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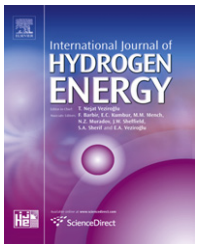

Bio-hydrogen production from thin stillage using conventional and acclimatized anaerobic digester sludge

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ABSTRACT

To assess the viability of biohydrogen production from thin stillage, a comparative evaluation of anaerobic digester sludge (ADS) and acclimatized anaerobic digester sludge (AADS) for biohydrogen production over a wide range of S^0/X^0 ratio (0.5–8 gCOD/gVSS) was performed. A maximum hydrogen yield of 19.5 L H_2/L thin stillage was achieved for the AADS while tests with ADS achieved a maximum yield of only 7.5 L H_2/L thin stillage. The optimum range of S^0/X^0 ratio for hydrogen production was found to be 1 to 2 gCOD/gVSS using conventional ADS and 3 to 6 gCOD/gVSS using AADS. The biomass specific hydrogen production rate for the AADS was 3.5 times higher than rate for the ADS throughout the range of S^0/X^0 ratio examined in this study. The DGGE profiles of the 16S rDNA gene fragments confirmed the superior performance of the AADS over the ADS, showing that the widely known hydrogen producers *Clostridium acetobutyricum*, *Klebsiella pneumonia*, *Clostridium butyricum* and *Clostridium pasteurianum* were the predominant species.

1. Introduction

Hydrogen production from renewable substrates is rapidly emerging as an alternative to fossil fuels, since it has triple the energy yield of hydrocarbon fuels [1] and produces only water with no CO, CO_2, hydrocarbons, or fine particles when combusted [2]. Hydrogen can be produced in many ways: electrolysis, photolysis, bio-photolysis, photo-fermentation, or dark fermentation. Fermentative technology is well established, and the co-products in dark fermentative hydrogen production are valuable (e.g. organic acids). Hence, dark fermentation is the most commonly used method in biological hydrogen production, especially when combined with waste treatment [3].

Thin stillage, the main by-product of the fermentation process in a conventional ethanol plant, is a strong candidate for biological hydrogen production. It is characterized by high chemical oxygen demand (COD) of up to 100 g/L, volatile solids (VS) of 60 g/L [4], volatile suspended solids (VSS) of 21 g/L, volatile fatty acids (VFAs) of 1.31 g/L [5], and total carbohydrates of 65% (based on dry mass) [6]. In a conventional ethanol plant, a portion of the thin stillage is re-circulated back to fermentation tanks in order to minimize waste discharge. The recirculation of thin stillage reduces water intake and subsequently waste disposal, increases corn processing capacity, and reduces nutrient and buffer requirements [7]. The main concern with thin stillage recirculation without any treatment is the accumulation of fermentation

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inhibitors (acetate, lactate, glycerol and ethanol) in the fermentation tank [8]. Therefore, treating thin stillage could facilitate the maximization of recirculation rates by improving its characteristics.

In the context of biohydrogen, the high suspended solids concentration of thin stillage is problematic, as it may necessitate long contact times to hydrolyze particulate carbohydrates. The optimum hydraulic retention time (HRT) for biohydrogen production ranges from 4 to 8 h [9,10]. Furthermore, the food-to-microorganisms (F/M) ratio is a critical parameter that affects hydrogen production with hydrogen yield increasing linearly at F/M ratios of 4–6.6 gCOD/gVSS.d [10]. For particulate wastes, the computation of F/M ratio is complicated as the VSS impacts both the food and microorganisms calculations. It is thus not surprising that given the challenges of biohydrogen production from thin stillage, searches on Google Scholar, Scifinder, and Engineering Village data bases with keywords "thin stillage, biohydrogen production, and particulate waste" revealed that no previous work has been conducted on hydrogen production from thin stillage. Furthermore, as apparent from Table 1 there are only a handful of studies on biohydrogen production from particulate wastes [11–14].

For batch experiments [11–25] the initial substrate concentration (S^0) represents the carbon and energy source for biosynthesis requirements and other energy purposes,

Table 1 – Hydrogen production potentials and yields for different S^0/X^0 ratios using different substrates and biomass in batch experiments.

