CHAPTER V
BIOLOGICAL HYDROGEN PRODUCTION FROM SWEET SORGHUM SYRUP BY MIXED CULTURES USING AN ANAEROBIC SEQUENCING BATCH REACTOR (ASBR)

5.1 Introduction

Hydrogen is an ideal energy due to its advantages including clean, efficient and non-polluting characteristics [1]. Nowadays, there has been an intensive research interest on biological hydrogen production in order to reduce global environmental concerns and energy insecurity [2]. In the biological process, hydrogen can be produced through dark fermentation from organic substances. Hydrogen production from various substrates, e.g., sugarcane bagasse hydrolysate [3], municipal solid waste [4], starch manufacturing wastewater [5], activated sludge [6] have been reported.

Recent studies on continuously production of hydrogen extensively used continuous stirred tank reactor (CSTR) process [7, 8, 9, 10]. However, the CSTR is difficult to maintain high levels of hydrogen-producing biomass at a short hydraulic retention time (HRT) due to its intrinsic structure [11, 12] that causes operational instability and limits the hydrogen production rate (HPR) [13]. In addition, CSTR system is time-consuming operation with continuous feeding because of a degradation of microorganisms [14, 15]. For that reason, anaerobic sequencing batch rector (ASBR) process has become the reactor option for producing hydrogen due to its advantages. Distinct advantages of ASBR when compared to CSTR system including high biomass concentration, a high degree of process flexibility, no requirement to apply a separate clarifier [16]. Moreover, ASBR system is efficient in operating control, without primary or secondary settling, with high organic removal efficiency and simple operation [17]. The ASBR cycle is composed of four steps which were

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This manuscript was published in International Journal of Hydrogen Energy (2010) In press.

filling, reacting, settling and decanting steps [16]. ASBR has been used to produce hydrogen from various kinds of substrates such as dairy wastewater and chemical wastewater with the maximum HPR of 0.0245 and 0.0089 mmol H_2/min-g COD, respectively [18, 19].

Investigations on important parameters for ASBR operation including solid retention time (SRT), organic loading rate (OLR), cyclic duration and hydraulic retention time (HRT) have been reported. Most of these, HRT is one of the important parameters that determines the economics of the hydrogen production process. The lower HRT attributed to a smaller reactor, cost reduction and therefore enhanced the productivity of the hydrogen production process [20]. Examples of ASBR operation to produce hydrogen including the findings of [21] whom found that ASBR with various HRTs (6-12 hr) used to produce hydrogen from sucrose by non-pretreated microflora gave the hydrogen yield of 0.7-2.6 mol H_2/mol sucrose. Another investigation reported that the maximum hydrogen yield of 0.73-2.5 mol H_2/mol sucrose was obtained when ASBR was operated at 4-13 hr HRTs [22].

Sweet sorghum (*Sorghum bicolor* var. Keller) is a new energy crop that can be used to produce renewable energy i.e. ethanol and hydrogen. It can be cultivated in most of temperate and tropical climate areas [23] and has high sugar productivity which mainly contains sucrose, fructose and glucose. These sugars are good substrates for ethanol [24] and hydrogen [24, 25] productions. Sweet sorghum extract was used as the substrate to produce hydrogen by non-pretreated microflora with the maximum yield of 0.86 mol H_2/mol glucose consumed [24]. Some pure culture *Ruminococcus albus* could produce hydrogen from sugars and sweet sorghum biomass with the maximum yield of 2-2.76 mol H_2/mol glucose [25].

Previous reports on ASBR and hydrogen production from sweet sorghum showed the promise on efficient hydrogen production process in large scale. Therefore, this study was an attempt to continuously produce hydrogen from sweet sorghum syrup based on the information obtained from the batch fermentation [26].

5.2 Materials and Methods

5.2.1 Seed sludge

Anaerobic seed sludge was obtained from a full scale anaerobic digester of Upflow Anaerobic Sludge Blanket (UASB) reactor of the brewery company in Khon Kaen, Thailand. The UASB is used to produce methane from the wastewater of beer production process. Prior to use, the anaerobic sludge was heated at 105 °C for 2 hr to inactivate methanogenic bacteria and then cooled at room temperature in dessicator. For inoculum preparation, the 20 g dry weight pre-treated sludge was cultivated in a 1.0 L glass bottle with a 700 mL working volume at room temperature for 2 days. The enrichment media comprised of the sweet sorghum syrup which was diluted to 20 g/L by sterile filtered water and supplemented with nutrient solution at a rate of 0.5 mL/L [27]. The volatile suspended solid of seed sludge is 5 g VSS/L.

