The role of hydraulic retention time oncontrolling methanogenesis and homoacetogenesis in biohydrogen production using upflow anaerobic sludge blanket (UASB) reactor and packed bed reactor (PBR)

Buchun Si^a, Jiaming Li^a, Baoming Li^a, Zhangbing Zhu^a, Ruixia Shen^a, Yuanhui Zhang^{b,*}, Zhidan Liu^{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, China;

^b Department of Agricultural and Biological Engineering, University of Illinois at Urbana-Champaign, Urbana, IL, USA

* Corresponding author.

Fax: +86-10-6273-7329; Tel: +86-10-6273-7329.

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

* Corresponding author.

Fax: 217-244-0323; Tel: 217-333-2693.

E-mail address: yzhang1@illinois.edu (Y. Zhang)

Abstract:

Methanogenesis and homoacetogenesis are two notorious hydrogen-consuming reactions during dark fermentation for biohydrogen production. The focus of this study was on the role of hydraulic retention time (HRT) to control methanogenesis and homoacetogenesis in an upflow anaerobic sludge blanket (UASB) reactor and a packed bed reactor (PBR). The HRT was changed from 24 to 4 h and 24 to 2 h in the UASB and PBR, respectively. A maximal hydrogen yield of 1.47 mol/mol glucose_{added} with a high hydrogen production rate of 4.38 L/L/d was achieved at 8 h HRT in UASB. In comparison, a maximal hydrogen yield of 0.89 mol/mol glucose_{added} with a high hydrogen production rate of 10.66 L/L/d was achieved at 2 h in PBR. With the reduction of the HRT, the volumetric hydrogen consumption due to methanogenesis in the UASB was decreased from 12.1 to 3.1%. As for PBR, the value was reduced from 66.9 to 31.4%. Homoacetogenesis in the UASB and PBR was dramatically suppressed when the HRT was decreased to 8 and 4 h, respectively. However, these hydrogen-consuming microbes cannot be completely removed. Microbial diversity analysis using Illumina MiSeq sequencing revealed the existence of *Clostridium ljungdahlii*, a homoacetogen, in UASB and PBR at low HRT. In addition, the low HRT reduced the relative abundance of *Clostridiaceae* and accelerated the proliferation of lactic acid producers and ethanol producers in the UASB and PBR, which were mainly from the families *Ruminococcaceae* and *Leuconostocaceae*.

Keywords:

Biohydrogen; Homoacetogenesis; Methanogenesis; Hydraulic retention time; Dark fermentation

1 Introduction

Biohydrogen production through dark fermentation is environmentally friendly and efficient. A mixed culture is recommended as inoculum in fermentative biohydrogen production because complex microbial communities are likely to contain a suite of necessary hydrolytic activities and potentially more robust to changing operational conditions [1]. However, hydrogen consumption through methanogenesis and homoacetogenesis were often observed in these cases [2, 3]. Specifically, produced hydrogen and carbon dioxide can be biologically converted into methane (methanogenesis, Eq.1) and acetic acid (homoacetogenesis, Eq.2) by hydrogenotrophic methanogens and homoacetogens, respectively.

These hydrogen consuming reactions including hydrogenotrophic methanogenesis ($\Delta G^0 = -135.0$ kJ) and homoacetogenesis ($\Delta G^0 = -104.0$ kJ), were more thermodynamically feasible than acetogenic reactions ($\Delta G^0 = -4.2 \sim -76.2$ kJ) under elevated hydrogen concentration [3]. Several strategies were attempted to suppress the hydrogen-consuming reactions, including pretreatment of inoculum, pH control, organic loading rate (OLR) shock and control of hydrogen partial pressure [4-6]. Various pretreatments of inoculum were found to be difficult in eliminating the hydrogenotrophic methanogens and homoacetogens. Heat-resistant spore-forming *Clostridium* sp. also existed in some homoacetogens, such as *Clostridium ljungdahlii*, *Clostridium aceticum* and *Clostridium scatologenes* [3]. Methanogens were also observed to be resistant to the pretreatment. Different pretreatment methods were reported, such as acid, heat and loading-shock. However, all of them failed to inhibit either methanogenesis or homoacetogenesis during long-term operation [7]. Hydrogenotrophic methanogens with a high tolerance to pH changes were found during the biohydrogen fermentation [8]. The strategy of pH control was considered more effective in changing the anaerobic metabolism pathway rather than the inhibition of hydrogen consumption [8]. Based on the principle of suppressing hydrogen consumption through the reduction of hydrogen partial pressure, Bakonyi et al. proposed an integrated system applying membrane technology that can simultaneously produce and purify fermentative hydrogen [9]. However, there were still some