Substrate	Seed	S^0/X^0[a]	H_2 Production Potential (mL)	Max. Hydrogen Yield			Ref.
				mol/mol_{subst}	L/L substrate	mL/$gCOD_{added}$	
Food waste	ADS[b]	1[c]	10				[11]
		2	25				
		3	55				
		4	163				
		5	250				
		6	360				
		7	175				
		8	30				
		9	10				
		10	5				
Food waste	ADS	7.8	70			101	[12]
Rice Winery	ADS			2.14			[13]
PFSS[d]	ADS	0.09		2.64			[14]
Brewery mixture	Grass compost	0.62				10.2	[15]
		1.08				12.8	
		2.12				19.3	
		4				24.9	
		6.4				19.8	
Sucrose	ADS				1.23		[16]
	ADS			3.18			[17]
	ADS			2.59			[17]
	ADS			2.73			[18]
	Compost						[19]
Glucose	ADS	1		3.09			[20]
	Sludge			1.6			[21]
	Sludge compost			2.1			[21]
	Clostridium sp.			2.8			[22]
	Enterobacter cloacae IIT-BT 08			2.2			[23]
	Actinomyces spp.			1.21			[24]
	Clostridium st.			1.17			[24]
	Porphyromonas sp.			1.08			[24]
Arabinose	Clostridium sp. Strain			2.3			[22]
Xylose	Clostridium sp. Strain			2.3			[22]
Cellobiose	Enterobacter cloacae IIT-BT 08			5.4			[23]
Fructose	Enterobacter cloacae IIT-BT 08			1.6			[23]
Cellulose	Sludge compost			2[e]			[25]

a S^0/X^0 ratio calculated based on $gTCOD_{substrate}/gVSS_{sludge}$.
b Anaerobic digester sludge.
c S^0/X^0 ratio was calculated based on $gVS_{substrate}/gVS_{sludge}$ in Ref. [11].
d PFSS: Preserved fruits soaking solution.
e mol/mol hexose.

while the initial biomass concentration (X^0) is the source of microorganisms responsible for substrate utilization [26]. The S^0/X^0 ratio reflects the initial energy level of batch cultivation. There is strong evidence that this ratio directly affects the growth patterns of microorganisms [27]. As apparent from Table 1, the extensive work by Pan et al. [11] indicated that as the value of S^0/X^0 ratio increases from 1 to 6 $gVS_{substrate}/gVS_{seed}$, hydrogen production potential increases then decreases beyond an S^0/X^0 ratio of 6.

The impact of microbial cultures on biohydrogen production from soluble substrates is well documented in the literature is evidenced in Table 1. For example, biohydrogen production from glucose varied from 1.08 mol H_2/mol glucose [24] to 3.09 mol H_2/mol glucose [20]. As expected, and due to lack of data on specific populations, hydrogen yields varied considerably even for a specific substrate/microorganism system, as demonstrated in Table 1. The hydrogen yields from glucose using *Clostridium* species varied from 1.17 mol H_2/mol glucose [24] to 2.8 mol H_2/mol glucose [22].

Typically, the design of biological treatment systems is predicated on batch and continuous-flow studies. For biohydrogen processes, the focus has been predominantly on batch studies due to concerns with long-term stability of continuous-flow systems associated with contamination due to methanogens in the feed. In such cases, batch studies are biased because they are conducted on pre-treated seed biomass as opposed to the enriched cultures that prevail in sustained continuous-flow systems. Pretreatment of anaerobic digester sludge is required primarily to restrain the hydrogen consuming bacteria and enrich the hydrogen producing bacteria, and this can be done by several methods such as heat, acid, base, aeration, or ultrasonication pretreatment [28]. Acclimatization of anaerobic digester sludge to enrich the hydrogen producers in a hydrogen bioreactor, where methanogens are washed out and hydrogen producers become the predominant community in the sludge in continuous-flow systems [10,29], is the most representative microbial culture for assessment of biohydrogen production potential from various substrates. An extensive search in Google Scholar, Scifinder, and Engineering Village data bases using keywords "biohydrogen production, acclimated sludge, acclimatized sludge, anaerobic digester sludge, fermentative hydrogen batches" revealed that no previous work has been conducted on hydrogen production in batch experiments using acclimatized anaerobic digester sludge from a continuous-flow biohydrogen system.

The main objectives of this study are threefold: assessment of the viability of biohydrogen production from thin stillage, comparative evaluation of anaerobic digester sludge (ADS) and acclimatized anaerobic digester sludge (AADS) for biohydrogen production, and determination of the optimal S^0/X^0 ratio and maximum hydrogen production potential.