5.2.2 Substrate

Sweet sorghum (*Sorghum bicolor* var. Keller) used as substrate in this study was obtained from the field experiment of Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand. Sweet sorghum syrup was prepared by concentrating sweet sorghum juice by heating to evaporate the water then it was sterilized at 110 °C for 28 min to prevent the contamination. Composition of sweet sorghum syrup was analyzed and showed in Table 1. Total sugar of sweet sorghum syrup was 75-80 °Brix determined by a hand refractometer (HRB-32 ATC) or 800-820 g/L total sugar determined by phenol sulphuric method. The syrup was diluted by sterile filtered water to obtain 25 g/L total sugar before used as the substrate. After dilution, the syrup composition consisted of, in mg/L, 0.94±1.3 acetone, 406±97.5 ethanol, 3.5±4.9 butanol, 9.58±0.7 acetic acid, 0.08±0.1 propionic acid and 8.90±4.6 butyric acid, respectively.

5.2.3 Reactor configuration

The reactor was made from "Acrylic" material with a total volume of 1.3 L (1 L liquid volume, 0.3 L gas holding capacity). Configuration of the reactor was shown in Figure 1. The ASBR was run at room temperature (30±3 °C) which operated in suspended mode using magnetic stirrer (Stuart heat-stir CB162, Keison International Ltd., USA). The feeding, decanting and settling of the ASBR were automatically controlled by digital time controller (TS-ET1, China). Two peristaltic pumps (Eyela roller pump RP-1000, Tokyo Rikakikai Co. Ltd., Japan) were used for transferring the influent and effluent of reactor. During the experiments, 2N NaOH solution was used to maintain pH within 5.0±0.1 using pH meter and controller (pH 190 series, Eutech Instruments, Singapore) while oxidation reduction potential (ORP) was monitored using the same model of pH meter.

Table 1 Sweet sorghum syrup compositions

Parameter	Value
Total COD (g/L)	566-570
Total solid (g/L)	295-300
Total sugar (g/L)	800-820
-Fructose (%)	32.19
-Glucose (%)	35.70
-Sucrose (%)	32.58
Total volatile fatty acid and alcohol (g/L)	25-28
-Acetic acid (%)	0.34
-Propionic acid (%)	-
-Butyric acid (%)	0.41
-Ethanol (%)	78.15
-Butanol (%)	2.25
-Acetone (%)	-

5.2.4 Reactor start up and operation

The reactor was started up by inoculating 100 mL of seed inoculum (equivalent to 500 mg as measured by VSS) into the ASBR containing 900 mL of enrichment medium. Contents in the ASBR were mixed by using magnetic stirrer and the reactor was operated at room temperature (30±3 °C). After 24 hr of reactor operation, 500 mL of the cultured medium was replaced by the fresh enrichment medium and the reactor was operated again for 24 hr. The medium replacement was repeated 5 times before starting the sequencing batch experiment at the 96 hr HRT. The reactor was initially fed with the OLR of 25 g/L-d sweet sorghum syrup containing 1.45 g/L $FeSO_4$ at controlled pH of 4.90-5.0 which was the optimum condition in batch experiment obtained from previous study [26]. Prior to use, the

ASBR was first purged with nitrogen for 15 min to create anaerobic condition. The reactor employed sequencing batch mode operation consisting of 20 min of filling period; 20 min of settling period; 20 min of decanting period and reacting period (varied by HRTs). The initial loading rate was increased stepwise by reducing HRT from 96, 48, 24 and 12 hr. The experimental design conditions in the ASBR system were tested at various HRTs as shows in Table 2. Constant substrate consumption and hydrogen production (±5% variation) were considered as indicators for the steady state conditions. The gas produced was collected daily and the biogas volume was measured by water replacement method.