challenges for this integrated system [9]. The reduction of hydraulic retention time (HRT) could be a potential strategy through increasing hydrogen yield and selection of mixed culture populations, such as *Clostridium* sp. and *Thermoanaerobacterium thermosaccharolyticum* [10-12]. In addition, low HRT could suppress the methane producers and inhibit the methanogenesis in a continuous stirred tank reactor (CSTR) for hydrogen production [13, 14]. Serious methanogenesis and homoacetogenesis were reported in high rate reactors, such as upflow anaerobic sludge blanket (UASB) and packed bed reactor (PBR) [15-18]. The HRT control may be a simple and practical approach to control methanogenesis and homoacetogenesis in high-rate biohydrogen reactors, although other methods, such as OLR shock [15] and heat treatment during the operation [19] had been attempted.

A number of studies have been conducted to determine the effect of HRT on hydrogen production and related microbial population [10-12]. However, few studies focused on the role of HRT on the control of hydrogen-consuming reactions. Methanogenesis and homoacetogenesis are two notorious hydrogen-consuming reactions for biohydrogen production. The purposes of this study were 1) to investigate the role of HRT on hydrogen-consuming reactions (methanogenesis and homoacetogenesis) in high-rate reactors; and 2) to analyze responses of microbial diversity to different HRTs using Illumina MiSeq sequencing.

2 Materials and methods

2.1 Substrate, inoculum and reactors operation

In this study, synthetic wastewater was used as substrate for biohydrogen production, which included glucose 8 g/L as a carbon source, NH_4Cl 300 mg/L as a nitrogen source and NaHCO_3 4 g/L as a buffer. In addition, the trace element was added as previously reported [20]. The initial pH of the synthetic wastewater was adjusted to 5.7 ± 0.1 using 2 mol/L HCl before feeding.

The UASB and PBR were established as previously described [21], both had a working volume of 2.5 L and operating temperature of 35°C. The carbon nanotubes (CNTs) were added into the UASB to accelerate the forming of granules [22]. The PBR was packed with polyethylene rings (10 mm diameter and 10 mm thick). The UASB and PBR were inoculated with heat treated (100°C,

15 min) anaerobic sludge, and previously operated at an HRT of 12 h for over 150 d to form the granules and biofilms, respectively. The reactors were stopped for 20 days. The reactors were restarted and until they were back to the previous conditions (i.e. the biogas production rate and gas composition) prior to the experimental data collection at an HRT of 24 h.

2.2 Analytical methods

The volumes of produced gases were measured using a gas meter and corrected to a standard temperature (0°C) and pressure (101.325 kPa, STP). The gas compositions, including hydrogen, methane and carbon dioxide, were determined using a gas chromatograph (GC1490, Agilent Technologies, USA) as previously reported [21]. The volatile fatty acids (VFAs) were analyzed using a high performance liquid chromatography (HPLC) (Shimadzu, Japan) as previously reported [21]. The microbe samples in the UASB at the HRT of 4 h and PBR at the HRT of 4 h and 2 h were collected and stored at -20°C for microbial morphology observation and phylogenetic diversity analysis. The microbial morphology was observed by scanning electron microscopy (SEM) (Quanta 200, FEI, USA) as previously described [22]. The phylogenetic diversity of the microbial consortium was analyzed via Illumina MiSeq sequencing [21]. Primers 515F (5'-barcode-GTGCCAGCMGCCGCGG-3') and 907R

