Effect of reaction mode on biohydrogen production and its microbial diversity

Buchun Si^a, Zhidan Liu^{a,*}, Yuanhui Zhang^{a,c}, Jiaming Li^a, Xin-Hui Xing^b,

Baoming Li^a, Na Duan^a, Haifeng Lu^a

^a Laboratory of Environment-Enhancing Energy (E2E), and Key Laboratory of Agricultural Engineering in Structure and Environment, Ministry of Agriculture, College of Water Resources and Civil Engineering, China Agricultural University, Beijing, 10083, China

^b Department of Chemical Engineering, Tsinghua University, 100084, Beijing, China

^c Department of Agricultural and Biological Engineering, University of Illinois at Urbana-Champaign, Illinois, 61801, USA

* Corresponding author.

Tel.: +086-010-6273-7329, Fax: +086-010-6273-7329

E-mail address: zdliu@cau.edu.cn (Z.Liu).

ABSTRACT

Biohydrogen production using the high-rate reactor is promising due to its ability of maintaining higher biomass concentrations through forming granules or biofilms. This study investigated the effect of reaction mode on hydrogen fermentation by comparing an upflow anaerobic sludge blanket (UASB) and a packed bed reactor (PBR). UASB and PBR were operated for 120 days at hydraulic retention time (HRT) of 24-12 h and organic loading rates (OLRs) of 0.96-15.36 g COD/L/d. Both UASB and PBR achieved maximal hydrogen production rates as 2.77 ± 0.18 and 1.28 ± 0.12 L/L/d, respectively, at 15.36 g COD/L/d, corresponding to hydrogen yields of 1.44 ± 0.01 and 0.67 ± 0.06 mol/mol glucose. Illumina MiSeq sequencing results revealed *Clostridium* sp. was the dominant microbial consortium for hydrogen production in UASB (92.1%) and PBR (71.7%). Compared to UASB, PBR showed a greater microbial diversity of ethanol and lactic acid producers, and may be more favorable for methanogenesis and homoacetogenesis. This study demonstrates that reaction mode significantly influenced microbial diversity and biohydrogen production.

Keywords: Biohydrogen production, Microbial diversity, Packed bed reactor, Reaction mode, Upflow anaerobic sludge blanket

1. Introduction

Hydrogen is a promising energy due to its rapid burning speed, high octane number, outstanding combustion efficiency and high energy content [1]. Currently, the overwhelming majority of hydrogen production comes from fossil fuels [2]. Biohydrogen

production through dark fermentation is promising due to the faster process and no direct requirement of solar energy [3].

Compared with conventional continuous stirred tank reactor (CSTR), the high-rate reaction mode has been recommended as a promising alternative for biohydrogen production from wastewater due to its ability of maintaining higher biomass concentrations by forming granules or biofilms [4]. Upflow anaerobic sludge blanket (UASB) which can form granules, has been widely used for biohydrogen production at high treatment efficiency and short hydraulic retention time (HRT) of 2-24 h with hydrogen yields of 0.75-2.57 mol/mol hexose [5-9]. Similarly, a packed bed reactor (PBR) can enhance hydrogen fermentation by forming biofilms on the packed materials at short HRT (0.5-24 h) and high organic loading rate (OLR) of 6.5-960 g COD/L/d with hydrogen yield of 0.35-2.11 mol/mol hexose [10-15]. Efficient hydrogen producing microbes were enriched using high-rate bioreactors. *Clostridium*, *Enterobacter*, *Ruminococcus*, *Klebsiella*, *Pantoea*, *Caloramator* were found in granules in UASB [7-9]. In comparison, the biofilm formed in PBR had a broader microbial diversity including *Clostridium*, *Ruminococcus*, *Enterobacter*, *Lactobacillus*, *Streptococcus*, *Pseudomonas*, *Klebsiella*, *Propionispira*, *Klebsiell* [11-14]. In addition, hydrogen production through PBR was reported unstable due to the existence of homoacetogenic organisms, such as *Clostridium ljungdahlii* [15, 16]. Lee et al. reported that hydrogen production in granules or biofilms system was related to the diversity of *Clostridia* [17]. Efforts have been made to compare the biofilm and granules system. Zhang et al. compared biofilm and granular system for biohydrogen production in two anaerobic fluidized bed reactors, and the hydrogen yields obtained in both reactors ranged from 0.4 to 1.7 mol H₂/mol glucose [18]. Alzate-Gaviria et al. studied the hydrogen production of the PBR and UASB fed with