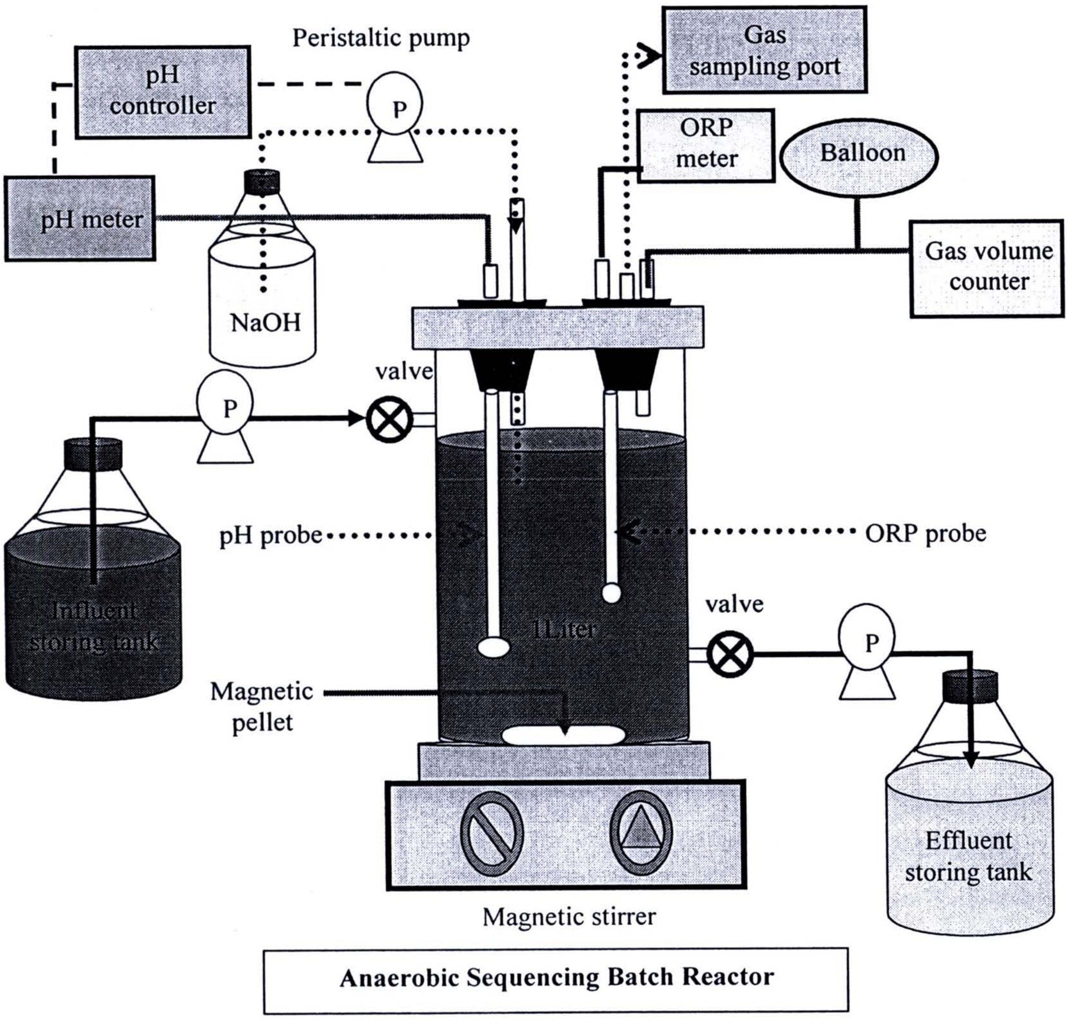

Figure 1 Schematic diagram of anaerobic sequencing batch reactor design (Not subject to scale).

5.2.5 Analytical methods

Biogas composition was measured by a gas chromatography (GC-2014, Shimadzu) equipped with a thermal conductivity detector (TCD) and 2 m stainless column packed with Shin carbon (50/80 mesh). The operational temperatures of the injection port, the column oven and the detector were 100, 120 and 150 °C, respectively. Helium was used as the carrier gas at a flow rate of 25 mL/min. For volatile fatty acids (VFAs), acetone and alcohols analysis, the liquid samples were first centrifuged at 10,000 rpm for 5 min, acidified by 0.2N oxalic acid and filtered through 0.45 μm cellulose acetate membrane. The same GC model with a flame ionization detector (FID) and a 30 m x 0.25 mm x 0.25 μm capillary column (Stabiwax) was used. The temperatures of the injector and detector were 250 °C. The initial temperature of column oven was 50 °C for 2 min followed with a ramp of 15 °C/min for 12.6 min and to final temperature of 240 °C for 1 min. Helium was used as a carrier gas with a flow rate of 66 mL/min. Sugars including fructose, glucose and sucrose were analyzed by a high performance liquid chromatograph (HPLC-SPD 10A, Shimadzu) equipped with a refractive index detector (RID) and Pinacle II Amino (5 μm) column. The column oven temperature was 35 °C. 75% (v/v). Acetonitrile was used as a mobile phase at a flow rate of 1.0 mL/min.

