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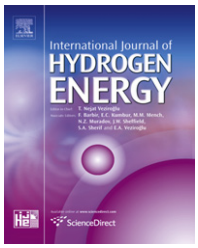

Effect of organic loading on a novel hydrogen bioreactor

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ABSTRACT

This study investigated the impact of six organic loading rates (OLR) ranging from 6.5 gCOD/L-d to 206 gCOD/L-d on the performance of a novel integrated biohydrogen reactor clarifier systems (IBRCSs) comprised a continuously stirred reactor (CSTR) for biological hydrogen production, followed by an uncovered gravity settler for decoupling of solids retention time (SRT) from hydraulic retention time (HRT). The system was able to maintain a high molar hydrogen yield of 2.8 mol H_2/mol glucose at OLR ranging from 6.5 to 103 gCOD/L-d, but dropped precipitously to approximately 1.2 and 1.1 mol H_2/mol glucose for the OLRs of 154 and 206 gCOD/L-d, respectively. The optimum OLR at HRT of 8 h for maximizing both hydrogen molar yield and volumetric hydrogen production was 103 gCOD/L-d. A positive statistical correlation was observed between the molar hydrogen production and the molar acetate-to-butyrate ratio. Biomass yield correlated negatively with hydrogen yield, although not linearly. Analyzing the food-to-microorganisms (F/M) data in this study and others revealed that, both molar hydrogen yields and biomass specific hydrogen rates peaked at 2.8 mol H_2/mol glucose and 2.3 L/gVSS-d at F/M ratios ranging from 4.4 to 6.4 gCOD/gVSS-d. Microbial community analysis for OLRs of 6.5 and 25.7 gCOD/L-d showed the predominance of hydrogen producers such as *Clostridium acetobutyricum*, *Klebsiella pneumonia*, *Clostridium butyricum*, *Clostridium pasteurianum*. While at extremely high OLRs of 154 and 206 gCOD/L-d, a microbial shift was clearly evident due to the coexistence of the non-hydrogen producers such as *Lactococcus sp.* and *Pseudomonas sp.*

1. Introduction

Hydrogen production from renewable substrates can reduce reliance on fossil fuels. It produces only water upon combustion, thus is considered as a clean energy source that can help mitigate pollution and global warming [1]. Biological hydrogen production is potentially regarded as one of the most promising alternatives for sustainable green energy production despite the feasibility of hydrogen production through water electrolysis and chemical cracking of hydrocarbons [2]. Among different biological processes for hydrogen production, dark fermentation is the most attractive one because of its potential of direct use of wastewater streams and organic wastes and its higher rate of hydrogen production in comparison with photo-fermentative processes [3].

Organic loading rate (OLR) is an important parameter in studying hydrogen bioreactors. In order to optimize a system for hydrogen production, it is essential to define either a range of the organic loading rates that the system can handle effectively, or an optimal organic loading rate for a maximum hydrogen yield. In the literature, there is no clear relationship between the hydrogen yield and the organic loading rate. In

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some cases higher OLRs decreased the hydrogen yield [4] whereas in some others higher OLRs increased the hydrogen yield [5]. For waste activated sludge as a seed material, it appears that increasing the OLR within the 40–160 gCOD/L-d increased hydrogen yield to an optimum of 1.6 mol H_2/mol glucose at an OLR of 120 gCOD/L-d [6], whereas hydrogen yield decreased with increasing OLR for both anaerobically digested sludge [7] and soil microorganisms [4]. Although lower molar H_2 yields at higher OLRs have been attributed to the inhibitory effect of higher H_2 partial pressures in the growth medium [4,8], variations in the composition of bacterial communities that become established at different OLRs [9] may be a major reason for lower yields. Hydrogen yield with digester sludge at an OLR of 45 gCOD/L-d was 1.3 mol H_2/mol glucose [7] as compared with 0.9 mol H_2/mol glucose with waste activated sludge [6]. Moreover, comparing the biomass concentration in two studies with continuously stirred tank reactors (CSTRs) utilizing agricultural soil as the seed and glucose as a substrate under approximately same OLRs, Van Ginkel and Logan [4] achieved much higher hydrogen yield (2.2 mol/mol) at a biomass concentration of 8 g/L compared to Zhang et al. [5] who reported 0.72 mol H_2/mol hexose with 0.9 g/L biomass. Oh et al. [10] achieved a hydrogen yield of 0.4 mol/mol at a biomass concentration of 2.2 g/L in a CSTR and Wu et al. [6] using a CSTR seeded with silicone-immobilized sludge realized a hydrogen yield of 1.6 mol/mol at 3.5 g/L of biomass compared to a hydrogen yield of 2.1 mol/mol achieved by Zhang et al. [5] at a similar OLR with a higher biomass concentration (4.6 g/L). It is thus clear that the higher biomass concentration in the reactors improved the hydrogen yield, which in essence shows that one of the key factors affecting the stability of hydrogen producing systems is maintaining higher biomass concentrations in the system. In addition, the low hydrogen yield and system failure was attributed to low concentrations of biomass due to washout [4].

This paper has two objectives; the first objective focuses primarily on the investigation of the effect of organic loading rate on the performance of a novel integrated biohydrogen reactor clarifier system (IBRCS) [11] and to specify an optimal range for organic loading rate that maximizes hydrogen yield. While the other objective of this paper is to assess the impact of organic loading rate on the physical and biochemical characteristics i.e. the various metabolic pathways and microbial shifts involved in biological hydrogen production, as well as particle size and settling properties. The premise of the IBRCS is decoupling of hydraulic retention time (HRT) from solids retention time (SRT), which has been demonstrated in a previous work [12].

2. Materials and methods

2.1. Systems set up and operations

Two lab-scale systems were operated at 37 °C for 220 days (Fig. 1), at six different organic loading rates ranging from 6.5 gCOD/L-d to 206 gCOD/L-d. Two integrated biohydrogen reactor clarifier systems (IBRCSs) comprised a continuously stirred reactor (CSTR) for biological hydrogen production (5 L working volume), followed by an uncovered gravity settler

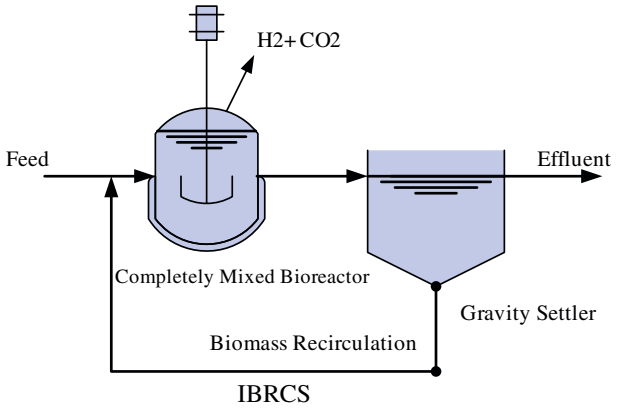

Fig. 1 – Experimental Setup for the integrated biohydrogen reactor clarifier system.