organic fraction of municipal solid waste (OFMSW) and synthetic wastewater, respectively. The UASB and PBR achieved 30% and 23% of the maximum hydrogen yield (4 mol H₂/mol glucose) [19]. However, the hydrogen production and microbial diversity in two reaction modes (UASB and PBR) under similar operational conditions have not been systemically compared.

The aim of the study was to investigate the effect of reaction mode on biohydrogen production through comparing the difference between the two typical high-rate reactors (UASB and PBR) under the same operational conditions including the feedstock, OLR, temperature and initial pH. In addition, the influence of reaction mode on microbial diversity and functions during hydrogen fermentation was examined and discussed.

2. Methods

2.1 Inoculum

The inoculum was obtained from an anaerobic digester at the Xiaohongmen Municipal Sewage Treatment Plant (Beijing, China). The volatile suspended solids (VSS) concentration and total suspended solids (TSS) concentration of the seed sludge were 8.12 and 24.7 g/L, respectively. The sludge was boiled at 100°C for 15 min to inhibit methanogenesis activity and harvest anaerobic spore-forming bacteria prior to use [20].

2.2 Substrate

Glucose and NH₄Cl were used as carbon and nitrogen sources to mimic the carbohydrate-rich wastewater with a carbon/nitrogen ratio of 47 based on nutrients requirement [21]. The concentrations of glucose and NH₄Cl were then altered to reach desired OLRs, and sodium bicarbonate was added (1 g/g COD) as a buffer. Besides these compositions, the

substrate contained (mg/L): K_2HPO_4 250, KH_2PO_4 250, MgCl_2 300, CoCl_2 25, ZnCl_2 11.5, CuCl_2 10.5, CaCl_2 5, MnCl_2 15, NiSO_4 16, FeCl_3 25 [22]. The initial pH of substrate was controlled in a desired interval (5-6.5), depending on the reactor performance.

2.3 Reactor design and experimental procedure

Fig 1 shows the schematic setup of the lab-scale high-rate reactors (UASB and PBR). The two reactors were made from transparent acrylic and both had a working volume of 2.5 L, and a height to diameter ratio of 6:1. To enhance the microbial retention and accelerate the enrichment, the CNTs at 100 mg/L were added into the UASB as the microbial carrier [8], whereas polyethylene rings (1 cm diameter and 1 cm wide) at 175 g/L were packed to the PBR with the void space ratio of 80%. Reactors were maintained at 37°C using water bath recirculation. Heat-treated anaerobic sludge was used as the inoculum at a volume ratio of 1:4. The reactors were flushed with nitrogen gas at 100 mL/min for 30 min to remove dissolved O_2 and obtain anaerobic conditions. The reactors were operated at OLRs of 0.96-15.36 g COD/L/d through changing the HRT and initial concentration of the substrate. Steady-state conditions were defined as the variations of hydrogen production rate and hydrogen concentration were constant ($\pm 10\%$) during 6 HRT of operation.

2.4 Analytical methods

Gas production was measured using a gas meter. Gas compositions were determined by a gas chromatographer (GC1490, Agilent Technologies, USA) equipped with a thermal conductivity detector (TCD) and a stainless steel column packed with TDX-01. Measured gas compositions included hydrogen, methane and carbon dioxide. The temperatures in the injector, column and detector were 150, 120 and 150°C, respectively. The nitrogen gas was

used as the carrying gas at a flow rate of 50 mL/min. Gas compositions, volume and pH of the effluents were monitored every day.

The volatile fatty acids (VFAs) produced during hydrogen fermentation were analyzed using a high performance liquid chromatography (HPLC) (Shimadzu, Japan) equipped with an ultraviolet detector and an acid analysis column (Phenomenex, USA). 5 mM H₂SO₄ was used as mobile phase at a flow rate of 1 mL/min. The oven temperature was 40°C. Samples were centrifuged (4000 rpm, 10 min) and the supernatant was filtered using a 0.45 µm membrane filter before use. Chemical oxygen demand (COD) was measured according to standard methods [23].