Table 2 ASBR operation

Parameters	HRT (hr)			
	96	48	24	12
Cycle period (hr)	48	24	12	6
Fill period (min)	20	20	20	20
React period (hr)	47	23	11	5
Settle period (min)	20	20	20	20
Decanting period (min)	20	20	20	20
OLR (g total sugar/L-Day)	6.25	12.5	25	50
Fill&Decanting volume (mL)	500	500	500	500

5.2.6 Microbial community analysis

Total genomic DNA was extracted from samples collected from the cyclic study using the Ultraclean Soil DNA Kit (MoBio Laboratory Inc., USA). The region of the 16S rRNA genes corresponding to position 340–518 in the 16S rRNA of *Escherichia coli* was PCR-amplified using the forward primer; L340GCf (50-CCTACGGGAGGCAGCAG-30) with a GC clamp at the 50 end and the reverse primer; K517r (50-ATTACCGCGGCTGCTGG-30) [28]. PCR amplification was conducted in an automated thermal cycler using the following protocol: initial denaturation for 5 min at 94 °C, 30 cycles of denaturation for 1 min at 95 °C, annealing for 30s at 55 °C, extension for 1 min at 72 °C, followed by a final extension for 7 min at 72 °C. The DGGE analysis of the PCR products was performed by electrophoresis for 20 min at 20V and 15 hr at 70V through a 7.5% polyacrylamide gel containing a linear gradient of denaturant (100% denaturant corresponds to 7M urea and 40% (v/v) formamide deionized with AG501-X8 mixed bed resin) ranging from 30% to 60% in 0.5 x TAE buffer at a constant temperature of 60 °C (DGGE unit, V20-HCDC, Scie-Plas Limited, UK). The gel was stained with Sybr-Gold (1000xconcentration) for 1 hr and visualized on a UV transilluminator. Most of the bands were excised from the gel and re-amplified with the same forward primer without a GC clamp and the reverse primer. After re-amplification, PCR products were purified using E.Z.N.A cycle pure kit (Omega Bio-tek, USA) and sequenced using primer K517r and an ABI PRISM Big Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, USA) in accordance with the manufacturer's instructions. Closest matches for partial 16S rRNA gene sequences were identified by database searches in GenBank using BLAST [29].

5.3 Results and discussion

5.3.1 Hydrogen production in ASBR with different HRTs

Continuous hydrogen production was operated in ASBR with different HRTs i.e. 96, 48, 24 and 12 hr. The OLR was increased to 6.25, 12.5, 25 and 50 g/L-d, respectively, when HRTs decreased. The average range of ORP was -271 to -385 mV which confirmed the anaerobic condition in ASBR. Results showed that the variation in HRTs affected metabolic products, which led to variation in hydrogen yield, hydrogen volume, hydrogen production rate and hydrogen content. Maximum hydrogen yield of 0.68 mol H_2/mol hexose consumed (Figure 2a) was obtained at 24 hr HRT, which coincided with the maximum hydrogen volume of 925.76 mL/L (Figure 2b), thus indicated that the optimum HRT was 24 hr.

Hydrogen content increased with an increase in HRT (Figure 2c). However, when HRT was greater than 24 hr, hydrogen yield and hydrogen volume decreased (Figures 2a, 2b, 2d) while hydrogen production rate gradually decreased from HRT 12 hr. Results showed the ranges of hydrogen gas content in the produced biogas was 21.05 to 44.68% (v/v) (Figure 2c) which were comparable to the other studies using ASBR. For example, hydrogen content of 20-35% was obtained when sucrose was used as substrate by acid-enriched sewage sludge microflora in ASBR [22]. Lin and Chou [22] also reported that hydrogen content of 15-35% was obtained from sucrose using non-pretreated microflora. However, the hydrogen content in this study is markedly lower than the results obtained from ASBR operating at short HRTs of 4-18 hr (32.19-63.17%) using starch as the substrate [30]. Variation of hydrogen content depended on substrate, microorganisms and bioreactor system [31]. Figure 2d illustrates the hydrogen production rate at various HRTs. Results showed maximum hydrogen production rate of 365.67 mmol H_2/L-d (Figure 2d) which was slightly higher than hydrogen production rate of 328 mmol H_2/L-d when sucrose was used as substrate [22].

Table 3 summarized the biohydrogen production using ASBR operation in literatures. When compared the hydrogen yield obtained from ASBR using sucrose as the substrate (Table 3), the hydrogen yield obtained from this study was lower but the hydrogen production rate was higher. The decrease in hydrogen yield might be due to the competition of other fermentation pathways in which substrate were used to produce other products such as ethanol, acetic and butyric acids rather than hydrogen [24]. The replacement of 50% (v/v) of fresh medium during ASBR operation could lower the substrate concentration in reactor resulted on a low hydrogen yield obtained. Sairan et al. [32] reported that the decanting/filling volume in ASBR is often limited to about 50% of total volume. In addition, at the lowest point in the decanting cycle, the liquid level should be an adequate distance above the top of the settled sludge to avoid the discharge of settled solids.