Table 2 – Raw thin stillage characteristics.

Parameter (mg/L)	Raw thin stillage quality (Av. ± SD)
TS	71500 ± 724
VS	64800 ± 595
TSS	36900 ± 486
VSS	35300 ± 437
TCOD	122000 ± 1400
SCOD	60600 ± 450
TBOD	68600 ± 800
SBOD	20800 ± 3300
TVFAs	12320 ± 860
Glucose	285 ± 10
Soluble Carbohydrates	35200 ± 1200
Total Carbohydrates	41200 ± 1600
NH_3-N	202 ± 6.7
NO_3^--N	16 ± 1.5
pH	3.46
Alkalinity ($CaCO_3$)	Not measured (pH < 4.3)

Table 3 – Volumes of seed and substrate used in bottles.

S^0/X^0	ADS		AADS	
(gCOD/gVSS)	V_t(mL)	V_s (mL)	V_t(mL)	V_s (mL)
0.5	15	235	9	241
1	30	220	16	234
2	50	200	30	220
4	80	170	54	196
6	100	150	73	177
8	120	130	89	161

2. Materials and methods

2.1. Seed sludge

ADS was collected from the primary anaerobic methane digester at Guelph's wastewater treatment plants (Guelph, Ontario, Canada) and used as seed sludge for the first run (sludge from methane reactor). The total suspended solids (TSS) and volatile suspended solids (VSS) concentrations of the ADS were 22.9 and 13.2 g/L respectively. Heat pretreatment for the ADS was conducted by heating the sludge at 70 °C for 30 min [10]. AADS was collected from a continuous-flow biohydrogen system with aforementioned ADS seed. The continuous system ran for 10 days with a flow of 15 L/d, using glucose as a substrate with a concentration of 30 g/L and anaerobic digester sludge as a seed at hydraulic retention time

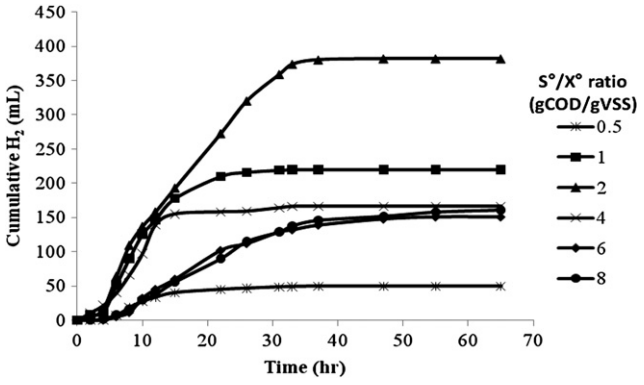

Fig. 1 – Cumulative hydrogen production using ADS.

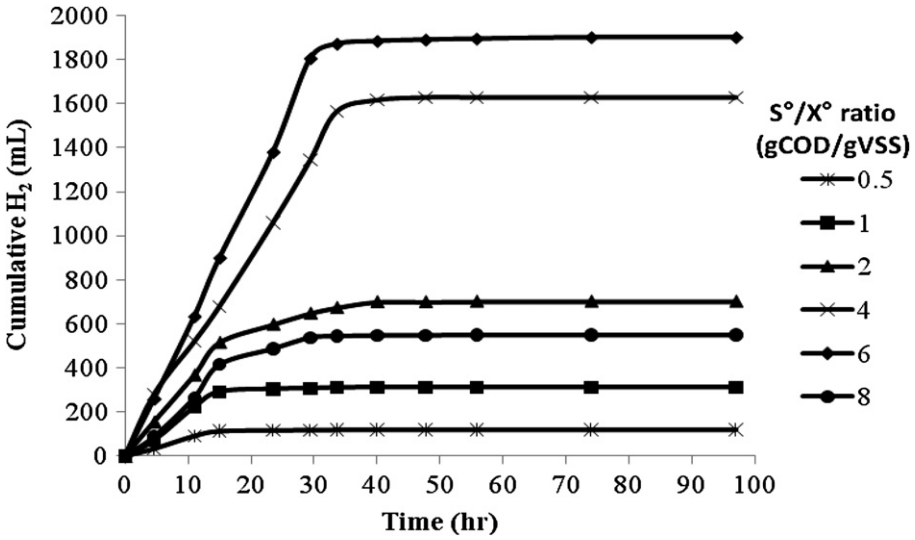

Fig. 2 – Cumulative hydrogen production using AADS.