After 120 days of operation (HRT 12 h, OLR 15.36g COD/L/d), hydrogen-producing granules in UASB and microbial carriers with hydrogen-producing biofilm in PBR were sampled. The microbial samples were then stored at -20°C prior to analysis together with inoculum. The microbial morphology was observed by scanning electron microscopy (Quanta 200, FEI, USA) as previously described [8].

The phylogenetic diversity of the microbial consortium was analyzed via Illumina MiSeq sequencing [24]. Genomic DNA was first extracted from microbial samples using the E.Z.N.A DNA Isolation Kit (Omega Biotec, USA). The V4-V5 regions of the bacteria 16S ribosomal RNA gene were then amplified by GeneAmp PCR System (ABI company, USA) using primers 515F (5'-barcode-GTGCCAGCMGCCGCGG-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3'), where the barcode was an eight-base sequence unique to each sample. The PCR process was conducted as follows: 95°C for 2 min, followed by 25 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final extension at 72°C for 5 min. PCR reactions were performed in triplicate. Each 20 µL PCR reaction mixture contained 4 µL of 5 × FastPfu Buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of each

primer (5 μ M), 0.4 μ L of FastPfu Polymerase, and 10 ng of template DNA. Amplicons were extracted from 2% agarose gels, purified using the AxyPrep DNA gel extraction kit (Axygen Biosciences, USA) and quantified using QuantiFluor ST (Promega, USA). Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq platform. Finally, the raw reads were deposited into the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database. Raw fastq files were demultiplexed and quality-filtered using Quantitative Insights into Microbial Ecology (QIIME). Reads which could not be assembled were discarded. Operational Taxonomic Units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1 <http://drive5.com/uparse/>) and chimeric sequences were identified and removed using UCHIME. The phylogenetic affiliation of each 16S rRNA gene sequence was analyzed by RDP Classifier (<http://rdp.cme.msu.edu/>) against the silva (SSU115) 16S rRNA database using confidence threshold of 70%.

2.5 Calculations of hydrogen fermentation

Hydrogen production was also calculated based on the biochemical reactions in order to better reveal the distribution of hydrogen. The theoretical biohydrogen production (H_2 theoretical, mM) was calculated [25]:

$$H_2 \text{ theoretical} = 2M_{\text{But}} + 2M_{\text{Ac}} - M_{\text{Prop}} \quad (1)$$

H_2 theoretical was calculated based on the production of VFAs (Eq. 1), where M_{But} , M_{Ac} and M_{Prop} represented the concentrations of butyric acid, acetic acid, and propionic acid (mM), respectively. Note that other products, including lactic acid and ethanol were not considered as previously described [25]. The activity of homoacetogenesis and methane production by hydrogenotrophic methanogens should be considered:

$$H_{2 \text{ homo}} = 4 M_{\text{Ac homo}} \quad (2)$$

$$H_{2 \text{ methane}} = 4 CH_{4 \text{ measured}} \quad (3)$$

Where $H_{2 \text{ methane}}$ and $H_{2 \text{ homo}}$ represented the hydrogen consumed by hydrogenotrophic methanogen and homoacetogenesis in mM, respectively, and $M_{\text{Ac homo}}$ and $CH_{4 \text{ measured}}$ represented the acetic acid (mM) produced by homoacetogenesis and CH_4 (mM) produced by hydrogenotrophic methanogens, respectively. Based on the above equation, $M_{\text{Ac homo}}$ can be calculated [26]:

$$M_{\text{Ac homo}} = (2M_{\text{But}} + 2M_{\text{Ac}} - M_{\text{Prop}} - H_{2 \text{ measured}} - 4CH_{4 \text{ measured}}) / 6 \quad (4)$$

Where $H_{2 \text{ measured}}$ represented the actually measured hydrogen (mM).

