H., Haller, D., Clavel, T., 2012. *Acetatifactor muris* gen. nov., sp. nov., a novel bacterium isolated from the intestine of an obese mouse. Arch. Microbiol. 194, 901–907. https://doi.org/10.1007/s00203-012-0822-1.
Prabhu, R., Altman, E., Eiteman, M.A., 2012. Lactate and acrylate metabolism by *Megasphaera elsdenii* under batch and steady-state conditions. Appl. Environ. Microbiol. 78, 8564–8570. https://doi.org/10.1128/AEM.02443-12.
R Core Team, 2023. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Rafieenia, R., Lavagnolo, M.C., Pivato, A., 2018. Pre-treatment technologies for dark fermentative hydrogen production: current advances and future directions. Waste Manag. 71, 734–748. https://doi.org/10.1016/j.wasman.2017.05.024.
Ren, N., Guo, W., Liu, B., Cao, G., Ding, J., 2011. Biological hydrogen production by dark fermentation: challenges and prospects towards scaled-up production. Curr. Opin. Biotechnol. 22, 365–370. https://doi.org/10.1016/j.copbio.2011.04.022.
Ren, N., Xie, T., Xing, D., 2009. Composition of extracellular polymeric substances influences the autoaggregation capability of hydrogen-producing bacterium *Ethanoligenens harbinense*. Bioresour. Technol. 100, 5109–5113. https://doi.org/10.1016/j.biortech.2009.05.021.
Ren, W., Wu, Q., Deng, L., Hu, Y., Guo, W., Ren, N., 2022. Simultaneous medium chain fatty acids production and process carbon emissions reduction in a continuous-flow reactor: Re-understanding of carbon flow distribution. Environ. Res. 212, 113294 https://doi.org/10.1016/j.envres.2022.113294.
Rossi, D.M., Berne da Costa, J., Aquino de Souza, E., Peralba, M. do C.R., Samios, D., Záchia Ayub, M.A., 2011. Comparison of different pretreatment methods for hydrogen production using environmental microbial consortia on residual glycerol from biodiesel. Int. J. Hydrogen Energy 36, 4814–4819. https://doi.org/10.1016/j.ijhydene.2011.01.005.
Ruan, D., Zhou, Z., Pang, H., Yao, J., Chen, G., Qiu, Z., 2019. Enhancing methane production of anaerobic sludge digestion by microaeration: enzyme activity stimulation, semi-continuous reactor validation and microbial community analysis. Bioresour. Technol. 289, 121643 https://doi.org/10.1016/j.biortech.2019.121643.
Shi, Z., Ma, L., Wang, Y., Liu, J., 2023. Abundant and rare bacteria in anthropogenic estuary: community co-occurrence and assembly patterns. Ecol. Indicat. 146, 109820 https://doi.org/10.1016/j.ecolind.2022.109820.
Simon, I., Arndt, M., 2002. Thermal and gas-sensing properties of a micromachined thermal conductivity sensor for the detection of hydrogen in automotive applications. Sens. Actuators Phys. 97–98, 104–108. https://doi.org/10.1016/S0924-4247(01)00825-1.
Stiles, M.E., 1994. Bacteriocins produced by *Leuconostoc* species. J. Dairy Sci. 77, 2718–2724. https://doi.org/10.3168/jds.S0022-0302(94)77214-3.
Su, X., Zhao, W., Xia, D., 2018. The diversity of hydrogen-producing bacteria and methanogens within an *in situ* coal seam. Biotechnol. Biofuels 11, 245. https://doi.org/10.1186/s13068-018-1237-2.
Sun, F., Wang, Youshao, Wang, Yutu, Sun, C., Cheng, H., Wu, M., 2024. Insights into the spatial distributions of bacteria, archaea, ammonia-oxidizing bacteria and archaea communities in sediments of Daya Bay, northern South China Sea. Mar. Pollut. Bull. 198, 115850 https://doi.org/10.1016/j.marpolbul.2023.115850.
Suzuki, S., Kuenen, J.G., Schipper, K., Van Der Velde, S., Ishii, S., Wu, A., Sorokin, D.Y., Tenney, A., Meng, X., Morrill, P.L., Kamagata, Y., Muyzer, G., Nealson, K.H., 2014. Physiological and genomic features of highly alkaliphilic hydrogen-utilizing Betaproteobacteria from a continental serpentinizing site. Nat. Commun. 5, 3900. https://doi.org/10.1038/ncomms4900.
Thompson, T.E., Conrad, R., Zeikus, J.G., 1984. Regulation of carbon and electron flow in Propionispira arboris : physiological function of hydrogenase and its role in homopropionate formation. FEMS Microbiol. Lett. 22, 265–271. https://doi.org/10.1111/j.1574-6968.1984.tb00739.x.
Walter, WilliamG., 1961. Standard methods for the examination of water and wastewater (11th ed.). Am. J. Public Health Nation's Health 51, 940. https://doi.org/10.2105/AJPH.51.6.940-a, 940.
Wang, B., Li, Y., Ren, N., 2013. Biohydrogen from molasses with ethanol-type fermentation: effect of hydraulic retention time. Int. J. Hydrogen Energy 38, 4361–4367. https://doi.org/10.1016/j.ijhydene.2013.01.120.
Wang, J., Qin, Z., Yi, M., 2018. Anaerobic fermentation reactor start-up operation and analysis of dominant bacteria in activated sludge. J. Shanghai Norm. Univ. Sci. 47, 704–712.
Wang, J., Yin, Y., 2017. Principle and application of different pretreatment methods for enriching hydrogen-producing bacteria from mixed cultures. Int. J. Hydrogen Energy 42, 4804–4823. https://doi.org/10.1016/j.ijhydene.2017.01.135.