(HRT) of 8 h and solids retention time (SRT) of 42 h. The TSS and VSS concentrations of the AADS were 10.9 and 9.4 g/L respectively.

2.2. *Microbial community analysis*

Biomass samples for the AADS were collected from the continuous-flow system at the end of the acclimatization period for microbial community analysis. 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). For further details refer to Hafez et al. [10].

2.3. *Raw thin stillage (substrate)*

Raw thin stillage was used as the substrate to assess the hydrogen production rates. Table 2 lists the different characteristics of the raw thin stillage measured in quadruplicates.

2.4. *Batch experiments*

Batch anaerobic studies were conducted in serum bottles with a liquid volume of 250 mL and head space volume of 60 mL. Experiments were conducted in triplicates for initial substrate-to-biomass (S^0/X^0) ratios of 0.5, 1, 2, 4, 6 and 8 $gCOD_{substrate}/gVSS_{seed}$. Volumes of thin stillage and sludge used in batches were calculated using the following equation:

$$S^0/X^0 = \frac{V_t(L) * \text{Thin stillage TCOD}\left(\frac{g}{L}\right)}{V_s(L) * \text{Sludge VSS}\left(\frac{g}{L}\right)}$$

Where V_t is the volume of thin stillage and V_s is the volume of sludge, and Table 3 shows the volumes used in bottles for each S^0/X^0 ratio. The initial pH value for the mixed solution in each bottle was adjusted using HCl and measured to be 5.47 ± 0.04 for both runs. A 5 g/L buffer solution ($NaHCO_3$) was also added for pH control.

Ten milliliter samples of the mixtures were collected initially. The head space was flushed with oxygen-free nitrogen gas for a period of 2 min and capped tightly with rubber stoppers. The bottles were then placed in a swirling-action shaker (Max Q4000, Incubated and Refrigerated Shaker, Thermo Scientific, CA) operating at 180 rpm and maintained at a temperature of 37 ^{0}C. Two control bottles were prepared using ADS and AADS without thin stillage for both runs respectively. Final samples were taken at the end of the batch experiment. The final pH for the mixed solution in each bottle was measured to be 5.05 ± 0.15 for both runs.

2.5. *Analytical methods*

The biogas production was measured using suitable sized glass syringes in the range of 5–100 mL where the gas was released from headspace of the serum bottles to equilibrate with the ambient pressure [30]. 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 (Mole sieve 5A, mesh 80/100, 6 ft × 1/8 in). Argon was used as the carrier gas at a flow

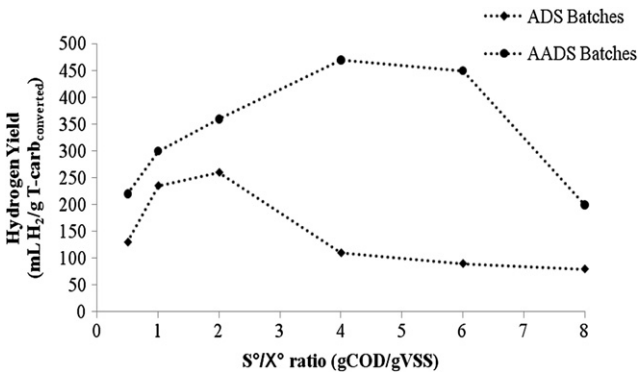

Fig. 3 – Hydrogen Yield.

Table 4 – Summary of initial and final batches data.