3. Results and discussion

3.1 Performance of UASB and PBR for biohydrogen production

3.1.1 Biohydrogen production

Fig. 2 showed the comparison of UASB and PBR for biohydrogen production. The HRT was set as 24 h in the first 37 d and then reduced to 12 h to avoid the risk of microorganisms washing-out. It was clear that the initial pH and concentration of substrate had an effect on the hydrogen production and composition [27]. The reported optimum initial pH values for hydrogen production ranged from 5 to 6.5 [28]. In this study, the initial pH was adjusted to 5.5 and 6.5 in the first 22 d, while significant methane production was observed (Fig. 2E). As a result, the initial pH was set as 5 at OLR of 0.96, 1.92 and 3.84 g COD/L/L, and then changed to 5.5 at OLR of 7.68 and 15.36 g COD/L/L to suppress methane production. As showed in Fig. 2, both UASB and PBR had a low hydrogen production in the first 56 d at OLRs of 0.96 g and 1.92 g COD/L/d. This suggested that low ORL was not desirable for the startup of hydrogen fermentation. With

the initial concentration increased to 1.92 g COD/L, corresponding to an OLR of 3.84 g COD/L/d, UASB and PBR achieved a steady hydrogen composition and a constant hydrogen production rate at 79 and 81 d, respectively. Fig. 2 revealed that reaction mode influenced the gas composition and gas production rate. Less than 1% methane content with a methane production rate (MPR) of 0.01-0.04 L/L/d was observed in UASB at the steady state. In comparison, the methane production in PBR was more significant (>12%) with a higher MPR of 0.04-0.49 L/L/d, which is consistent with the findings by Penteado et al.[29]. The hydrogen contents in UASB (47.5-54.7%) were higher than those in PBR (26.4-34.0%) at the steady-states after each OLR, corresponding to hydrogen production rate in UASB (0.22-2.77 L/L/d) and PBR (0.50-1.28 L/L/d). Alzate-Gaviria et al. also found the UASB and PBR systems had different performance of hydrogen production [19]. The better hydrogen production in UASB may partially result from the mass transfer. Another study compared hydrogen production using biofilm and granular sludge system in two anaerobic fluidized bed reactors [18]. However, no significant difference was observed, probably due to the similar mass transfer in two fluidized bed reactors. In this study the limited mass transfer in PBR made hydrogen difficult to release from packed carriers. The increase of hydrogen partial pressure would inhibit the hydrogen production and may limit the use of higher feeding strengths [4, 30]. Hence, a short HRT or back-mixing might be required for the PBR in order to strengthen the mass transfer [4, 16]. Compared to PBR, UASB had a better mass transfer for the effective contact between the microbial consortia and the substrate. Besides these, the three phase separator used in UASB made the biogas easy to release, and decreased the hydrogen partial pressure, therefore leading to a higher total gas and hydrogen production. ORL had an influence on the hydrogen yield. The yields of hydrogen in UASB increased from 4 ± 6 mmol/mol to

1.44 $\pm$ 0.01 mol/mol glucose (Fig. 3), with the increase of the OLR from 0.96 to 15.36 g COD/L/d. The PBR exhibited a similar trend with an increase in hydrogen yields from 6 $\pm$ 22 mmol/mol to 0.67 $\pm$ 0.06 mol/mol glucose with the increase of OLR. The results suggested increasing OLR may be an appropriate way to enrich hydrogen-producing bacteria. However, the methanogenesis was not effectively suppressed as methane yields in the PBR increased from 5 $\pm$ 8 mmol/mol to 0.26 $\pm$ 0.01 mol/mol glucose. The significant methanogenesis in PBR may be caused by the active hydrogenotrophic methanogens to consume the produced hydrogen.

3.1.2 Variation of VFAs concentrations and COD removal rate

Acetic acid, lactic acid and butyric acid are the common metabolites and main VFAs during the hydrogen production (Fig. 2H, I). The different distribution of metabolism products in two reactors revealed different metabolic pathways caused by different microbial communities. As showed in Fig. 2H and Fig. 2I, the yields of acetic acid were 0.51 $\pm$ 0.06, 0.57 $\pm$ 0.09 and 0.47 $\pm$ 0.08 mol/mol glucose at the steady state of 3.84, 7.68, 15.36 g COD/L/d in PBR, respectively. In comparison, the yields of acetic acid in UASB were much lower as 0.27 $\pm$ 0.08, 0.34 $\pm$ 0.04 and 0.29 $\pm$ 0.01 mol/mol glucose, respectively. The difference may be caused by homoacetogenesis, which would convert hydrogen and carbon dioxide into acetic acid [16].