(5'-CCGTCAATTCMTTTRAGTTT-3') were used to amplify the V4-V5 regions of the bacteria 16S ribosomal RNA gene by GeneAmp PCR System (ABI company, USA). The PCR process was conducted as previously described [21]. Amplicons were extracted from 2% agarose gels and 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. 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). The phylogenetic affiliation of each 16S rRNA gene sequence was analyzed by a RDP Classifier (<http://rdp.cme.msu.edu/>) against the SILVA (SSU115) 16S rRNA database using a confidence threshold of 70%.

2.3 Quantification of methanogenesis and homoacetogenesis

Quantification analysis was conducted in order to better understand the effects of HRT on methanogenesis and homoacetogenesis in the UASB and PBR. The theoretical hydrogen yield ($H_{2\text{-theoretical}}$), the hydrogen consumed by homoacetogenesis ($H_{2\text{-homo}}$) and the hydrogen consumed by methanogenesis ($H_{2\text{-methane}}$) were calculated respectively as previously described[21]. The ratios of $R_{\text{methane}} = H_{2\text{-methane}}/H_{2\text{-theoretical}}$ and $R_{\text{homo}} = H_{2\text{-homo}}/H_{2\text{-theoretical}}$ were proposed to represent methanogenesis and homoacetogenesis, respectively.

3 Results and discussion

3.1 Effect of HRT on hydrogen production in the UASB and PBR

The UASB was operated at an HRT of 24h, 12h, 8h and 4h, and then back to 8h and 12h (Fig. 1A). The hydrogen production rate (HPR) increased from 1.24L/L/d to 2.90L/L/d as the HRT decreased from 24h to 12 h. A maximal HPR value of 4.38L/L/d was achieved at 8h with a hydrogen content of 46.0% (v/v) (Fig. 1A). The HPR decreased to 4.22 L/L/d when the HRT decreased from 8 h to 4 h. Compared to HRT at 8 h, although the HPR at 4 h was only decreased by 4 %, the hydrogen yield (in terms of mol of H_2 per mol of glucose) was substantially decreased because the OLR was doubled at 4 h HRT. As shown in the Table 1, the optimal HRT of the UASB was 8h which was similar to the literature reported[23]. Methanogenesis was observed all through the operation even though the methane concentration was only around 1% (v/v) (Fig. 1A). The produced VFAs mainly consisted of butyric acid, acetic acid and lactic acid (Fig. 1A). The accumulation of lactate was observed at the HRT of 4 h, accompanied with decrease of HPR.

Similar to the UASB, the PBR was first operated at HRT 24 h, 12 h, 8 h, 4 h and 2h, and then went back to 12 h (Fig. 1B). A significant methane production was observed in the PBR (Fig. 1B). The methane concentration increased slightly as the HRT decreased from 24h (10.7%) to 8h (13.0%), accompanied with the decrease of hydrogen content from 38.8% to 24.9%. Further reduction of HRT to 4 h and 2 h resulted in the reduction of methane content to 8.3 and 4.4%, respectively. Similarly, the HPR was increased as the decrease of HRT in PBR. A maximum

HPR of 10.66 L/L/d was achieved at 2 h with a hydrogen content of 39.6% (Fig. 1B). The accumulation of lactic acid was also observed in PBR at 2 h, similar to that in UASB. The PBR seemed overloaded although the lower HRT was not attempted. The optimal HRT of the PBR in this study was similar to the previous report [19], in which poor hydrogen production at the HRT of less than 2 h. A maximum HPR of 10.66 L/L/d was achieved in PBR in this study owing to the short HRT adopted. The UASB and PBR in this study achieved their highest hydrogen yield of 1.47 and 0.89 mol/mol glucose at 8 and 2 h, respectively. Table 1 showed the comparison of hydrogen production in this study and the literature.