	S^0/X^0 (gCOD/gVSS)	$Tcarb_i$[a] (g/L)	$Tcarb_f$[b] (g/L)	Carbohydrates removal (%)	$Tcarb_{converted}$ (g)	$TVFAs_i$[c] (gCOD/L)	$TVFAs_f$[d] (gCOD/L)	Final TVFAs/TCOD (%)	H_2 yield (mL/gTcarb$_{converted}$)	H_2 yield (L H_2/$L_{thin\ stillage}$)
ADS	0.5	2.5	1.0	60	0.38	0.7	2.7	8.6	130	3.3
	1	5.0	1.3	74	0.94	1.5	6.4	20.3	235	7.3
	2	8.4	2.5	70	1.47	2.5	11.8	31.6	260	7.6
	4	13.4	7.4	45	1.51	3.9	12.9	26.8	110	2.1
	6	16.8	10.1	40	1.68	4.9	16.2	27.9	90	1.5
	8	20.2	12.1	40	2.02	5.9	14.5	23.3	80	1.3
AADS	0.5	4.4	2.2	50	0.55	0.4	3.6	9.8	220	8.1
	1	6.4	2.2	65	1.04	0.8	6.8	17.5	300	11.3
	2	10.0	2.2	78	1.95	1.5	11.8	27.6	360	14.0
	4	15.4	1.5	90	3.47	2.7	24.0	47.1	470	19.5
	6	19.2	2.3	88	4.22	3.6	29.5	53.6	450	17.7
	8	22.0	11.0	50	2.75	4.4	21.4	31.5	200	4.4

a Initial total carbohydrates.
b Final total carbohydrates.
c Initial total volatile fatty acids.
d Final total volatile fatty acids.

rate of 30 mL/min and the temperatures of the column and the TCD detector were 90 °C and 105 °C, respectively. Total volatile fatty acids (TVFAs), as well as total and soluble chemical oxygen demand (TCOD, SCOD) were measured using HACH methods and test kits (HACH Odyssey DR/2500 spectrophotometer manual) [31]. TSS and VSS concentrations were analyzed using standard methods [32]. Soluble parameters were determined after filtering the samples through 0.45 μm filter paper.

2.6. Data analysis

Hydrogen gas production was calculated from head space measurements of gas composition and the total volume of biogas produced at each time interval, using the mass balance equation:

$$V_{H,i} = V_{H,i-1} + C_{H,i} * V_{G,i}$$

Where $V_{H,i}$ and $V_{H,i-1}$ are cumulative hydrogen gas volumes at the current (i) and previous (i – 1) time intervals, $V_{G,i}$ is the total biogas volume in the current time intervals, $C_{H,i}$ is the fraction of hydrogen gas in the headspace of the bottle measured using gas chromatography in the current time interval.

3. Results and discussion

3.1. Hydrogen production

Figs. 1 and 2 show the cumulative hydrogen production at different S^0/X^0 ratios for both runs using ADS and AADS, respectively. Standard deviation values were not shown on the curve since the coefficients of variation (calculated as standard deviation divided by the average) in both runs were approximately less that 10%. In the ADS batches as the S^0/X^0 ratio increased from 0.5 to 2 gCOD/gVSS, hydrogen production rapidly increased from 49 mL at S^0/X^0 ratio of 0.5 gCOD/gVSS to a maximum of 386 mL at S^0/X^0 ratio of 2 gCOD/gVSS after which it decreased to 163 mL with further increase in S^0/X^0 ratio. This behavior is consistent with another study [11] that used food waste as a substrate and anerobic digester sludge as the seed, where a wide range of S^0/X^0 ratios from 1 to 10 gVS_{feed}/gVS_{seed} was studied in mesophilic batch fermentation tests. In the aforementioned study, hydrogen production initially increased at high S^0/X^0 ratios and reached a maximum of 357 mL at an S^0/X^0 ratio of 6 gVS_{feed}/gVS_{seed}, then decreased at S^0/X^0 ratios greater than 6 gVS_{feed}/gVS_{seed}. In the AADS batches, the same behavior was observed and a maximum hydrogen production of 1974 mL (5 times the ADS batches) was achieved at an S^0/X^0 ratio of 6 gCOD/gVSS. The type of sludge also affected the biogas composition, with the maximum hydrogen content of the headspace in batches using ADS and AADS reaching 54% and 69%, respectively.

3.2. Hydrogen yields

Fig. 3 shows the hydrogen yield based on the total carbohydrates converted for batches using both ADS and AADS. As depicted in Fig. 3, for the ADS batches, a low hydrogen yield of 130 mL H_2/gT-carb.$_{converted}$ was obtained at S^0/X^0 ratio of 0.5 gCOD/gVSS which is due to insufficient feed, after which

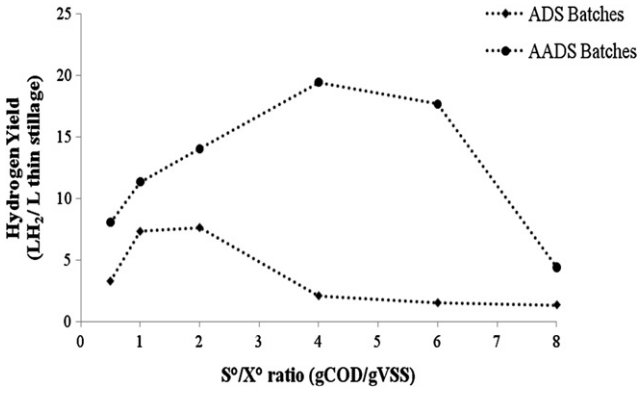

Fig. 4 – Specific Hydrogen Yield.