(volume 8 L) i.e. open to atmosphere for the decoupling of solids retention time (SRT) from the hydraulic retention time (HRT). Details of the operational conditions for the six runs are listed in Table 1. In order to enrich hydrogen producing bacteria, the sludges were heat treated at 70 °C for 30 min. Following the completion of each run and the attainment of steady-state conditions, the systems were cleaned and inoculated with pre-treated sludges. OLR-1 and 2 were run simultaneously, followed by OLR-3 and 4, and lastly OLR-5 and 6. The systems were monitored for total chemical oxygen demand (TCOD), soluble COD, volatile fatty acids (VFA), ethanol, lactate, glucose, volatile suspended solids (VSS), total suspended solids (TSS) and biogas composition including hydrogen, methane and nitrogen. The quantity of produced biogas was recorded daily using a wet-tip gas meter (Rebel wet-tip gas meter company, Nashville, TN, USA).

2.2. Inocula and media compositions

Anaerobically digested sludge from the St.Marys wastewater treatment plant (St.Marys, Ontario, Canada) was used as the seed. The two systems operated in parallel at the same time under two different OLRs for a total of six OLRs (three consecutive runs). The systems were seeded with 5 liters of sludge and started up in a continuous mode with the feed containing glucose at different concentrations as highlighted in Table 1. The same startup technique was repeated for the three runs. It must be emphasized that there was no sludge wastage from the clarifier throughout the operation, and the

Table 1 – Operational conditions.

	Glucose (g/L)	HRT (h)	SRT (h)	OLR (gCOD/L-d)	pH
OLR -1	2	8	50 ± 5	6.5	5.5–6.5
OLR -2	8	8	45 ± 4	25.7	5.5–6.5
OLR -3	16	8	45 ± 6	51.4	5.5–6.5
OLR -4	32	8	42 ± 6	103	5.5–6.5
OLR -5	48	8	27 ± 3	154	5.5–6.5
OLR -6	64	8	26 ± 2	206	5.5–6.5

Note. Values represent average ± standard deviation.

values of SRTs presented in Table 1 represent the average ± standard deviation (SD) during steady-state operation. It is noteworthy that the reactors operation was consistent over time and accordingly the average SRT with SD of less than 10% of the mean SRT is representative of the overall SRT during the run. As expected, the clarifier effluent VSS was substantially lower than the reactor VSS and remained unchanged during steady-state operation. The feed contained sufficient inorganics (mg/L): $NaHCO_3$, 2000–16000; $CaCl_2$, 140; $MgCl_2.6H_2O$, 160; NH_4HCO_3, 600; $MgSO_4.7H_2O$, 160; urea, 500–2000; Na_2CO_3, 124–300; $KHCO_3$, 156; K_2HPO_4, 15–20; trace mineral solution, 500; H_3PO_4, 250–1500.

2.3. *Analytical methods*

The biogas composition including hydrogen, methane, and nitrogen was determined by a gas chromatograph (Model 310, SRI Instruments, Torrance, CA) equipped with a thermal conductivity detector (TCD) and a molecular sieve column (Molesieve 5A, mesh 80/100, 6 ft X 1/8 in). Argon was used as carrier gas at a flow rate of 30 mL/min. The temperatures of the column and the TCD detector were 90 and 105 °C, respectively. The concentrations of volatile fatty acids (VFAs) were analyzed using a gas chromatograph (Varian 8500, Varian Inc., Toronto, Canada) with a flame ionization detector (FID) equipped with a fused silica column (30 m × 0.32 mm). Helium was used as carrier gas at a flow rate of 5 mL/min. The temperatures of the column and detector were 110 and 250 °C, respectively. Lactic acid concentrations were measured using a high-performance liquid chromatography system (1200 series, Agilent Technologies) equipped with Aminex HPX-87H ion exclusion column (300 mm × 7.8 mm I.D.; BIO-RAD), and a UV-detector at 210 nm. The column temperature was adjusted to 30 °C. The same instrument with a refractive index detector (RID) was used to measure the concentrations of glucose. The temperature of the RID detector was set to 35 °C. The amount of volatile suspended solids (VSS) and chemical oxygen demand (COD) were measured according to standard methods [13]. Particle size distribution was determined by Malvern Mastersizer 2000 (version 5.22) laser beam diffraction granulometer.

2.4. *Microbial community analysis*

For OLR-1, 2, 5 and 6 biomass samples were collected during the last week of steady-state operation. The total genomic community DNA was extracted using UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) and after PCR amplification were analyzed by denaturing gradient gel electrophoresis (DGGE). The primer sets of 357FGC (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGC-CTACGGGAGGCAGCAG-3′) and 518R (5′–ATTACCGCGGCTG-CTGG-3′) at the annealing temperature of 53 °C were used for the PCR amplification of the variable V3 region of 16S rDNA from the purified genomic DNA. Denaturing gradient gel electrophoresis (DGGE) of PCR products was performed with a DCode universal mutation system (Bio-Rad laboratories, Hercules, CA, USA). The PCR products were applied directly to 8% (w/v) polyacrylamide gel with 15%–55% denaturant gradients. Electrophoresis was performed at a constant voltage of 130 V at 58 °C for 5 h. The DNA templates of the bands of interest were re-amplified and the PCR products were purified using QIAquick PCR purification Kit (QIAGEN Sciences, Maryland, USA) in accordance with the manufacturer's protocol. The sequences of re-amplified DNA fragments were determined by dideoxy chain termination (Sequencing Facility, John P. Robarts Research Institute, London, Ontario) and compared with available sequences in GenBank database using the BLAST program [14].

3. Results

Fig. 2 (a and b) shows the diurnal variation of hydrogen production rate and yield (based on the amount of glucose converted). Although steady-state was observed in all runs after 3–7 days of startup, the systems were kept in operation at steady-state for 55–75 days. The systems showed stable hydrogen production during the experimental period. The coefficient of variation (calculated as standard deviation divided by the average) for hydrogen production rate and yield in all runs was approximately less than 10%. As summarized in Table 2, the two integrated biohydrogen reactor clarifier system (IBRCS) were operated at OLRs of 6.5 gCOD/L-d and 25.7 gCOD/L-d for 55 days in steady-state. The hydrogen production rate averaged 12 L/d and 48 L/d for OLR-1 and OLR-2, respectively. The two IBRCSs were then restarted and tested under OLRs of 51.4 gCOD/L-d and 103 gCOD/L-d. The operational period was extended for a period of 75 days. The hydrogen production rate increased to 97 L/d and 179 L/d for OLR-3 and OLR-4, respectively. The glucose conversion in the system under the three OLRs was almost 100% and decreased to approximately 95% at OLR-4.

Fig. 3 depicts the steady-state volumetric hydrogen production and molar yields, calculated based on the data of the last 55 days for OLR-1 and 2, and 75 days for OLR 3–6. As illustrated in Fig. 3a linear increase in the hydrogen production rate with the increase of the OLR was observed up to 103 gCOD/L-d. On the other hand, the hydrogen yield of 2.8 mol H_2/mol glucose was almost constant during the same range of OLRs. To determine the optimum OLR that maximizes hydrogen production, the systems were restarted under an OLRs of 154 gCOD/L-d and 206 gCOD/L-d. The average hydrogen yields after 75 days of steady-state operation were 1.2 mol H_2/mol glucose and 1.1 mol H_2/mol glucose for OLR-5 and OLR-6, respectively. The increase in OLR not only decreased the hydrogen yield but also the hydrogen production rate dropped to approximately 65 L/d (13 L/L/d). The hydrogen content in the biogas was around 72% in OLR-1 and 2, 66% in OLR-3 and 4, and 42% in OLR-5 and 6 with the balance in all cases CO_2. It is apparent from Fig. 3 that the maximum OLR at the system HRT of 8 h in terms of hydrogen production is 103 gCOD/L-d.