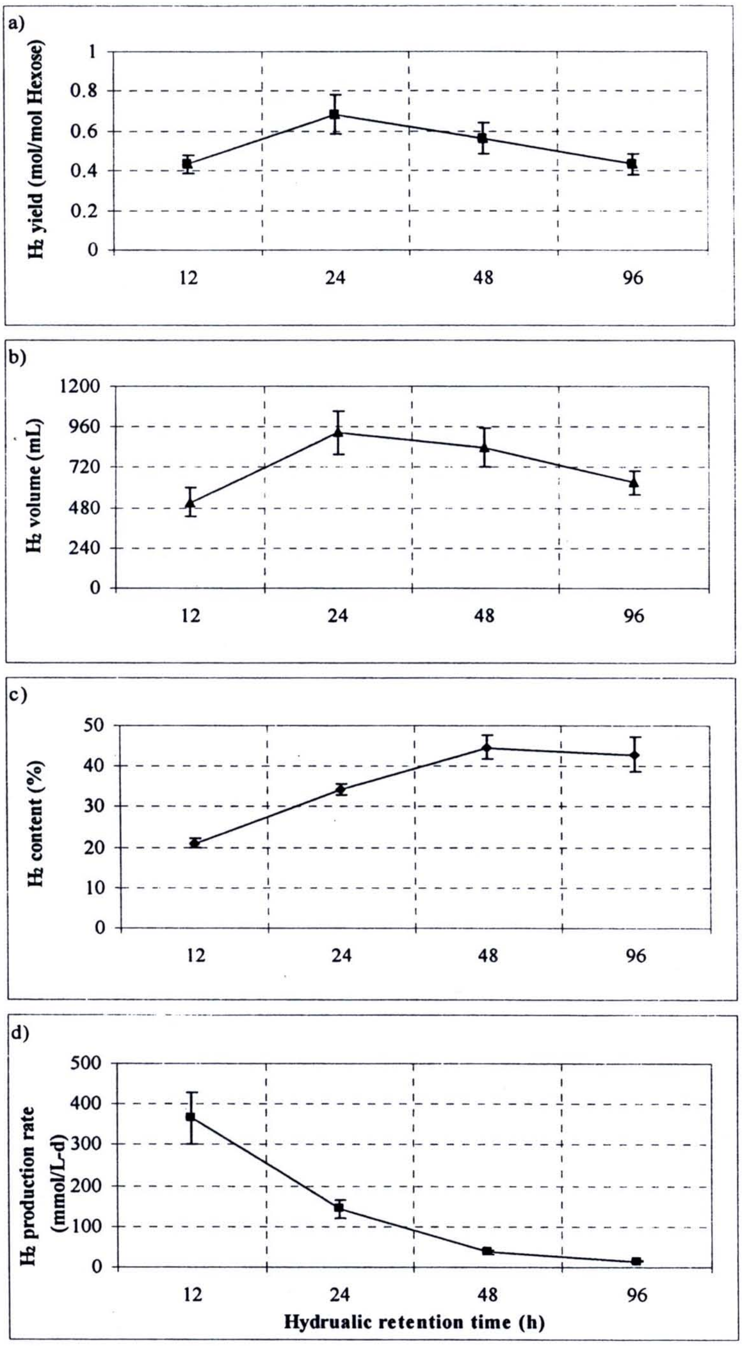

Figure 2 Effect of HRTs on a) hydrogen yield, b) hydrogen volume, c) hydrogen content and d) hydrogen production rate.

5.3.2 Substrate removal efficiency and soluble metabolites

Effect of HRTs on biohydrogen production from sweet sorghum syrup by mixed cultures was investigated. The substrate removal efficiency and soluble metabolites production including acetone, ethanol, butanol, acetic, propionic and butyric acids were summarized in Table 4. The removal efficiency of substrate was 75.47-95.83% and trended to decrease with a decrease in HRTs. Results indicated that a reduction of HRT led to a low substrate consumption resulted in a reduction of the amount of soluble metabolites produced i.e. ethanol, butanol, acetic and butyric acids.