Wang, N., Yang, Y., Xu, K., Long, X., Liu, Haibo, Zhang, Y., Liu, Hongzhou, Chen, T., Li, J., 2023. Insight into the metabolic pathway of EAD based on metabolic flux, microbial community, and enzyme activity. Biochem. Eng. J. 196, 108938 https://doi.org/10.1016/j.bej.2023.108938.
Wang, X., Dong, T., Zhang, A., Fang, Y., Chen, D., Zhao, C., Luo, Q., Yang, H., 2019. Isolation of bacteria capable of hydrogen production in dark fermentation and intensification of anaerobic granular sludge activity. Int. J. Hydrogen Energy 44, 15853–15862. https://doi.org/10.1016/j.ijhydene.2018.07.034.
Wu, Y., Wang, C., Zheng, M., Zuo, J., Wu, J., Wang, K., Yang, B., 2017. Effect of pH on ethanol-type acidogenic fermentation of fruit and vegetable waste. Waste Manag. 60, 158–163. https://doi.org/10.1016/j.wasman.2016.09.033.
Xin, X., She, Y., Hong, J., 2021. Insights into microbial interaction profiles contributing to volatile fatty acids production via acidogenic fermentation of waste activated sludge assisted by calcium oxide pretreatment. Bioresour. Technol. 320, 124287 https://doi.org/10.1016/j.biortech.2020.124287.
Ye, B., Zhang, J., Zhou, Y., Tang, M., You, F., Li, X., Yang, Q., Wang, D., Liu, X., Duan, A., Liu, J., 2023. Pretreatment of free nitrous acid combined with calcium hypochlorite for enhancement of hydrogen production in waste activated sludge. Sci. Total Environ. 900, 165774 https://doi.org/10.1016/j.scitotenv.2023.165774.
Yi, X.-H., Wan, J., Ma, Y., Wang, Y., Guan, Z., Jing, D.-D., 2017. Structure and succession of bacterial communities of the granular sludge during the initial stage of the simultaneous denitrification and methanogenesis process. Water. Air. Soil Pollut. 228, 121. https://doi.org/10.1007/s11270-016-3168-5.
Yin, T., Wang, W., Zhuo, S., Cao, G., Ren, H., Li, J., Xing, D., Xie, G., Liu, B., 2023. g. Fuel 334, 126748. https://doi.org/10.1016/j.fuel.2022.126748.
Yin, Y., Wang, J., 2019. Optimization of fermentative hydrogen production by *Enterococcus faecium* INET2 using response surface methodology. Int. J. Hydrogen Energy 44, 1483–1491. https://doi.org/10.1016/j.ijhydene.2018.11.154.
Zhang, C., Lv, F.-X., Xing, X.-H., 2011. Bioengineering of the *Enterobacter aerogenes* strain for biohydrogen production. Bioresour. Technol. 102, 8344–8349. https://doi.org/10.1016/j.biortech.2011.06.018.
Zhang, H., John, R., Peng, Z., Yuan, J., Chu, C., Du, G., Zhou, S., 2012. The relationship between species richness and evenness in plant communities along a successional gradient: a study from Sub-Alpine meadows of the Eastern Qinghai-Tibetan Plateau, China. PLoS One 7, e49024. https://doi.org/10.1371/journal.pone.0049024.
Zhang, J., Kobert, K., Flouri, T., Stamatakis, A., 2014. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics 30, 614–620. https://doi.org/10.1093/bioinformatics/btt593.
Zhang, Z., Yu, Y., Xi, H., Zhou, Y., 2021. Review of micro-aeration hydrolysis acidification for the pretreatment of toxic and refractory organic wastewater. J. Clean. Prod. 317, 128343 https://doi.org/10.1016/j.jclepro.2021.128343.
Zhao, W., Yan, B., Ren, Z.J., Wang, S., Zhang, Y., Jiang, H., 2022. Highly selective butyric acid production by coupled acidogenesis and ion substitution electrodialysis. Water Res. 226, 119228 https://doi.org/10.1016/j.watres.2022.119228.
Zhao, X., Xing, D., Qi, N., Zhao, Y., Hu, X., Ren, N., 2017. Deeply mechanism analysis of hydrogen production enhancement of *Ethanoligenens harbinense* by Fe^{2+} and Mg^{2+}: monitoring at growth and transcription levels. Int. J. Hydrogen Energy 42, 19695–19700. https://doi.org/10.1016/j.ijhydene.2017.06.038.
Zheng, Y., Li, L., Jin, Z., An, P., Yang, S.-T., Fei, Y., Liu, G., 2021. Characterization of fermented soymilk by *Schleiferilactobacillus harbinensis* M1, based on the whole-genome sequence and corresponding phenotypes. LWT 144, 111237. https://doi.org/10.1016/j.lwt.2021.111237.
Zhong, Y., Nyman, M., Fåk, F., 2015. Modulation of gut microbiota in rats fed high-fat diets by processing whole-grain barley to barley malt. Mol. Nutr. Food Res. 59, 2066–2076. https://doi.org/10.1002/mnfr.201500187.
Zhou, L., Gao, Y., Yu, K., Zhou, H., De Costa, Y.G., Yi, S., Zhuang, W.-Q., 2020. Microbial community in *in-situ* waste sludge anaerobic digestion with alkalization for enhancement of nutrient recovery and energy generation. Bioresour. Technol. 295, 122277 https://doi.org/10.1016/j.biortech.2019.122277.
Zhou, Z., Ming, Q., An, Y., Ruan, D., Chen, G., Wei, H., Wang, M., Wu, Z., 2021. Performance and microbial community analysis of anaerobic sludge digestion enhanced by *in-situ* microaeration. J. Water Process Eng. 42, 102171 https://doi.org/10.1016/j.jwpe.2021.102171.