The biomass concentration in the reactor is an important operational parameter that affects both system stability and hydrogen yield. The average concentration of VSS in the biohydrogen reactor increased ten-fold from 1.5 g/L to 15.7 g/L with the increase in OLR from 6.5 gCOD/L-d to 103 gCOD/L-d. In OLR-5 and OLR-6 the concentrations of VSS were 18.4 g/L and 17 g/L, respectively (see Table 3). Using steady-state data

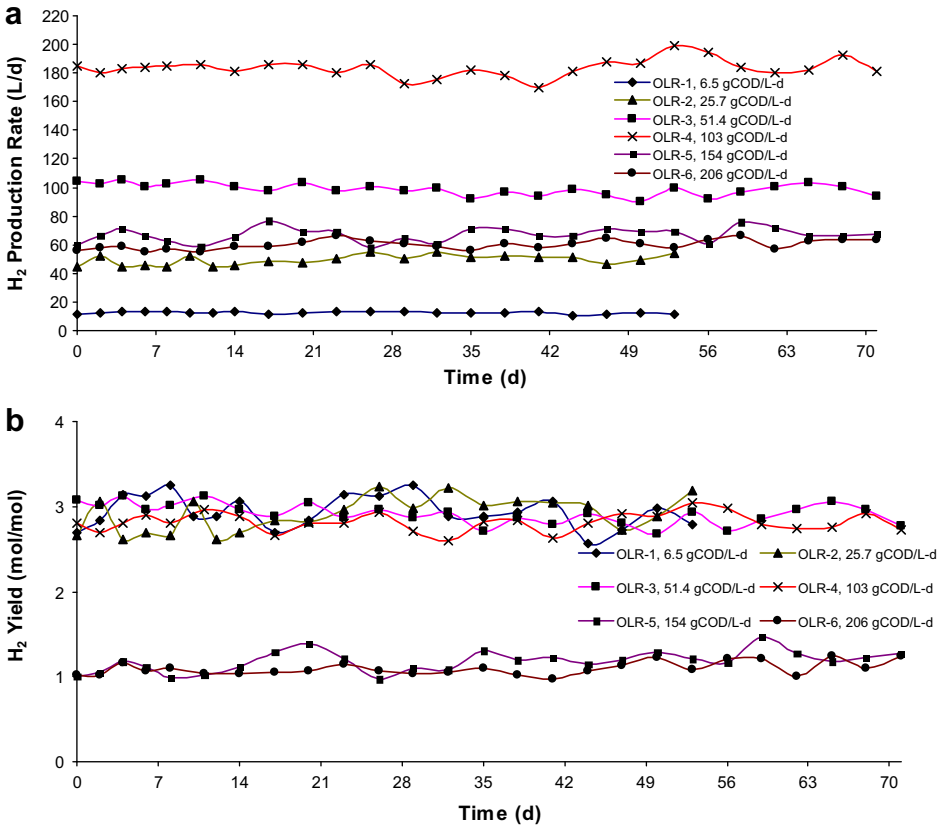

Fig. 2 – Diurnal variation for: a) hydrogen production rate, b) hydrogen yield.

for both VSS (g/L) and hydrogen production rate (L/d) at each OLR, the biomass specific hydrogen production rate was calculated. During the first four OLRs i.e. 6.5 to 103 gCOD/L-d, the average biomass hydrogen production rate was 2.1 ± 0.3 L H_2/gVSS-d and the average food-to-microorganisms' ratio (F/M) was 5.7 ± 0.9 gCOD/gVSS-d. When the OLR was increased to 154 gCOD/L-d and 206 gCOD/L-d the biomass specific hydrogen production rate dropped to 0.7 L H_2/gVSS-d with average F/M ratios of 8.5 and 12.1 gCOD/gVSS-d, respectively.

The COD mass balances for the six runs, computed considering the measured influent and effluent CODs, and the equivalent CODs for both gas and biomass are shown in Table 3. The closure of COD balances at 96%–109% validates the reliability of the data. During the first four OLRs acetate and butyrate were the main liquid products with trace concentrations of ethanol and no detection of lactate, while in the last two OLRs propionate, isovalerate, valerate and ethanol concentrations increased markedly. VFAs accounted for 100%, 100%, 87%, 85%, 47%, and 35% of the bioreactor effluent SCOD in OLR-1 to 6, respectively. Using the stoichiometric yield of 4 and 2 mol H_2/mol glucose from the Eq. (1 and 2), and according to the measured average concentrations of acetate and butyrate, the contribution of the two pathways was estimated.

Table 2 – Summary of steady-state data.

	OLR -1	OLR -2	OLR -3	OLR -4	OLR -5	OLR -6
Total Gas (L)	17 ± 2.7	66 ± 8.0	151 ± 12.4	264 ± 18	155 ± 7.9	153 ± 7.4
Hydrogen Gas (%)	71 ± 0.9	73 ± 2.7	65 ± 3.3	67 ± 2.7	43 ± 2.8	39 ± 1.5
Hydrogen Gas (L/d)	12 ± 1.3	48 ± 4.7	97 ± 5.0	179 ± 12	67 ± 5.0	60 ± 3.4
Hydrogen Gas (L/L/d)	2.4 ± 0.2	9.6 ± 0.9	19.6 ± 0.8	35.6 ± 2.7	13.4 ± 1.0	12 ± 0.7
Yield (mol/mol)	2.8 ± 0.3	2.8 ± 0.3	2.9 ± 0.1	2.8 ± 0.3	1.2 ± 0.1	1.1 ± 0.1
Glucose Conversion (%)	99.9 ± 1.0	99.9 ± 1.5	99.9 ± 1.2	94.8 ± 2.0	56.1 ± 3.3	40.5 ± 2.4
% COD removed	30	30	30	37	20	14
Biomass Yield (gVSS/gglucose)	0.12 ± 0.02	0.09 ± 0.01	0.10 ± 0.01	0.1 ± 0.01	0.21 ± 0.02	0.2 ± 0.02
Specific H2 Production Rate (L/gVSS-d)	1.7 ± 0.2	2.4 ± 0.2	2.2 ± 0.2	2.3 ± 0.2	0.7 ± 0.1	0.7 ± 0.1
F/M (gCOD/gVSS-d)	4.4 ± 0.4	6.2 ± 0.4	5.8 ± 0.6	6.4 ± 0.2	8.5 ± 0.6	12.1 ± 0.6

Note. Values represent average ± standard deviation.