The performance of hydrogen production in the reactors suggested that methanogenesis and homoacetogenesis were suppressed at low HRT. In order to further reveal the reaction mechanism of both methanogenesis and homoacetogenesis during biohydrogen production, hydrogen production and consumption at different HRTs was quantified in the following section.

3.2 Effect of HRT on hydrogen-consuming reactions (methanogenesis and homoacetogenesis)

As shown in Fig. 2, there were some differences between $H_{2\text{-theoretical}}$ and measured hydrogen yield ($H_{2\text{-measured}}$) in UASB and PBR. Both biotic (homoacetogenesis and methanogenesis) and abiotic (improved mass transfer) factors contributed to these differences. Although hydrogen gas is almost insoluble in water and would escape from aqueous medium, insufficient liquid-to-gaseous phase component transport may take place in the UASB and PBR. For instance, the produced hydrogen might adhere to the granules in UASB and biofilms in PBR. This would partly contribute to deviations between the measured and the theoretical yields [24-26]. Moreover, it was assumed that the hydrogen-consuming reactions (homoacetogenesis and methanogenesis) might have played a more important role. Fig. 2 illustrates the influence of HRT on methanogenesis and homoacetogenesis in the UASB and PBR. As for the UASB, the $H_{2\text{-theoretical}}$ was increased from 1.50 to 1.62 mol/mol glucose_{added} as the HRT decreased from 24 h to 8 h. The increase of $H_{2\text{-measured}}$ from 1.25 to 1.47 mol/mol glucose_{added} was also observed. Both the maximal $H_{2\text{-theoretical}}$ and $H_{2\text{-measured}}$ were achieved at 8 h. The increased hydrogen yield mainly was attributed to improved mass transfer and enhanced contact of microbe and bulk solution. In addition, the reduction of R_{methane} and R_{homo} with the decrease of HRT suggested the low HRT might enhance

the release of hydrogen gas, and avoid further conversion of produced hydrogen through methanogenesis and homoacetogenesis. However, methane production in the UASB was observed during the experiments, which suggested hydrogenotrophic methanogens could not be completely removed through the decrease of the HRT. This might be due to the specific structure of hydrogen-producing granules in the UASB. The layer structure of granules in the UASB for methane production was observed. Specifically, the external layer mostly consisted of acidogens, and the inside layer was composed of syntrophic microbial consortia and acetoclastic methanogens [27]. The similar structure might also exist in hydrogen-producing granules, where hydrogen-consuming microbes are surrounded by hydrogen producers, and thus formed tight syntrophic microbial consortia. Such structure may encourage the hydrogenotrophic methanogens unless the whole granule is washed out. As shown in Fig. 2, compared with methanogenesis, homoacetogenesis could be fully inhibited at a HRT of 8 h. A drastic decrease of $H_{2\text{-theoretical}}$ and $H_{2\text{-measured}}$ was observed at 4 h. Overloading occurred as hydrogen producers were washed out and non-hydrogen-producing pathways such as lactic acid production were enhanced (Fig. 1A). The R_{homo} was increased to $9.5 \pm 6.4\%$ at 4 h. The increase of homoacetogenesis might result from its competitive relationship with methanogenesis [15]. The recovery of methanogenesis and homoacetogenesis with the HRT back to 12 h, suggested both could not be completely removed from the UASB, only temporarily suppressed.