COD removal rate was around 20% for both reactors during the steady-state process (Fig. 2G), similar to the previous study (19.2-38.8%) [15, 31, 32]. Specifically, the COD removal rates of PBR (22.2 $\pm$ 2.4%) and UASB (22.0 $\pm$ 3.7%) were similar at an OLR 15.36g COD/L/d although the significant difference in methane production in UASB and PBR was obtained. The conflict may be because the methane production were mainly

conducted by hydrogenotrophic methanogens, as acetoclastic methanogens will increase the COD removal rate through converting acetic acid into methane and carbon dioxide.

3.2 Microbial morphology and diversity in UASB and PBR

Fig.4 showed the SEM images of the inoculum, granules in UASB and biofilm in PBR. The inoculum originally consisted of flocculent and dispersed microbial community (Fig. 4A, D). The mature hydrogen-producing granules were observed covered with cracks in UASB after 120 d's operation (Fig. 4B). These granules were primarily rod shape bacteria, which bounded with each other tightly as shown in Fig. 4E. A small amount of coccus-shaped bacteria were also observed. Inner porous structure of the granules was observed. In comparison, biofilms were formed on the polyethylene carrier in PBR (Fig. 4C). More microbial communities with broader diversity were observed on the biofilm, where microbial consortia were connected each other through extracellular secretions (Fig. 4F).

Illumina MiSeq sequencing results revealed the significant difference of microbial diversity in inoculum, UASB and PBR (Table 1). [As showed in the Table 1, Ace and Chao indexes indicate the richness of the microbial communities, whereas Shannon and Simpson indexes represent the microbial diversity \[33\].](#) Of the three samples, the inoculum had the highest indexes of Shannon, ACE and Chao, and the lowest Simpson index, indicating the biggest bacterial species diversity [and highest species richness](#) of the three samples. This suggested that the specific microbes in UASB and PBR had been selectively enriched through the long term operation. Compared to UASB, PBR had higher [species richness and bigger](#) of microbial diversity. Fig.5 illustrated the relationship of microbial diversity among the three communities. The sum of total observed OTUs in the inoculum (373), UASB (45) and PBR (93) were in accord with their richness. The overlap between UASB

(31, 69%) and PBR (31, 30%) showed a strong similarity of microbial diversity between them. The extra unique parts of UASB and PBR may be owing to the limitation of sample detection, included in the unclassified part in inoculum (13%). Although only 7 OTUs were shared by three of them, the majority microbial species in UASB (92%) and PBR (76%) were included. The OTUs ratios of UASB/inoculum (12%) and PBR/inoculum (25%) were proposed to represent the effect of specific microbe enrichment in the reactors. It is clear that the two reaction mode had different biomass enrichment process. Compared to UASB, PBR may have a stronger ability to retain the microbial consortia, owing to its high speed of incubating the biofilm attached on the carrier in the early operational stage [10, 34]. Moreover, the biofilm system were more resistant to process imbalances [35-37] could favor the slow growing of microorganisms. This would have a negative effect on eliminating the methanogens. The current study confirmed the significant activity of methanogens in PBR under high OLR and short HRT (Fig.2E). In comparison, the formation of active microbial consortia enriched in UASB through anaerobic sludge granulation required a relatively long time (2-8 months) [38]. Chang and Lin observed visible granules after 4 months in UASB for biohydrogen production [6]. The granulation process is a selective evolution of the microbial community [9, 39], and hence methanogens were easily washed out under specific operation conditions such as short HRT and low initial pH.