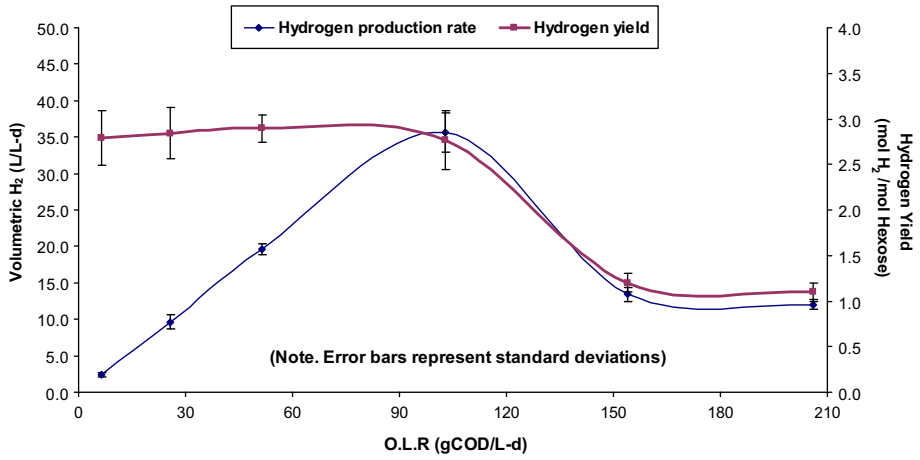

Fig. 3 – Relationship between hydrogen production rate and hydrogen yield versus OLR.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (2)$$

In OLR-1, 65% and 35% of the hydrogen produced were through the acetate and butyrate pathways, respectively. The main liquid products in OLR-2 were acetate and butyrate at steady-state concentrations of 2494 mg/L and 1594 mg/L respectively, with approximately 68% and 32% of the hydrogen yield through the acetate and butyrate pathways, respectively. For OLR-3 and OLR-4, the steady-state acetate concentrations ranged from 4135 mg/L to 7575 mg/L while the butyrate varied from 3008 mg/L to 5035 mg/L, with acetate and butyrate pathways contributing 66%, 34% of the hydrogen production in OLR-3 and 69%, 34% in OLR-4, respectively. Furthermore, in OLR-5 and OLR-6 the acetate concentrations decreased to 4647 mg/L and 4426 mg/L, respectively, and both acetate and butyrate pathways equally contributed to hydrogen production at 50% each.

Since the theoretical hydrogen yield from glucose with acetate formation of 4 mol H_2/mol glucose is twice that of butyrate formation, previous studies indicated that the hydrogen yield increases with the molar ratio of acetate/butyrate [15,16]. Fig. 4 (a and b) shows the diurnal variation of acetate and butyrate concentrations in the bioreactors. The steady-state average molar ratios of acetate/butyrate were 2.3, 2.3, 2.0 and 2.2 for OLRs 1, 2, 3 and 4, respectively, but dropped to 1.1 for OLR-5 and OLR-6.

As depicted in Table 2, the biomass yields for the six runs, calculated as the slope of the cumulative biomass produced versus the cumulative glucose converted, were 0.12, 0.09, 0.10, 0.1, 0.21 and 0.2 for OLR-1 to 6, respectively. It should be noted that biomass production incorporated both the temporal changes in bioreactor mixed liquor volatile suspended solids (MLVSS) and the solids leaving in the clarifier liquid effluent The average observed biomass yield for OLRs 1, 2, 3 and 4 was 0.1 g VSS/g $glucose_{conv.}$ half of the 0.2 g VSS/g $glucose_{conv.}$ observed for OLRs 5 and 6. The inverse relationship between the biomass and hydrogen yields emphatically demonstrates that the higher biomass yield is attributed to different microorganisms other than hydrogen producers. The IBRCS as a new configuration proved to be selectively enriching the hydrogen producers and minimizing the growth of other competitors that decrease the hydrogen yield through decoupling of SRT from HRT. Using Eq. (3) and the biomass yield reported in the literature for hydrogen producers of 0.1 g VSS/g glucose [17], it was estimated that the non-hydrogen producing bacteria constituted about 0–15% of the measured bioreactor VSS at OLR-1, OLR-2, OLR-3, and OLR-4 and approximately 50–60% at OLR-5 and OLR-6.

$$X_v = X_a + X_i = X_{HP} + X_{nHP} + X_i = \theta_c \cdot Y_{obs} \cdot OLR \cdot \eta + X_{nHP} + X_i \quad (3)$$

where X_v is the total biomass, X_a is the active microbial population in the reactor, which in this case consists of hydrogen producers (X_{HP}) and non-hydrogen producers (X_{nHP}), X_i is the inert remains of microorganisms in the reactor, θ_c is solid retention time, Y_{obs} is the observed yield of hydrogen producers, OLR is the organic loading rate and η is the substrate conversion efficiency.

3.1. *Biomass particle size and settling characteristics*

In order to study the effect of the OLRs on the particle size and settling characteristics, five samples from both the biohydrogen reactor and the clarifier liquid effluent were collected during the last 10 days of steady-state operation of each run. As summarized in Table 4, although the mean particle size varied narrowly from 38 μm to 48 μm during OLRs 1–4, it increased slightly to 52 μm in OLR-5 and drastically to 78 μm in OLR-6. The similar particle size in OLRs 1–4 can be attributed to the F/M ratio (5.7 gCOD/gVSS-d) that was almost the same during these runs, while the increase in particle size in the last two runs is accompanied by a significant increase in the F/M ratio (8.5–12.1 gCOD/gVSS-d). This finding contradicts with Zhang et al. [5] who reported an increase in the particle size from 12 μm to 58 μm with the increase in the OLR from 27 gCOD/L-d to 80 gCOD/L-d. To evaluate the settling characteristics of the biomass, both zone settling velocity (ZSV) and sludge volume index

Table 3 – Summary of products and COD mass balance.

Measured parameters	OLR -1	OLR -2	OLR -3	OLR -4	OLR -5	OLR -6
VSS reactor (mg/L)	1489 ± 116	4190 ± 308	8915 ± 972	15703 ± 926	18472 ± 1404	17038 ± 883
VSS out (mg/L)	247 ± 46	744 ± 50	1578 ± 141	3073 ± 397	5565 ± 581	5240 ± 372
VSS out (mgCOD/L) [a]	350 ± 65	1056 ± 71	2241 ± 200	4364 ± 563	7902 ± 825	7441 ± 528
SCOD out (mg/L)	1492 ± 79	6023 ± 194	11922 ± 1230	21267 ± 1627	40960 ± 1624	59091 ± 1358
Acetic (mg/L)	638 ± 105	2494 ± 217	4135 ± 490	7575 ± 509	4647 ± 632	4426 ± 553
Propionic (mg/L)	29 ± 16	132 ± 61	156 ± 29	206 ± 50	584 ± 78	988 ± 257
Isobutyraic (mg/L)	0	0	0	0	0	0
Butyric (mg/L)	398 ± 55	1594 ± 126	3008 ± 471	5035 ± 636	6876 ± 550	6651 ± 729
Isovaleric (mg/L)	6 ± 7	4 ± 3	45 ± 20	71 ± 21	2 ± 3	286 ± 98
Valeric (mg/L)	0	0	0	0	10 ± 6	172 ± 61
Ethanol (mg/L)	14 ± 7	73 ± 46	57 ± 29	123 ± 56	411 ± 56	642 ± 155
Lactic (mg/L)	0	0	0	0	0	0
VFA (mgCOD/L)	1491 ± 87	5924 ± 257	10344 ± 1114	17976 ± 1444	19232 ± 1156	20582 ± 1686
Glucose Out (mg/L)	0	0	0	1670 ± 615	21073 ± 1564	38066 ± 1508
Hydrogen Gas (L/d)	12 ± 1.3	48.1 ± 4.7	97 ± 5	179 ± 12	67 ± 5	60 ± 3.4
Hydrogen Gas (gCOD/d) [b]	7.6 ± 0.8	30 ± 3	61 ± 3.1	113 ± 7.6	42.2 ± 3.1	38 ± 2
COD balance (%)[c]	109 ± 5	106 ± 3	98 ± 6	96 ± 5	101 ± 4	101 ± 2

a Based on 1.42 gCOD/gVSS.
b Based on 8 gCOD/g H_2.
c COD balance (%) = ((VSSout (gCOD/d) + H_2 (gCOD/d) + SCOD out (gCOD/d))/(TCODin (gCOD/d)).