Regarding PBR, the decrease of HRT from 24 to 8 h resulted in an increase of $H_{2\text{-theoretical}}$ from 1.31 to 1.55 mol/mol glucose_{added}. However, the methane content and R_{methane} were also increased, leading to the unstable performance of the measured hydrogen yield. This result suggested that the HRT range of 24–8 h could enhance the $H_{2\text{-theoretical}}$ by strengthening the contact of microbes and substrate. However, the HRT was not low enough to affect the strong association in the microbial consortia consisting of hydrogenotrophic methanogens and hydrogen producers. Further decrease of HRT to 4 h resulted in an increase of $H_{2\text{-theoretical}}$ to 1.62 mol/mol glucose_{added} and decreases of R_{methane} and R_{homo} , suggesting that the HRT not only enhanced the mass transfer, but also affected the microbial consortia. Both R_{methane} and $H_{2\text{-theoretical}}$ were reduced, accompanied with the accumulation of lactic acid, at a further decrease of HRT to 2 h, indicating the overloading of PBR. Similar to the UASB, recovery of methanogenesis and homoacetogenesis was observed in

PBR with HRT back to 12 h from 2 h.

3.3 Microbial morphology and diversity

SEM images indicated microbial morphology of granules in the UASB (Fig. 3A) and biofilms in PBR (Fig. 3B, C). The granules and biofilms both consisted of rod-shaped bacteria, but the coccus-shaped bacteria were also observed in the granules. Compared with the biofilms at 4 h, the sample at 2 h had fewer microbes probably due to the washout at low HRT.

The microbial diversity was characterized by Illumina MiSeq sequencing. As shown in Table 2, Shannon, Simpson, Chao, ACE and operational taxonomic units (OTUs) were used to represent the bacterial diversity. The granules in the UASB at 4 h had a higher Shannon and lower Simpson index than biofilms in PBR at 4 h, indicating a higher diversity of bacterial species. However, biofilms had a greater bacterial richness in terms of more OTUs, higher Chao and ACE richness estimators than granules owing to the inhomogeneous environment in PBR. Compared to biofilms at 4 h, the sample at 2 h showed the lower bacteria richness and higher bacterial diversity. These results indicated that the low HRT in the UASB and PBR might reduce microbial richness through the washout of microbes and increase microbial diversity through accelerating the proliferation of non-hydrogen-producing microorganism.

The phylum *Firmicutes* was the dominant bacteria under all the conditions, including granules at 4 h (99.5%), biofilms at 4 h (99.2%) and 2 h (99.2%). *Firmicutes* consisted of the families *Clostridiaceae*, *Ruminococcaceae* and *Leuconostocaceae* (Fig. 4A). The relative abundance of the families *Ruminococcaceae* and *Leuconostocaceae* in the UASB at 4 h and PBR at 2 h was higher than that of PBR at 4 h (Fig. 4A). From the perspective of genera (Fig. 4B), *Clostridium* sp. which belongs to the family *Clostridiaceae* was reported as the main hydrogen producer [28]. Specifically, the relative abundance of *Clostridium* sp. in biofilms at 4 h (92.5%) was higher than granules at 4 h (84.1%) and biofilms at 2 h HRT (76.0%) (Fig. 4B). *Ruminococcaceae* *Incertae Sedis* sp. and *Ethanoligenens* sp. which come from the family *Ruminococcaceae* were reported as ethanol producers [29]. *Weissella* sp. which belongs to the family *Leuconostocaceae* was a heterofermentative lactic acid bacterium able to produce lactic acid, acetic acid, and ethanol [30]. These results indicated that the low HRT would decrease the relative

abundance of the hydrogen producer *Clostridiaceae*, and accelerate the proliferation of lactic acid producers and ethanol producers in both UASB and PBR, mainly belonging to the family *Ruminococcaceae* and *Leuconostocaceae*.

It appeared that the hydrogenotrophic methanogens and homoacetogen population might decrease as the reduced hydrogen consumption with decrease of HRT (Fig. 2). However, they were not fully removed from the reactors. The species *Clostridium ljungdahlii*, a homoacetogen, were found in the UASB and PBR. The hydrogenotrophic methanogens could also survive in the reactors even at low HRT. These hydrogenotrophic methanogens may consist of the genera *Methanobrevibacter* and *Methanobacterium* as previously described [31].