Table 3 Comparison on hydrogen production using ASBR operation

HRT (hr)	Reactor	Substrate	Seed	H_2 yield (mol H_2/mol substrate)	H_2 production rate (mol H_2/L-d)	Reference
6-12	ASBR	Sucrose	Non-pretreated microflora	0.7-2.6 mol H_2/mol sucrose	0.07-0.33	[21]
4-18	ASBR	Starch	Heat-treated sludge	0.06-0.51 mol H_2/mol hexose	0.04-0.18	[30]
24-42	ASBR	Food waste	Heat-treated sludge	0.18-1.12 mol H_2/mol hexose	0.01-0.12	[20]
4-12	ASBR	Sucrose	Acid-enriched sewage sludge	0.73-2.5 mol H_2/mol sucrose	0.07-0.33	[22]
12-96	ASBR	Sweet sorghum syrup	Heat-treated sludge	0.43-0.68 mol H_2/mol hexose	0.01-0.36	This study

Low concentration of propionic acid (0.31-0.61 mg/L) was observed. The highest hydrogen yield of 0.68 mol H_2/mol hexose (Figure 2a) was found at 24 hr HRT, which consistent with low propionic acid concentration (0.31 mg/L) produced. Propionic acid was produced in methane-producing phase by hydrogenotropic methanogen which consumed hydrogen from acidogenesis process to produce methane through hydrogen consuming reaction [33]. Results implied that the ASBR operation with a pH control could inhibit propionic acid production. In this study, pH was adjusted to 5.0±0.1 which was optimal pH for hydrogen production by mixed cultures [34]. Han et al. [35] reported that pH involves with three important facts i.e.

methanogen growth limitation, hydrogen production performance and regulation of shift to solventogenesis. In general, hydrogen production process without pH control could produce solvents as the end products [36].

Operation of ASBR at short HRT could reduce solvent production as well as VFAs production (Table 4). Ranges of butyric acid/acetic acid (B/A) ratio was found to be 0.31-1.43 (Table4). The B/A ratio could be a quantitative indicator of substrate metabolism and hydrogen production by anaerobic microflora [20]. In this study, the maximum B/A ratio was 1.43 which implied that low hydrogen producing metabolism was observed. It has been reported that the B/A ratio greater than 2.60 indicated an efficient hydrogen production by anaerobic microflora [35].

Table 4 Soluble metabolites production at steady state for each hydraulic retention time (HRT).

HRT (hr)	Substrate removal efficiency (%)	Soluble metabolites (mg/L)						TVFA[a] (mg/L)	B/A[b]
		Acetone	Ethanol	Butanol	Acetic acid	Propionic acid	Butyric acid		
96	93.16	10.77	1621.49	78.25	23.80	0.60	33.97	58.37	1.43
48	95.83	4.50	1769.43	5.95	16.70	0.32	6.80	23.82	0.41
24	75.87	3.63	1544.56	4.45	17.07	0.31	7.68	25.06	0.45
12	75.47	2.67	1040.57	0	13.47	0.40	4.16	18.03	0.31

[a] Total volatile fatty acid

[b] Butyric acid/Acetic acid

5.3.3 Cyclic study

Figure 3 illustrates the study on single cycle period (12 hr) at optimum HRT (24 hr) including ORP, pH, hydrogen yield, hexose consumed, hydrogen production and content. The ORP ranged from -232 to -403 mV (Figure 3a) which indicated the anaerobic condition in ASBR, while pH was maintained between 4.92 to 4.98. The hydrogen yield increased along with cyclic period and stabilized at maximum hydrogen yield of 0.43 mol H_2/mol hexose (9 to 10 hr). Results showed the maximum hydrogen volume of 62.50 mL at 7 hr, while maximum hydrogen content of 21.90% was observed at 10 hr of cycle period (Figure 3c). Results showed a low hydrogen production efficiency in single cycle operation (12 hr) as well as hydrogen volume and hydrogen content