Fig.6 detailed the microbial diversity at the levels of phyla, families and genera. The inoculum had the highest diversity with 25 identified phyla. The main phyla were *Bacteroidetes* (7.4%), *Chloroflexi* (23.2%), *Proteobacteria* (20.4%) and *Spirochaetae* (31.5%), and the phylum *Firmicutes* only accounted for 2.5%. Compared to the inoculum, UASB and PBR exhibited much simple microbial consortium with only 6 and 7 identified

phyla, indicating a selection process occurred for each reactor system. The significant difference between UASB and PBR was the distribution of phyla *Bacteroidetes*, *Firmicutes* and *Proteobacteria* (Fig. 6A). The phylum *Firmicutes* was the main microbial consortium in UASB (99.7%) and PBR (91.4%). PBR had a higher richness than UASB, including *Bacteroidetes* (0.2%), *Proteobacteria* (2.9%) and unclassified (5.3%). Further analysis at the families level showed that the phylum *Firmicutes* in UASB and PBR consisted of *Clostridiaceae*, *Leuconostocaceae*, *Streptococcaceae* and *Ruminococcaceae* (Fig 6B). Among these, *Clostridiaceae* was the dominant family in UASB (93.3%) and PBR (73.4%). The relative abundance of families *Leuconostocaceae*, *Ruminococcaceae*, *Streptococcaceae*, *Enterobacteriaceae* in PBR was higher than that in UASB. From the perspective of genus (Fig 6C), *Clostridium* sp. was found as the main genus in PBR (71.7%) and UASB (92.1%) which was regarded as the most common microbial consortium in biohydrogen fermentation [9, 13, 17, 40]. As showed in the Table 2, butyrate and acetate were reported as the primary soluble metabolites produced by *Clostridium* sp. for hydrogen fermentation [41]. In particular, *Ethanoligenens* sp. and *Ruminococcaceae Incertae Sedis* sp., which belong to family *Ruminococcaceae* were found in both reactors. Ethanol was also produced although *Ruminococcaceae* were hydrogen producers [42]. Guo et al. reported *Ethanoligenens harbinense* B49 from the family *Ruminococcaceae* was an effective hydrogen producer by ethanol type fermentation [43]. *Weissella* sp. and *Lactococcus* sp. were also identified, which belong to lactic acid-producing family *Leuconostocaceae* and *Streptococcaceae*, respectively [44, 45]. *Leuconostocaceae* were found in the biofilm of PBR by Chojnacka et al. [46], *Leuconostocaceae* are heterofermentative bacteria able to produce lactic acid, acetic acid, and ethanol [46], whereas *Streptococcaceae* are facultatively anaerobic bacteria able to

produce lactic acid by fermentation [47]. The higher relative abundance of *Ruminococcaceae Incertae Sedis* sp., *Ethanoligenens* sp., *Weissella* sp., and *Lactococcus* sp. in PBR than UASB may be responsible for the lower hydrogen yield since their main metabolic products were lactic acid and ethanol, which will reduce the hydrogen yield [48]. Microbial diversity results supported the different performance of biohydrogen production through UASB and PBR, due to their differentiation of reaction mode.

3.3 Side reaction of hydrogen fermentation: methanogenesis and homoacetogenesis

The maximum hydrogen yields in UASB (1.44 ± 0.01 mol/mol glucose) and PBR (0.67 ± 0.06 mol/mol glucose) observed in this study were still lower than the expected maximum value (2 mol/mol glucose [49]) for mesophilic hydrogen fermentation. The low hydrogen yield was partly caused by methanogenesis and homoacetogenesis. In order to understand the distribution of hydrogen gas, the quantification of two side biochemical reactions including methanogenesis and homoacetogenesis were conducted.

The methanogenesis was considered as the activity of hydrogenotrophic methanogens in this study due to the following reasons. Firstly, compared with the acetoclastic methanogens, hydrogenotrophic methanogens have a shorter generation time for easier survival from short HRT (12-24 h) [50]. Secondly, the final pH values in UASB and PBR were below 5 (Fig. 2D), which would inhibit acetoclastic methanogens rather than hydrogenotrophic methanogens due to the latter's high tolerance to acidic shock as previously observed [51]. As showed in the Table 3, the ratio of H_2 methane/ H_2 theoretical in PBR was higher than that of UASB, suggesting that PBR was more favorable for methanogenesis.