4 Conclusion:

This study verified that reduction of HRT successfully suppressed methanogenesis and homoacetogenesis in the UASB and PBR. By manipulating HRT, hydrogen consumption due to methanogenesis was decreased from 12.1 to 3.1% in the UASB, and from 66.9 to 31.4% in PBR. Homoacetogenesis in the UASB and PBR was completely suppressed when the HRT was reduced from 24 h to 8 and 4 h, respectively. However, it was difficult to completely remove the hydrogen-consuming microbes from high-rate reactors. Microbial diversity analysis using Illumina MiSeq sequencing revealed that low HRT would decrease the relative abundance of the hydrogen producer *Clostridiaceae* and accelerate the proliferation of non-hydrogen-producing microorganism, primarily attributed to the families *Ruminococcaceae* and *Leuconostocaceae*.

Acknowledgement

This work was financially supported by the National Natural Science Foundation of China (21106080), National Key Technology Support Program of China (2014BAD02B03), NSFC-JST Cooperative Research Project of China (21161140328) and the Chinese Universities Scientific Fund (2012RC030).

References:

- [1] Hung CH, Chang YT, Chang YJ. Roles of microorganisms other than *Clostridium* and *Enterobacter* in anaerobic fermentative biohydrogen production systems-A review. *Bioresour Technol* 2011; 102: 8437-44.
- [2] Dinamarca C, Bakke R. Apparent hydrogen consumption in acid reactors: Observations and implications. *Water Sci Technol* 2009; 59: 1441-7.
- [3] Saady NMC. Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: Unresolved challenge. *Int J Hydrogen Energy* 2013; 38:13172-91.
- [4] Shanmugam SR, Chaganti SR, Lalman JA, Heath DD. Statistical optimization of conditions for minimum H₂ consumption in mixed anaerobic cultures: Effect on homoacetogenesis and methanogenesis. *Int J Hydrogen Energy* 2014; 39:15433-45.
- [5] Liu D, Zeng RJ, Angelidaki I. Effects of pH and hydraulic retention time on hydrogen production versus methanogenesis during anaerobic fermentation of organic household solid waste under extreme-thermophilic temperature (70°C). *Biotechnol Bioeng* 2008; 100:1108-14.
- [6] Bakonyi P, Nemestothy N, Simon V, Belafi-Bako K. Review on the start-up experiences of continuous fermentative hydrogen producing bioreactors. *Renew Sust Energy Rev* 2014; 40: 806-13.
- [7] Luo G, Karakashev D, Xie L, Zhou Q, Angelidaki I. Long-term effect of inoculum pretreatment on fermentative hydrogen production by repeated batch cultivations: Homoacetogenesis and methanogenesis as competitors to hydrogen production. *Biotechnol Bioeng* 2011; 108: 1816-27.
- [8] [7] Kim IS, Hwang MH, Jang NJ, Hyun SH, Lee ST. Effect of low pH on the activity of hydrogen utilizing methanogen in bio-hydrogen process. *Int J Hydrogen Energy* 2004; 29: 1133-40.
- [9] Bakonyi P, Nemestothy N, Belafi-Bako K. Biohydrogen purification by membranes: An overview on the operational conditions affecting the performance of non-porous, polymeric