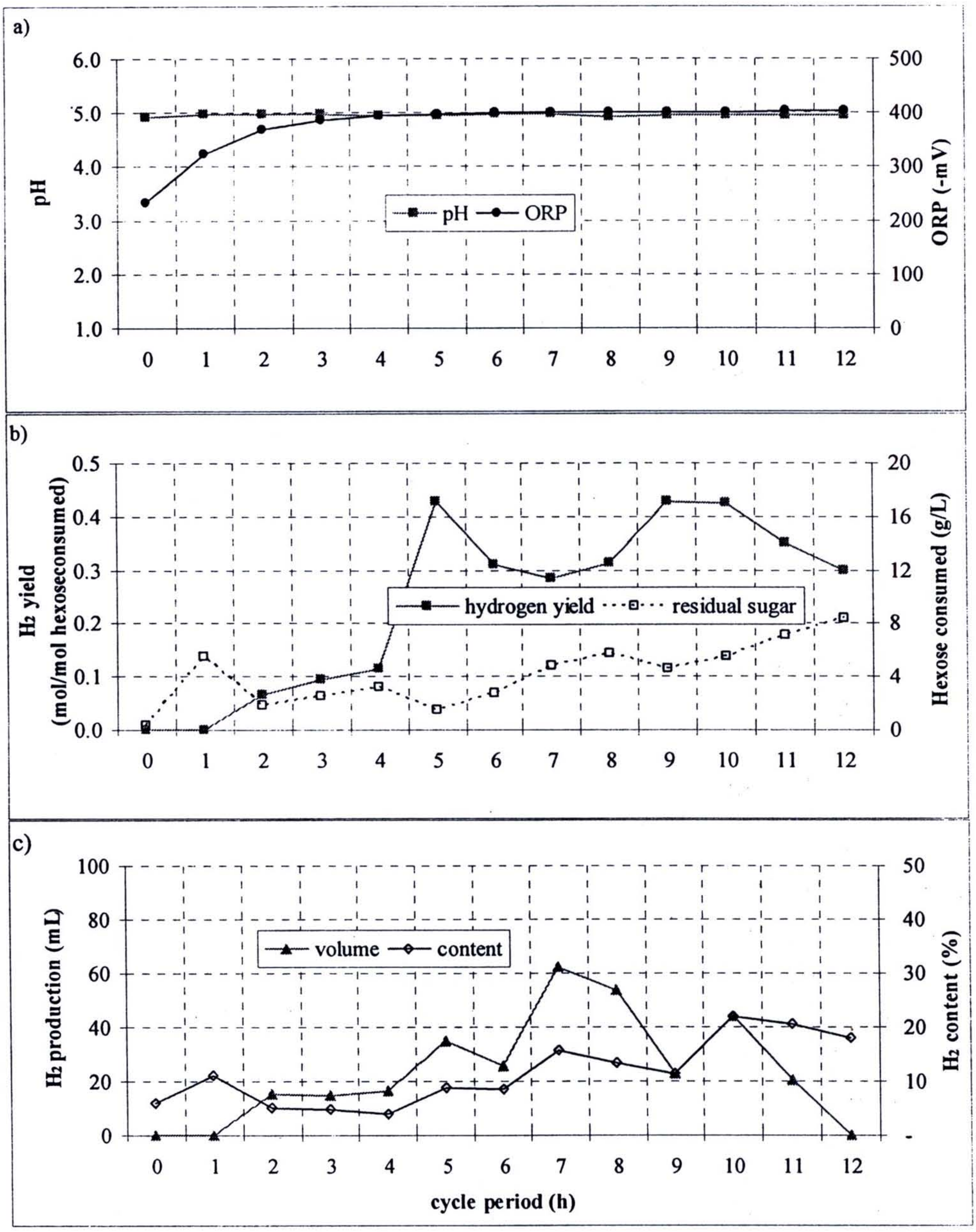

Figure 3 Single cyclic study (12 hr) for hydrogen production.

The microbial community in the culture broth from ASBR operation in a cyclic study was analyzed by DGGE and the results were shown in Figure 4 and Table 5. Results indicated that the microbial population was operating step dependent. *Bacillus* sp., *Sporolactobacillus* sp. and *Clostridia* including *C.butyricum*, *C.acetobutyricum* and *Clostridium* sp. were dominant in culture broth at the filling and reacting steps. *Clostridia* species have been reported to be responsible for hydrogen production via butyrate type fermentation [31, 5]. Hydrogen fermentation by *Clostridia* species is accompanied with VFAs and/or solvent production [31]. The relatively high hydrogen content in the range of 40-45% could be found in the biogas produced by *Clostridia* species [31]. The presence of *Sporolactobacillus* sp. in the reacting step might be responsible for the relatively low hydrogen content (21.90%) obtained from ASBR system. Lactic acid bacteria such as *Lactobacillus* sp. had been reported to decrease hydrogen content in biogas production due to its inhibitory effect caused by the excreted bacteriocins which have an adverse effect on hydrogen producing bacteria [37]. In the settling and decanting steps, only *Prochlorococcus* sp. was dominant. The results suggested that the time for settling step of 20 min was long enough to settle and maintain the hydrogen producer with in the ASBR.