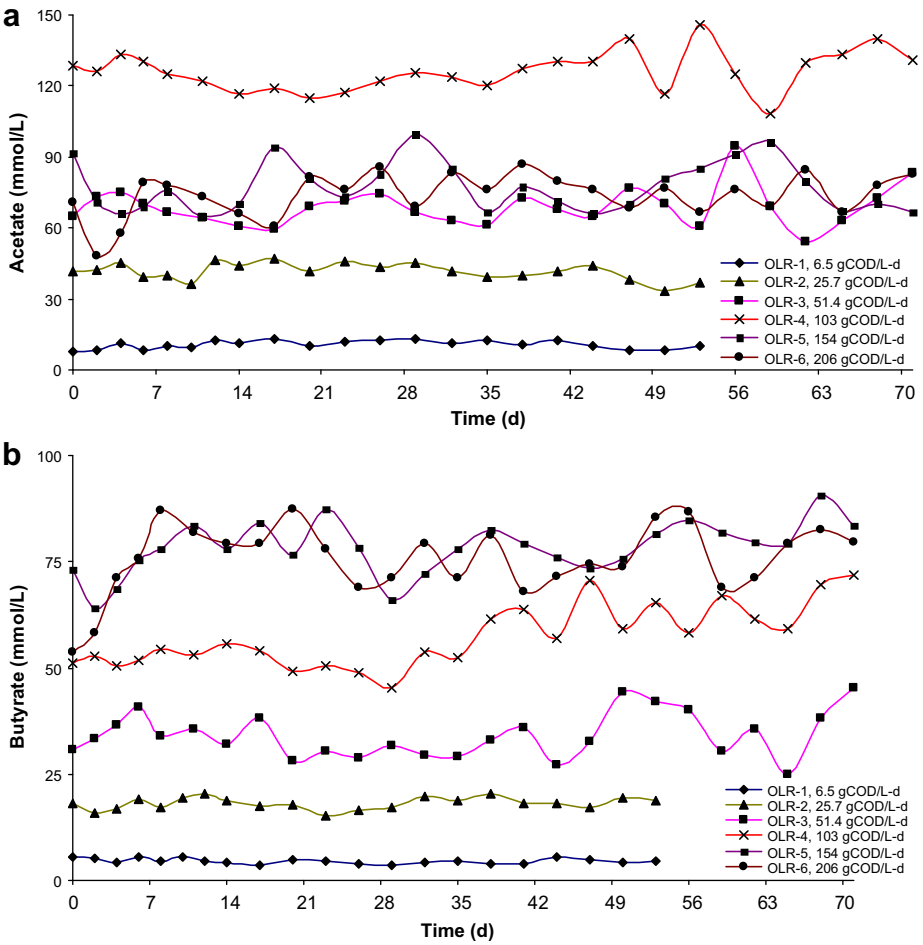

Fig. 4 – Diurnal variation for: a) Acetate, b) Butyrate.

Table 4 – Summary of particle size and settling characteristics data.

	OLR -1	OLR -2	OLR -3	OLR -4	OLR -5	OLR -6
Bioreactor Mean Particle Size (μm)	38 ± 4	48 ± 3	47 ± 4	48 ± 5	52 ± 5	78 ± 8
Clarifier Effluent Mean Particle Size (μm)	8 ± 1	8 ± 0.7	10 ± 3	12 ± 4	15 ± 2	16 ± 3
ZSV (m/d)	120 ± 16	200 ± 24	195 ± 13	190 ± 21	220 ± 26	240 ± 15
SVI (mL/g)	110 ± 12	87 ± 10	92 ± 8	85 ± 16	90 ± 10	82 ± 11

(SVI) were performed on a weekly basis throughout the study. The ZSV ranged from 120–240 m/d and SVI from 82 to 110 mL/g. It is noteworthy that the zone settling velocity increased almost linearly with biomass average particle size up to 52 μm, and stabilized at around 240 m/d thereafter. Similarly SVI decreased linearly with particle size up to 52 μm and stabilized at around 80 mL/g thereafter. The relationship between the ZSV and SVI (not shown) was inversely linear with R^2 of 0.825. The settleability of the hydrogen producers was considered to be superior to activated sludge since SVI of 100 mL/g and ZSV of 100 m/d are considered typical for good settling activated sludge. Furthermore, the consistent settling characteristics in all runs, with ZSV as high as 240 m/d in OLR-6, indicates that the high effluent VSS concentrations of 5565 and 5240 mg/L, in OLR-5 and 6 are primarily due to the clarifier limitations, rather than settling characteristics. Additionally, the improved performance of the IBRCS is not due to physical changes in biomass i.e. preferential settling of hydrogen producers, but is solely attributed to microbial changes resulting from the decoupling of SRT from HRT.

3.2. *Microbial community analysis*

The DGGE profiles of the 16S rDNA gene fragments at four organic loading rates are demonstrated in Fig. 5. Table 5 shows the results of the sequence affiliation. Comparing the results from OLR-1 and OLR-2, revealed that the relatively higher OLR-2 (25.7 gCOD/L-d) resulted in a significant increase in microbial diversity. *Clostridium acetobutyricum* (band A), *Klebsiella pneumonia* (band B) and uncultured bacterium (DQ464539.1) (band F) were the only observed bands at OLR-1. *C. acetobutyricum*, *K. pneumonia* are well known hydrogen producers that have been frequently used for hydrogen production [18,19] or detected as active microorganisms in mixed cultures of hydrogen producing bioreactors [20,21]. The uncultured bacterium DQ464539.1 (band F) had also been reported in an acidophilic ethanol-H_2-coproducing system. At OLR-2 another hydrogen producers including *Clostridium butyricum* (band C), a *C. acetobutyricum* affiliated strain (band D) and *Clostridium pasteurianum* (band E) were detected. High yields of hydrogen have been reported in the literature with *C. butyricum* and *Clostridium pasteurianum* [22,23]. Band G which was available only at OLR-2 identified as an uncultured bacterium (DQ414811.1). This band was 97% similar to a strain which had been reported in a hydrogen production bioreactor by Koskinen et al. [24]. Increasing the OLR to OLR-5 and OLR-6 resulted in formation of different microbial community in the reactors. Although most of the hydrogen producers were also present at OLR-5 and OLR-6 some new bands also appeared with increasing the OLR. Two of these bands which were identified were *Lactococcus sp.* (band H) and *Pseudomonas sp.* (band I). *C. acetobutyricum* (band A) was absent at both OLR-5 and OLR-6. It should be noted that some of the DGGE bands were not identified due to presence of a lot of bands in a small area could also be related to hydrogen producers. The increase in the microbial diversity with the increase in the OLR from 6.5 to 25.7 gCOD/L-d is in agreement with the findings of Luo et al. [9], while at the extremely high OLR-5 and OLR-6 clear microbial shifts *only* were identified.