The homoacetogenesis also had an effect on the decrease of the hydrogen yield, although heat pretreatment of seed sludge was conducted in this study, Luo et al. found that inhibition of homoacetogenesis during the long-term biohydrogen production was not dependent on the pretreatment of inoculum [25]. Illumina MiSeq sequencing results confirmed the existence of homoacetogenesis as there was a small amount of homoacetogenic microorganism *Clostridium ljungdahlii* in UASB and PBR, and might function as the conversion of hydrogen and carbon dioxide into acetate [52]. The quantification results showed (Table 3) that PBR had a higher value of $H_2 \text{ homo}/H_2 \text{ theoretical}$, indicating more favorable conditions in PBR for homoacetogenesis.

4. Conclusion

This study demonstrated that the reaction mode significantly affected hydrogen fermentation and microbial diversity. UASB had a better performance than PBR for biohydrogen production with a maximal hydrogen production rate of 2.77 ± 0.18 L/L/d and a hydrogen yield of 1.44 ± 0.01 mol/mol glucose, whereas PBR achieved 1.29 ± 0.09 L/L/d and 0.67 ± 0.06 mol/mol glucose, respectively. Illumina MiSeq sequencing results revealed much simple microbial diversity of UASB and PBR compared to the inoculum after 120 days' operation. PBR showed the lower proportion of hydrogen-producing microorganism *Clostridium* sp., higher distribution of ethanol and lactic acid producer, and its more favorable conditions for methanogenesis and homoacetogenesis.

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Figure captions:

Fig. 1 Schematic description of the UASB (A) and PBR (B) for continuous biohydrogen production.

Fig. 2. Performance of UASB and PBR for biohydrogen production. OLR (A), HRT (B), initial pH (C), final pH (D), gas composition (E), gas production rate (F), COD removal rate (G) and VFAs yields (H, I).

Fig. 3 The relationships between gas yields and OLR.

Fig. 4 SEM images of inoculum (A, D), granules in UASB (B, E) and biofilms in PBR (C, F).

Fig. 5 Venn chart of the inoculum, microbial consortia in granules in UASB and biofilms in PBR. The numbers represents OTUs (operational taxonomic units). The number of OTUs and their overlaps among cycles illustrate the logical relations between microbial samples.

Fig. 6 Taxonomic classification bacterial communities of inoculum, UASB and PBR at the phyla (A), families (B) and genera (C) levels through Illumina Miseq sequencing.

Table 1 Diversity analysis of the microbial community for clustering at 97% identity.

Sample	Chao	Shannon	Simpson	ACE
Inoculum	416	3.57	0.0829	401
UASB	65	1.48	0.4013	137
PBR	105	2.29	0.1879	106

Table 2 Representative microorganisms involved in biohydrogen production and their fermentation products

Microorganisms	Family	Fermentation products	Reference
<i>Clostridium</i> sp.	<i>Clostridiaceae</i>	H ₂ , butyrate , acetate	[41]
<i>Weissella</i> sp.	<i>Leuconostocaceae</i>	lactate, acetate , ethanol	[46]
<i>Ethanoligenens</i> sp.	<i>Ruminococcaceae</i>	H ₂ , ethanol, acetate	[42, 43]
<i>Ruminococcaceae Incertae</i>	<i>Ruminococcaceae</i>	H ₂ , ethanol, acetate	[42, 43]
<i>Sedis</i> sp.			
<i>Lactococcu</i> sp.	<i>Streptococcaceae</i>	lactate	[47]

Table 3 Quantification for methanogenesis and homoacetogenesis in UASB and PBR under an OLR of 15.36 g COD/L/L

	$Ac_{\text{homo}}/Ac_{\text{total}} (\%)$	$H_2 \text{ methane}/H_2 \text{ theoretical} (\%)$	$H_2 \text{ homo}/H_2 \text{ theoretic} (\%)$
UASB	1.5 ± 4.2	5.2 ± 3.8	1.3 ± 0.5
PBR	11.1 ± 3.4	49.4 ± 6.0	12.4 ± 0.8

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