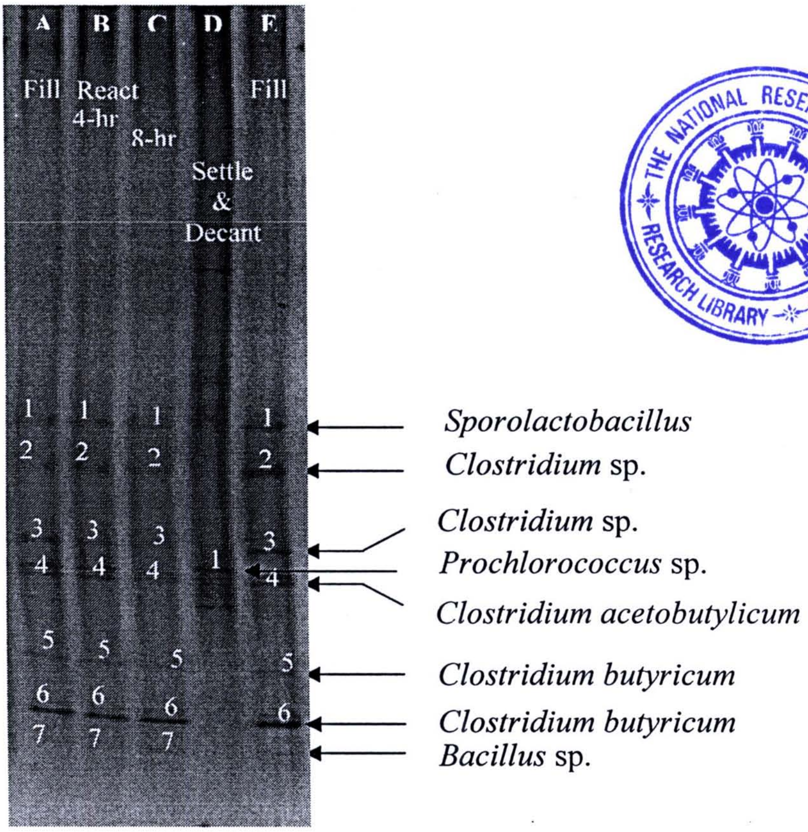

Figure 4 DGGE profiles of 16s rRNA gene fragments from sludge samples of ASBR operation in a single cycle at optimum HRT (24 hr).

Table 5 Summary of DGGE analysis results for microbial community from hydrogen production in single cyclic study (12 hr) at optimum HRT 24 hr and the control initial pH 5.0.

Lane	Operating step	Closest relative of sequenced band	
		DGGE band	Affiliation
A	Filling period	A1	*Sporolactobacillus* sp.
		A2	*Clostridium* sp.
		A3	*Clostridium* sp.
		A4	*Clostridium acetobutylicum*
		A5	*Clostridium butyricum*
		A6	*Clostridium butyricum*
		A7	*Bacillus* sp.
B	Reaction period at 4 hr	B1	*Sporolactobacillus* sp.
		B2	*Clostridium* sp.
		B3	*Clostridium* sp.
		B4	*Clostridium acetobutylicum*
		B5	*Clostridium butyricum*
		B6	*Clostridium butyricum*
		B7	*Bacillus* sp.
C	Reaction period at 8 hr	C1	*Sporolactobacillus* sp.
		C2	*Clostridium* sp.
		C3	*Clostridium* sp.
		C4	*Clostridium acetobutylicum*
		C5	*Clostridium butyricum*
		C6	*Clostridium butyricum*
		C7	*Bacillus* sp.
D	Settling and decanting period	D1	*Prochlorococcus* sp.
E	Filling period at new cycle	E1	*Sporolactobacillus* sp.
		E2	*Clostridium* sp.
		E3	*Clostridium* sp.
		E4	*Clostridium acetobutylicum*
		E5	*Clostridium butyricum*
		E6	*Clostridium butyricum*
		E7	*Bacillus* sp.

5.4 Conclusion

Hydraulic retention time (HRT) plays an important role in production hydrogen continuously using ASBR. Results showed that hydrogen content decreased with a reduction in HRT i.e. from 42.93% (96 hr HRT) to 21.06% (12 hr HRT). Decrease in HRT resulted in a decrease of soluble metabolite production, especially acetone and butanol. HRT of 24 hr was the optimum condition for ASBR operation indicated by the maximum hydrogen yield of 0.68 mol H_2/mol hexose. Results indicated that the decreasing of HRT led to a reduction in substrate consumption. The microbial determination in DGGE analysis indicated that the well known hydrogen producers *Clostridia* species were dominant in the reacting step. However, the relatively low hydrogen content of 21.90% in the react step was obtained which might be resulted from the presence of *Sporolactobacillus* sp. that could excrete the bacteriocins causing the adverse effect on hydrogen producing bacteria in the system.

5.5 References

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