4. Discussion

The fundamental reasons for the relatively low hydrogen yields of 1.1–1.2 mol H_2/mol glucose in OLR-5 and 6 were investigated. Since in this study, as apparent from Table 1, the

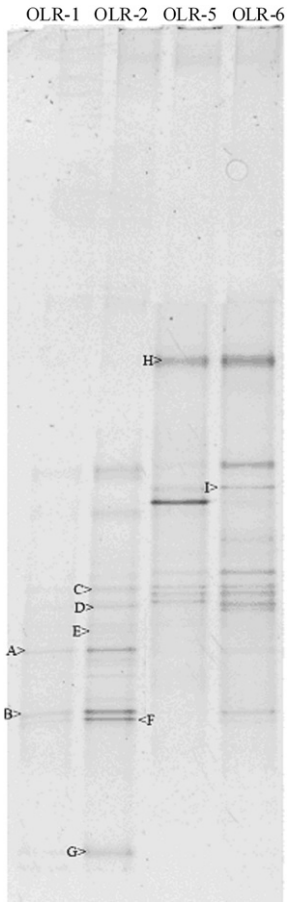

Fig. 5 – DGGE profile of the 16S rDNA gene fragment at four different organic loading rates.

Table 5 – Affiliation of denaturing gradient gel electrophoresis (DGGE) fragments determined by their 16S rDNA sequence.

Affiliation (accession no.)	Bands	Similarity (%)	OLR-1	OLR-2	OLR-5	OLR-6
Clostridium acetobutyricum (FM994940.1)	A	99	×	×		×
Klebsiella pneumonia (GQ214541.1)	B	100	×	×		×
Clostridium butyricum (DQ831124.1)	C	99		×	×	×
Clostridium acetobutyricum (FM994940.1)	D	95		×	×	×
Clostridium pasteurianum (GQ214541.1)	E	99		×		
Uncultured bacterium (DQ464539.1)	F	96	×	×		×
Uncultured bacterium (DQ414811.1)	G	97		×		
Lactococcus sp. (YM05004.1)	H	95			×	×
Pseudomonas sp. (AJ846267.1)	I	96			×	×

pH was maintained in the range of 5.5–6.5 in all runs, the differences in reactor performances can be attributed to substrate inhibition, and product inhibition which affect the fermentation products, predominantly acetate and butyrate. Table 3 clearly reveals that at ORL-5 and OLR-6 of 154 and 206 gCOD/L-d, acetate concentrations averaged 4647 and 4426 mg/L, well below the 7575 mg/L in OLR-4, and therefore the observed reduction in hydrogen yield in OLR-5 and OLR-6 cannot be attributed to acetate inhibition. In fact, Biowin® specifies an acetate inhibition threshold for propionate acidogenesis of 10 000 mg/L. Rodriguez et al. [25] who developed a complex anaerobic mixed culture fermentation model for estimating the stoichiometry of different products from glucose established theoretically that acetate fermentation is conducive for hydrogen production. Substrate inhibition is widely reported in the literature. Rodriguez et al. [25] reported that the modeling of a chemostat reactor operating at an HRT of 8 h and influent glucose concentration at above 0.2 mole glucose (36 g/L i.e. OLR of 115 gCOD/L-d) would shift to ethanol fermentation, with a precipitous drop in hydrogen yield from 2 mol H_2/mol glucose with a biomass concentration of 0.045 M ($C_5H_7O_2N$)/L i.e. 5.13 gVSS/L corresponding to an F/M of 22.5 gCOD/gVSS-d. Wang and Wan [16] reported that in batch tests at 35 °C and initial S/X of 14 g glucose/gVSS, both glucose conversion efficiency and hydrogen yield increased with increasing substrate concentration up to 25 g/L, but decreased thereafter. Kim et al. [20] observed from CSTRs at 12 h HRT, that the molar hydrogen yield decreased at glucose concentrations above 35 gCOD/L (i.e. 70 gCOD/L-d) while Van Ginkel and Logan [4] reported that CSTR at 10 h HRT sustained stable operation up to 40 g/L (100 gCOD/L-d). Thus there is a consensus that the upper limit of substrate concentration without a marked decrease in molar hydrogen yield is around 30 g/L [26]. The marked decrease during OLR-5 and OLR-6 in molar hydrogen and specific hydrogen production at glucose concentrations and OLRs greater than 32 g/L and 103 gCOD/L-d, coupled with the observation of average *residual* (not influent) glucose concentration of 21 073 and 38 066 mg/L seems to confirm literature findings that substrate inhibition occurs and result in either changes in metabolic pathways or microbial shifts occur at ambient glucose concentrations of 20 g/L and above, clearly suggesting that real-time measurement of reactor glucose concentrations might be a valuable tool in averting glucose inhibition of fermentative hydrogen production.

Fig. 6 showing the correlation of molar hydrogen yield with acetate/butyrate molar ratios corroborates that indeed low molar hydrogen yields are associated with low acetate-to-butyrate ratios in the range of 0.8–1.3 whereas acetate-to-butyrate ratios of 2–3 result in high molar hydrogen yields. The literature is contradictory on the acetate and butyrate molar yields and their impact on hydrogen yield. Both Thong et al. [15] who did a comparative batch experiments on hydrogen production performance observed that the hydrogen yield increased from 0.3 to 2 mol H_2/mol glucose with the molar acetate-to-butyrate ratio in the range of 0.3–1.2. Wang and Wan [16] observed that at initial concentrations of 1–25 g/L when hydrogen production peaked, acetate-to-butyrate ratios were >1. Rodriguez et al. [25] established through modeling that at hydrogen yields of < 2.5 mol H_2/mol glucose, the butyrate concentration is greater than the acetate concentration, while at hydrogen yields above 2.5 mol H_2/mol glucose acetate concentration is greater than butyrate. Varma et al. [27] who ran a CSTR at influent glucose concentrations of 10, 20, 40 and 50 g/L corresponding to OLR of 40, 48, 160 and 92 gCOD/L-d, respectively observed that although hydrogen yield in general increased with increasing acetate and butyrate concentrations, butyrate was at all times higher than acetate. Furthermore, increased acetate production was not associated with increased hydrogen production due to homoacetogenesis, whereby CO_2 and H_2 are converted to acetic acid [20,28].

Despite the extensive research in fermentative biohydrogen production, there is sparsity of information related to two important operational and design parameters namely, the food-to-microorganisms ratio (F/M), and the relationship between hydrogen yield and biomass yield as most of the literature is focused on molar hydrogen yield, biomass specific hydrogen production rate, and volumetric hydrogen production rate. In aerobic systems, biomass yields are high enough to sustain biomass growth and the relationship between the main gaseous product (CO_2) and biomass yield has not been of

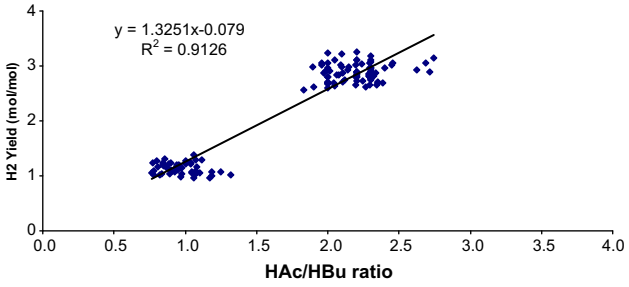

Fig. 6 – Relationship between molar hydrogen yield and acetate/butyrate molar ratios.