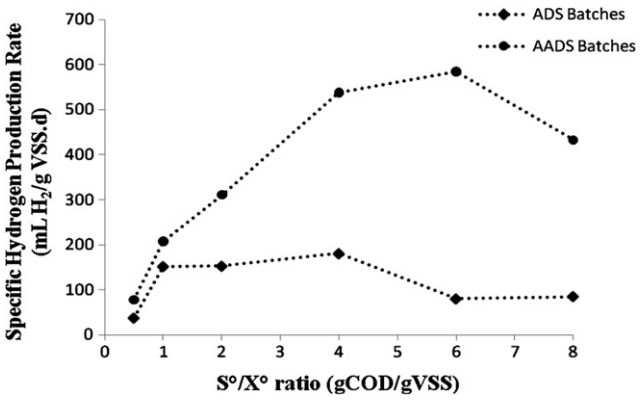

Fig. 5 – Biomass Specific Hydrogen Production Rate.

hydrogen yield stabilized at an average of 248 mL H_2/gT-carb.$_{converted}$ within the S^0/X^0 ratio of 1–2 gCOD/gVSS before declining to an average of 90 mL H_2/gT-carb.$_{converted}$ at S^0/X^0 ratios of 4–8 gCOD/gVSS. On the other hand, the hydrogen yields for the AADS batches followed the same aforementioned trend but the optimum range of S^0/X^0 ratio was 3–6 gCOD/gVSS and a maximum yield of 470 mL H_2/gT-carb.$_{converted}$ was achieved. However, considering the 5% standard deviation of hydrogen gas production, it is likely that the optimum S^0/X^0 range is between 3 and 6 gCOD/gVSS. This trend is similar to that observed by Jinming et al. [11] who used food waste as the substrate and anaerobic digester sludge as the seed, where the hydrogen yield increased slowly to a maximum of 39 mL H_2/gVS at S^0/X^0 ratio of 6 gVS_{feed}/gVS_{seed} prior to decreasing to almost zero at S^0/X^0 ratio of 8 gVS_{feed}/gVS_{seed} and higher. In addition, in another study [12], the same trend was observed in batches using seed sludge from a local anaerobic digester and food waste as the substrate, with a maximum yield of 101 mL H_2/gCOD at S^0/X^0 ratio of 7.68 gCOD/gVSS. The differences in the optimum S^0/X^0 ratios in the literature can be attributed to the differences in the waste type and characteristics as well as the anaerobic digester sludges.

Carbohydrates, which represent 30% of thin stillage (Table 2), play the main role in H_2 production [33]. It is obvious that with the low percentage of hydrogen producers in the ADS, only a part of the carbohydrates in thin stillage was converted to hydrogen with a maximum conversion efficiency of 74% at S^0/X^0 ratio of 1 gCOD/gVSS, while in batches using AADS the carbohydrates conversion efficiency reached 90% at S^0/X^0 ratio of 4 gCOD/gVSS as illustrated in Table 4.

To assess the acidification efficiency, total volatile fatty acids (TVFAs) were measured for both sets of batches. The maximum final TVFAs concentrations were 16.2 gCOD/L and 29.5 gCOD/L for the ADS and the AADS, respectively corresponding to the maximum hydrogen yield and carbohydrates conversion efficiency at an S^0/X^0 ratio of 6 gCOD/gVSS. On the other hand, TVFAs constituted 10% of the TCOD of the raw thin stillage, (Table 2). However, the percentage of TVFAs increased to 27.9% and 53.6% of the TCOD at the end of the batches for ADS and AADS, respectively, at S^0/X^0 ratio of 6 gCOD/gVSS (Table 4).