- and ionic liquid based gas separation membranes. *Int J Hydrogen Energy* 2013; 38: 9673-87.
- [10] Zhang Z, Show K, Tay J, Liang DT, Lee D, Jiang W. Effect of hydraulic retention time on biohydrogen production and anaerobic microbial community. *Process Biochem* 2006; 41: 2118-23.
- [11] Júnior ADN, Wenzel J, Etchebehere C, Zaiat M. Effect of organic loading rate on hydrogen production from sugarcane vinasse in thermophilic acidogenic packed bed reactors. *Int J Hydrogen Energy* 2014; 39: 16852-62.
- [12] Li S, Whang L, Chao Y, Wang Y, Wang Y, Hsiao C, Tseng I, Bai M, Cheng S. Effects of hydraulic retention time on anaerobic hydrogenation performance and microbial ecology of bioreactors fed with glucose-peptone and starch-peptone. *Int J Hydrogen Energy* 2010; 35: 61-70.
- [13] Chen CC, Lin CY, Chang JS. Kinetics of hydrogen production with continuous anaerobic cultures utilizing sucrose as the limiting substrate. *Appl Microbiol Biotechnol* 2001; 57: 56-64.
- [14] Pakarinen O, Kaparaju P, Rintala J. The effect of organic loading rate and retention time on hydrogen production from a methanogenic CSTR. *Bioresour Technol* 2011; 102: 8952-7.
- [15] Carrillo-Reyes J, Celis LB, Alatríste-Mondragón F, Razo-Flores E. Decreasing methane production in hydrogenogenic UASB reactors fed with cheese whey. *Biomass Bioenergy* 2014; 63: 101-8.
- [16] Lima DMF, Zaiat M. The influence of the degree of back-mixing on hydrogen production in an anaerobic fixed-bed reactor. *Int J Hydrogen Energy* 2012; 37: 9630-5.
- [17] Penteado ED, Lázaro CZ, Sakamoto IK, Zaiat M. Influence of seed sludge and pretreatment method on hydrogen production in packed-bed anaerobic reactors. *Int J Hydrogen Energy* 2013; 38: 6137-45.
- [18] Lima DMF, Moreira WK, Zaiat M. Comparison of the use of sucrose and glucose as a substrate for hydrogen production in an upflow anaerobic fixed-bed reactor. *Int J Hydrogen Energy* 2013; 38: 15074-83.
- [19] Chang J, Lee K, Lin P. Biohydrogen production with fixed-bed bioreactors. *Int J Hydrogen Energy* 2002; 27: 1167-74.

- [20] Mohan SV, Chiranjeevi P, Mohanakrishna G. A rapid and simple protocol for evaluating biohydrogen production potential (BHP) of wastewater with simultaneous process optimization. *Int J Hydrogen Energy* 2012; 37: 3130-41.
- [21] Si B, Liu Z, Zhang Y, Li J, Xing X, Li B, Duan N, Lu H. Effect of reaction mode on biohydrogen production and its microbial diversity. *Int J Hydrogen Energy* 2015; 40: 3191-200.
- [22] Liu Z, Lv F, Zheng H, Zhang C, Wei F, Xing X. Enhanced hydrogen production in a UASB reactor by retaining microbial consortium onto carbon nanotubes (CNTs). *Int J Hydrogen Energy* 2012; 37: 10619-26.
- [23] Chang F, Lin C. Biohydrogen production using an up-flow anaerobic sludge blanket reactor. *Int J Hydrogen Energy* 2004; 29: 33-9.
- [24] Fang Z, Yan Z, Man C, Zeng RJ. Hydrogen supersaturation in thermophilic mixed culture fermentation. *Int J Hydrogen Energy* 2012; 37: 17809-16.
- [25] Tapia-Venegas E, Esteban Ramirez J, Donoso-Bravo A, Jorquera L, Steyer J, Ruiz-Filippi G. Bio-hydrogen production during acidogenic fermentation in a multistage stirred tank reactor. *Int J Hydrogen Energy* 2013; 38: 2185-90.
- [26] Ramirez-Morales JE, Tapia-Venegas E, Nemestothy N, Bakonyi P, Belafi-Bako K, Ruiz-Filippi G. Evaluation of two gas membrane modules for fermentative hydrogen separation. *Int J Hydrogen Energy* 2013; 38: 14042-52.
- [27] Huang J, Chou H, Ohara R, Wu C. Consecutive reaction kinetics involving a layered structure of the granule in UASB reactors. *Water Res* 2006; 40: 2947-57.
- [28] Lee D, Show K, Su A. Dark fermentation on biohydrogen production: Pure culture. *Bioresour Technol* 2011; 102: 8393-402.
- [29] Veeravalli SS, Chaganti SR, Lalman JA, Heath DD. Optimizing hydrogen production from a switchgrass steam exploded liquor using a mixed anaerobic culture in an upflow anaerobic sludge blanket reactor. *Int J Hydrogen Energy* 2014; 39: 3160-75.
- [30] Chojnacka A, Blaszczyk MK, Szczesny P, Nowak K, Suminska M, Tomczyk-Zak K, Zielenkiewicz U, Sikora A. Comparative analysis of hydrogen-producing bacterial biofilms and granular sludge formed in continuous cultures of fermentative bacteria. *Bioresour Technol* 2011; 102: 10057-64.
- [31] Carrillo-Reyes J, Celis LB, Alatríste-Mondragón F, Montoya L, Razo-Flores E. Strategies to