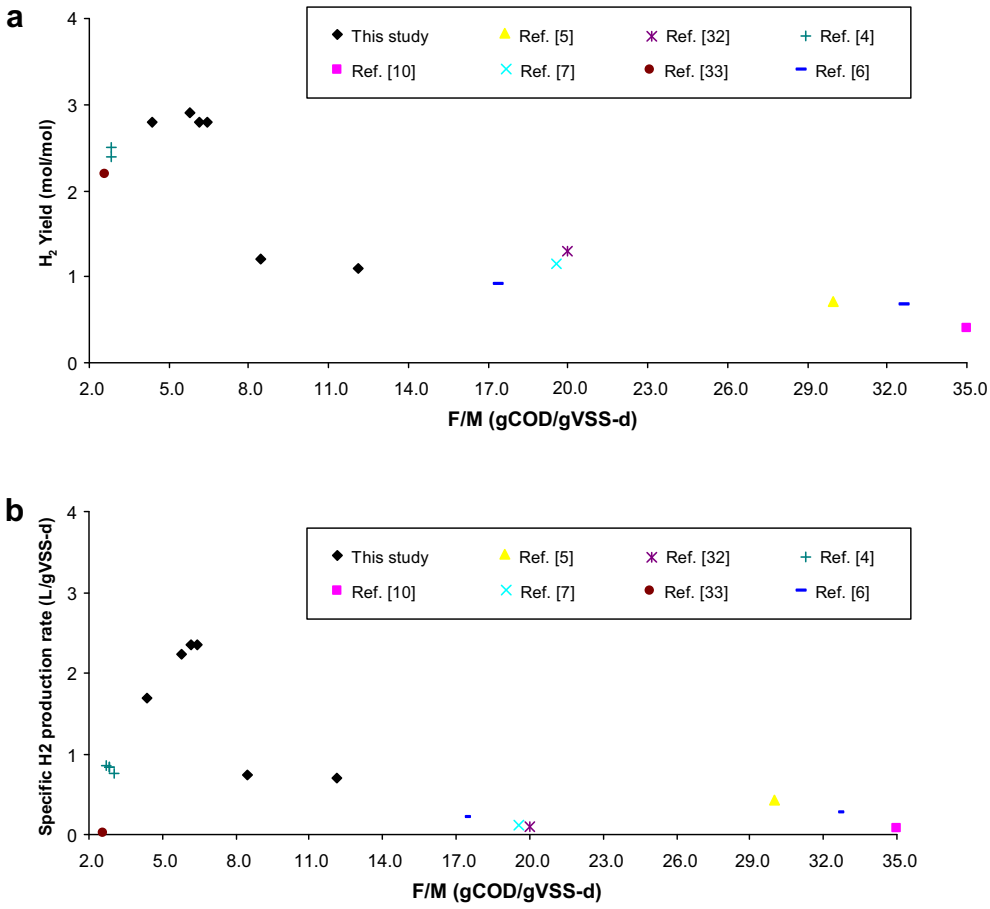

Fig. 7 – Relationship between: a) F/M ratio and Hydrogen yield, b) F/M ratio and Biomass specific hydrogen production rate.

interest, until recently, with the inception of carbon credits. In anaerobic systems, the low biomass yields are compensated for by the long system SRTs, a strategy that proved to be futile for biohydrogen production in chemostat reactors due to contamination with methanogens.

Figs. 7a and b illustrate the relationship between F/M ratio on one hand and both molar hydrogen yield and biomass specific hydrogen production rate for the six OLRs with the overall averages and standard deviations during steady-state presented in Table 2 as well as seven literature studies [4,5,6,7,10,32,33]. Fig. 7a clearly demonstrates that, based on this and literature studies, the molar hydrogen yield increased from 2.2 mol H_2/mol glucose at F/M ratio around 2 and peaked at 2.8 mol H_2/mol glucose at F/M ratio of 6.4 gCOD/gVSS-d, followed by a precipitous drop to around 1.0–1.2 mol H_2/mol glucose over a wide range of F/M ratios from 8.5 to 20 gCOD/gVSS-d, with hydrogen yields dipping below 0.8 mol H_2/mol glucose at F/M ratios of 30 gCOD/gVSS-d and above. It is interesting to note that the seven literature studies compared in Fig. 7a seem to agree with the general trend of this study. It is thus evident that to optimize hydrogen production, the bioreactor F/M ratio should be in the rage of 4.4–6.4 gCOD/gVSS-d. The specific hydrogen production rate depicted in Fig. 7b seems to confirm to the same pattern of a mild increase to a maxima followed by a sharp decline and stabilization over a wide range of F/M ratios. Once again, the specific hydrogen production rate peaked at 2.4 L/gVSS-d at F/M ratio of 6.2 gCOD/gVSS-d, consistent with the molar hydrogen yield. Furthermore, if the F/M was calculated on the basis of glucose converted rather than influent, denoted henceforth as (F'/M), an interesting observation is readily discernible. While for OLR-2, OLR-3 and OLR-4 F'/M is around 6.0 gCOD/gVSS-d, F'/M for OLR-1, OLR-5 and 6 are 4.4, 4.6 and 4.9 gCOD/gVSS-d, respectively. Thus despite operating at approximately similar F'/M, molar hydrogen yield and biomass specific hydrogen production in OLR-5 and OLR-6, were 58% lower than in OLR-1. This clearly emphasizes that not only did the glucose conversion efficiency drop by 44% and 60%, respectively in OLR-5 and OLR-6 relative to OLR-1 to 4 but also that the glucose fermentation followed different pathways in the presence of excess (unconverted) glucose of around 21 073 and 38 666 mg/L (Table 3) in OLR-5 and OLR-6. This clearly emphasizes the importance of the F/M for biohydrogen production, with bioreactor performance deteriorating sharply at F/M >6.4. Recalculating the F/M ratios based on only the hydrogen producers estimated above, in accordance with Eq. (3), as F/M' clearly emphasizes that the F/M' ratios of 17 and 24 gCOD/gVSS-d are multiples of the 8.5 and 12.1 gCOD/gVSS-d observed for OLR-5 and 6, highlighting the gross overloading of hydrogen producers.