Fig. 4 shows the relationship between the S^0/X^0 ratio and the ultimate hydrogen yield per liter of thin stillage for batches using both ADS and AADS. As illustrated in Fig. 4, the hydrogen yield per liter of thin stillage followed the same trend as in Fig. 3 with a maximum yield of 7.6 and 19.5 L H_2/L thin stillage at S^0/X^0 ratio of 2 and 4 gCOD/gVSS in batches using ADS and AADS respectively. The much higher observed hydrogen yields, both in terms of per unit waste volume or carbohydrates converted in the AADS relative to the ADS clearly highlighted the limitation of the most widely used approach for assessing biohydrogen production, i.e. using unacclimatized and unenriched ADS in batch studies and emphasize the need for continuous-flow studies.

3.3. *Specific hydrogen production rate*

Fig. 5 shows the specific hydrogen production rate (SHPR) for the six different S^0/X^0 ratios. The maximum SHPR for batches using AADS was 585 mL H_2/gVSS.d at an S^0/X^0 ratio of 6 gCOD/gVSS while in batches using ADS the maximum only reached 181 mL H_2/gVSS.d at S^0/X^0 ratio of 4 gCOD/gVSS. Hafez et al. [10] observed the same pattern of a maximum SHPR followed by a sharp decline at high S^0/X^0 ratio using the authors' data as well as seven other literature studies.

The findings of this study contradicts the observations of Jiunn et al. [34] who studied hydrogen production using organic fraction of municipal solid waste (OFMSW) as the substrate and two types of sludges at different mixing ratios;

Table 5 – Gompertz data for both ADS and AADS batches.

S^0/X^0 (gCOD/gVSS)	ADS				AADS			
	P[a] (mL)	R_m[b] (mL/hr)	λ[c] (hr)	SHPR (mL/gVSS.d)	P (mL)	Rm (mL/hr)	λ (hr)	SHPR (mL/gVSS.d)
0.5	49	4.8	4.5	37	121	11.5	1.7	78
1	220	18.4	3.3	152	311	28.9	2.2	208
2	386	16.8	2.8	153	704	38.9	2.3	311
4	159	16.9	3.6	181	1676	57.9	2.4	538
6	150	6.6	6.1	80	1974	52.1	2.5	585
8	163	6.1	6.1	85	550	33.8	2.6	433

a P: Ultimate hydrogen production.
b R_m: Rate of hydrogen production.
c λ: Lag phase duration.

Table 6 – Summary of COD balance.

	S^0/X^0 (gCOD/gVSS)	$COD_{initial}$ g/L	COD_{final} g/L	COD removed g/L	cumulative H_2 mL	H_2 gCOD/L	COD balance[a] %
ADS	0.5	32.7	30.7	2.0	49	0.14	94
	1	38.4	31.5	6.9	220	0.63	84
	2	46.0	37.3	8.7	386	1.10	84
	4	57.4	48.2	9.2	159	0.48	85
	6	65.0	58.1	6.9	150	0.43	90
	8	72.6	62.1	10.5	163	0.46	86
AADS	0.5	39.0	36.6	2.4	121	0.35	95
	1	43.3	38.8	4.4	311	0.90	92
	2	51.0	42.7	8.3	704	2.02	88
	4	62.7	51.0	11.7	1676	4.68	89
	6	70.9	55.0	14.1	1974	5.46	85
	8	76.9	68.0	8.9	550	1.58	90

a COD balance (%) = [H_2 (gCOD) + COD_{final} (gCOD)]/[$COD_{initial}$ (gCOD)].

Fig. 6 – DGGE profile of the 16S rDNA gene fragments for the AADS.

pre-treated anaerobic digester sludge and acclimatized sludge from a hydrogen producing chemostat reactor with an HRT of 10 h and sucrose as the substrate, and reported no trend for the SHPR with increasing the acclimated sludge percentage.

Table 5 shows the kinetics from the Gompertz model [34] for both batches using ADS and AADS. The coefficient of determination R^2 was 0.999 for all Gompertz data. It is apparent that the lag phase in the AADS batches with an average of 2.3 h is much lower than that in the ADS batches with an average of 4.4 h and this also can be related to the increase in the percentage of hydrogen producers in the AADS relative to the ADS. The maximum hydrogen production rate in batches using ADS was 18.4 mL/h at S^0/X^0 ratio of 1 gCOD/gVSS which is one third the 57.9 mL/h in batches using AADS at S^0/X^0 ratio of 4 gCOD/gVSS. The trend of an increase to the maximum followed by a decline at higher S^0/X^0 ratio is consistent with the findings of Jinming et al. [11] who observed an increase in the hydrogen production rate with the increase of S^0/X^0 ratio to a maximum of 19.5 mL/h at an S^0/X^0 ratio of 5 gVS_{feed}/gVS_{seed}, followed by a decrease with further increase in the S^0/X^0 ratio. A correlation (not shown) of the biomass specific production rate for ADS and AADS (R^2 of 0.72) revealed that over the range of S^0/X^0 ratios that was studied, the active biomass (hydrogen producers) in the AADS is 3.5 times than that of the ADS calculated based on the specific hydrogen production rates for both sludges.