cope with methanogens in hydrogen producing UASB reactors: Community dynamics. Int J Hydrogen Energy 2014; 39: 11423-32.

Figure captions:

Fig. 1 Performance of UASB (A) and PBR (B) at different HRTs, including HRT, pH of the effluent, biogas content, hydrogen production rate (HPR), methane production rate (MPR) and VFAs concentration

Fig. 2 Influence of HRT on methanogenesis and homoacetogenesis in UASB (A) and PBR (B).

Fig. 3 SEM images of granules in UASB at 4 h (A), biofilms in PBR at 4 h (B) and 2 h (C)

Fig. 4 Taxonomic classification bacterial communities of UASB at 4h, PBR at 4h and 2h at the families (A) and genera (B) levels through Illumina MiSeq sequencing. Families and genera less than 1% of total composition were classified as “others”.

Table 1 Comparative study of operating conditions and efficiency of dark fermentative hydrogen production.

<i>Reactor</i>	<i>Substrate</i>	<i>Temperature</i> (°C)	<i>HRT range,</i> <i>optimal value</i> (h)	<i>Maxi. hydrogen</i> <i>yield</i> (mol H ₂ /mol hexose)	<i>Maxi.HPR</i> (L/L/d)	<i>References</i>
CSTR	Glucose	37	6-12, 10	1.95	6.11	[10]
PBR	Sucrose	25	2, na ^a	0.7 ^b	2.99	[16]
PBR	Sucrose	25	2, na	1.05 ^b	1.14	[17]
PBR	Sucrose	35	0.5-5, 2	na	9.96	[19]
UASB	Glucose	na	24, na	2.45	5.55	[22]
UASB	Sucrose	35	4-24, 8	0.75 ^b	6.06	[23]
UASB	Glucose	37	4-24, 8	1.47	4.38	This study
PBR	Glucose	37	2-24, 2	0.89	10.66	This study

a na= not available.

b Calculated by per mole of hexose equivalent.

Table 2 Statistical analysis of non-parametric diversity indexes of Illumina MiSeq sequencing

<i>Sample</i>	<i>Shannon</i>	<i>Simpson</i>	<i>ACE</i>	<i>OTUs</i>	<i>Chao</i>
UASB (4 h)	2.00	0.2162	41	37	42
PBR (4 h)	1.49	0.3629	109	58	101
PBR (2 h)	2.15	0.1511	68	48	59

Fig. 1 Performance of UASB (A) and PBR (B) at different HRTs, including HRT, pH of the effluent, biogas content, hydrogen production rate (HPR), methane production rate (MPR) and VFAs concentration

Fig. 2 Influence of HRT on methanogenesis and homoacetogenesis in UASB (A) and PBR (B).

Fig. 3 SEM images of granules in UASB at 4 h (A), biofilms in PBR at 4 h (B) and 2 h (C)

Fig. 4 Taxonomic classification bacterial communities of UASB at 4 h,PBR at 4 h and 2 h at the families (A) and genera (B) levels through Illumina MiSeq sequencing. Families and genera less than 1% of total composition were classified as “others”.