Screening of the biomass yield data in Table 2 reveals that the yield for OLR-5 and OLR-6 was twice that of OLR-1 through 4, which implies that in principle the bioreactors in OLR-5 and OLR-6 should have produced more biomass, in effect decreasing the F/M ratio. However, comparing the bioreactor VSS in OLR-4, OLR-5

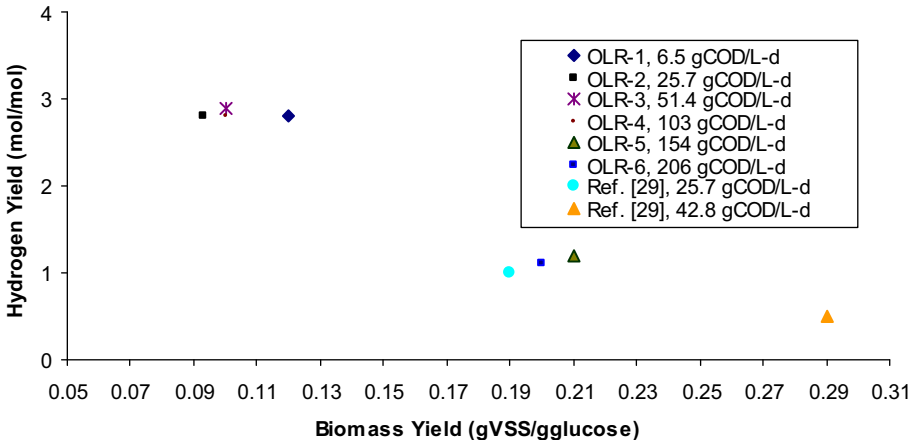

Fig. 8 – Relation between the biomass yield and the hydrogen yield.

and OLR-6 clearly indicates that the biomass in OLR-5 and OLR-6 were marginally (8.5% and 17.5% on average basis) than in OLR-4. Thus, it is postulated that higher reactor biomass concentrations in OLR-5 and OLR-6 would have inevitably improved overall performance i.e. glucose conversion efficiency, hydrogen yield and biomass specific hydrogen production.

Fig. 8 shows the inverse relationship between the biomass and hydrogen yields using the results reported in this study and values from two CSTRs operated at OLRs of 25.7 and 42.8 gCOD/L-d and HRTs of 8 h and 12 h reported in a previous study [29]. The hydrogen yield was around 2.8 mol H_2/mol glucose when the average biomass yield was 0.1 g VSS/g glucose, while hydrogen yields of 1.1 and 0.5 mol H_2/mol glucose corresponded to average biomass yields of 0.2 and 0.3 g VSS/g glucose, respectively. Comparing both the biomass yield and hydrogen yield in the CSTR and IBRCS at an OLR of 25.7 gCOD/L-d reveals that the IBRCS hydrogen yield of 2.8 mol H_2/mol glucose was 160% higher than the CSTR with a biomass yield of 0.09 g VSS/g glucose which was 50% lower. Similarly, despite the comparability of OLR of 51.4 gCOD/L-d (OLR-3) with the 42.8 gCOD/L-d in the CSTR, the hydrogen yield in the IBRCS was much higher while the biomass yield was markedly lower than the CSTR. These results emphatically demonstrate that the OLRs did not significantly affect the biomass yield while the increase in biomass yield could be attributed to the relatively high F/M ratios. Thus, it appears that at high F/M ratios a microbial shift occur leading to an increase in the biomass yield which is not related to hydrogen producers that are characterized by low biomass yields (0.1 g VSS/g glucose) [17]. While it is arguable that the biomass yield is indeed a stoichiometric parameter and should remain constant, the results of this study confirm the findings of the experimental data from CSTRs operated at influent glucose concentrations of 10–50 g/L and HRTs of 6–13 h that point to a variable biomass yield Varma et al. [27], and also corroborate the modeling work of Rodriguez et al. [25]. However, although the two aforementioned studies indicate a simultaneous increase in biomass and hydrogen yields, the results of this study indicate that an inverse relationship exists between biomass and hydrogen yields. The importance of this disparity in biomass and hydrogen yields and its influence on hydrogen bioreactor design must be stressed, as the positive relationship does not reflect the challenge of this opposing trend in that the former suggests that sufficient biomass can be generated readily to maximize hydrogen production while the later concept rationalized a more delicate balance and trade-off that has to be maintained.

While SRTs were in the range of 42–50 h on average in OLR-1 to 4 (Table 1), SRTs were only 27 h and 26 h in OLR-5 and 6 due to biomass washout and clarifier limitations. The criticality of decoupling HRT from SRT in biohydrogen production need not be emphasized, as literature studies indicated that short HRTs of 3–8 h despite being conducive to hydrogen production result in biomass washout [30] while long HRTs of 1–2 d diminished the hydrogen due to methanogenesis [31]. In our previous work the decoupling of SRT from HRT using clarification has been very successful in preventing methanogenesis and producing a stable hydrogen yield of 2.8 mol/mol from ethanol fermentation waste product [12].

Biohydrogen production is indeed a very sensitive process, in that it requires careful balancing of various parameters, namely HRT, SRT, OLR and F/M. Design of biohydrogenator reactors is further complicated by the inverse relationship of hydrogen yield and biomass yield, wherein to optimize hydrogen production, the relatively low biomass yield is pertinent, but a low yield translates to low biomass concentrations that increase the F/M ratio which adversely impact hydrogen production. On the other hand, high biomass yields are usually concomitant with low hydrogen production, and may be indicative of drastic changes in microbial communities. Biohydrogen reactor systems must thus be designed not only to facilitate decoupling of SRT from HRT and F/M control but also to operate within a very narrow range of process parameters, with respect to influent loadings, and volatile acids, emphasizing the need for pre-equalization in highly variable industrial applications.

5. Summary and conclusions

Based on the findings of this study within the range of OLRs investigated (6.5–206 gCOD/L-d) and at HRT of 8 h and SRT of 1–2 d, the following conclusions can be drawn:

- The optimum volumetric hydrogen production rate occurred at an OLR of 103 gCOD/L-d

- Molar hydrogen yield remained relatively stable at 2.8 mol H_2/mol glucose at OLRs in the range of 6.5–103 gCOD/L-d, but declined rapidly thereafter to 1.1 mol H_2/mol glucose.
- Molar hydrogen yield correlated linearly with the acetate-to-butyrate molar ratios, and was mostly around 1 and 2.8 mol H_2/mol glucose at acetate-to-butyrate ratios ranging from 0.8 to 1.3 and 2 to 3, respectively.
- Glucose conversion decreased drastically from 995 at OLRs of 6.5–103 gCOD/L-d, to only 56% and 40% at OLRs of 154 and 206 gCOD/L-d, not due to acetate inhibition, but primarily due to residual glucose concentrations of 21 000 and 38 000 mg/L.
- Analysis of impact of F/M ratio on molar hydrogen yield and biomass specific hydrogen production rate clearly showed that the optimum F/M ratio for biohydrogen reactors is 4.4–6.4 gCOD/gVSS-d.
- The stoichiometric biomass yield for hydrogen producers not only was variable but also exhibited a negative correlation with the hydrogen yield contradictory to some literature, due to microbial shifts.
- The biomass yield was not significantly impacted by the OLR, but by the F/M ratio, with the best hydrogen production at a biomass yield of 0.1 g VSS/g glucose.
- Microbial community analysis on OLR-1 and OLR-2 showed the predominance of hydrogen producers such as *C. acetobutyricum, K. pneumonia, C. butyricum, and C. pasteurianum*. While at extremely high OLRs of 154 and 206 gCOD/L-d, a microbial shift was clearly evident due to the coexistence of the non-hydrogen producers such as *Lactococcus sp*. and *Pseudomonas sp*.

This study indicates that due to the trade-off between biomass and hydrogen yields, and the criticality of maintaining F/M ratio within a narrow range of 4.4–6.4 gCOD/gVSS-d, decoupling of the system SRT from HRT is not only beneficial but necessary. Thus, an intricate relationship between the various process design parameters i.e. HRT, SRT, OLR, F/M, biomass and hydrogen yields exist, practically that inhibition of glucose conversion that can basically impact hydrogen yield may occur at *residual* glucose concentrations of >21 g/L.

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