3.4. *COD balance*

COD mass balance data is presented in Table 6. The closure of COD balances at 88 ± 4% verifies the reliability of the data. The percentage average COD reduction was 12 ± 4% for the ADS batches and 16 ± 7% for the AADS batches. COD reduction increased at S^0/X^0 ratios from 0.5 to 2 gCOD/gVSS and reached a maximum of 16% at S^0/X^0 ratio of 2–4 gCOD/gVSS in batches using ADS, and 24% at S^0/X^0 ratio of 4 gCOD/gVSS in batches using AADS after which it decreased at higher S^0/X^0 ratios. As apparent from Table 6, in batches using ADS, although at an S^0/X^0 ratio of 8 gCOD/gVSS, the COD removed was 10.5 g/L (14%), at an S^0/X^0 ratio of 4 gCOD/gVSS the COD removed was 9.2 g/L (16%).

Table 7 – Affiliation of denaturing gradient gel electrophoresis (DGGE) fragments determined by their 16S rDNA sequence.

Affiliation (accession no.)	Bands	Similarity (%)	AADS
Clostridium acetobutyricum (FM994940.1)	A	99	×
Klebsiella pneumonia (GQ214541.1)	B	100	×
Clostridium butyricum (DQ831124.1)	C	99	×
C. acetobutyricum (FM994940.1)	D	95	×
Clostridium pasteurianum (GQ214541.1)	E	99	×
Uncultured bacterium (DQ464539.1)	F	96	×
Uncultured bacterium (DQ414811.1)	G	97	×

3.5. *Microbial community*

The DGGE profiles of the 16S rDNA gene fragments for the AADS are demonstrated in Fig. 6. Table 7 shows the results of the sequence affiliation. The results revealed that *Clostridium acetobutyricum* (band A), *Klebsiella pneumonia* (band B), uncultured bacteria (DQ464539.1) and (DQ414811.1) for bands F and G, respectively, were the main identified bands for the AADS. *C. acetobutyricum and K. pneumonia* are frequently reported as candidates for hydrogen production [10,35–38]. In addition, another hydrogen producers including *Clostridium butyricum* (band C), a *C. acetobutyricum* affiliated strain (band D) and *Clostridium pasteurianum* (band E) were detected. In a continuous system for biohydrogen production, Hafez et al. [10] have shown that high hydrogen yields can be achieved using *C. butyricum* and *C. pasteurianum*.

4. Conclusions

The outcome of this study revealed the importance of using AADS over the conventional ADS in hydrogen batches. It is highly recommended to use acclimatized sludges from a continuous-flow system to assess biohydrogen production from a particular waste as opposed to the most widely used technique of batch studies with pre-treated anaerobic digester sludge. Based on the findings of this study, the following conclusions can be drawn:

- Thin stillage has a potential for hydrogen production with a yield of 19.5 L H_2/L thin stillage with AADS while tests with ADS only revealed a maximum potential of 7.5 L H_2/L thin stillage.
- The optimum experimental range of S^0/X^0 ratio for hydrogen production is 1–2 $gCOD_{substrate}/gVSS_{seed}$ using conventional ADS.
- The optimum experimental range of S^0/X^0 ratio for hydrogen production within the investigated range is 3–6 $gCOD_{substrate}/gVSS_{seed}$ using AADS.
- The biomass specific hydrogen production rate for the AADS was 3.5 times higher than that of the ADS throughout the range of S^0/X^0 ratio that was studied.
- The DGGE profiles of the 16S rDNA gene fragments for the AADS confirmed its superior performance over the ADS where, hydrogen producers such as *C. acetobutyricum*, *K. pneumonia, C. butyricum* and *C. pasteurianum* were the predominant species that were detected.

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