CHAPTER IV

OPTIMIZATION OF HYDRAULIC RETENTION TIME AND MICROBIAL COMMUNITY ANALYSIS OF HYDROGEN PRODUCTION FROM SWEET SORGHUM SYRUP IN AN ANAEROBIC SEQUENCING BATCH REACTOR

4.1 Introduction

Research on clean fuel production has been received much interest with the hope to reduce greenhouse gases in the environment and to replace the fossil fuel. Hydrogen is an ideal energy due to its advantages including clean, efficient and non-polluting characteristics [Lin et al., 2007]. Biologically, hydrogen can be produced by direct biophotolysis, indirect biophotolysis, photo fermentation and dark fermentation. Among the biological hydrogen production processes, dark fermentation shows a high potential for practical application [Levin et al., 2001] due to lower energy requirements, process simplicity, utilization of low value waste as raw materials and higher rates of hydrogen production [Levin et al., 2001].

Various types of microorganisms have been found in dark fermentation system which both responsible for hydrogen production and stoppage. *Clostridium* sp. could produce hydrogen from sucrose with the yield ranged between 2.0 and 4.8 mol of hydrogen/mol sucrose [Chen et al., 2005] which is higher than that of the other fermentative bacteria such as *Enterobacter* sp. (1mol hydrogen/mol hexose) [Kapdan and Kargi, 2006]. Hydrogen producing microfloras obtained from natural sources contain mostly *Clostridium* ssp. was widely to use as seed inoculums for biohydrogen production under non sterile conditions [Kim et al., 2006; Oh et al., 2003]. However, the metabolism of substrate in continuous hydrogen production under non sterile conditions caused a population shift from hydrogen producing bacteria such as acetogens, propionate producers, lactate producers, and methanogens [Oh et al.,

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2003]. For continuously hydrogen production, stable hydrogen production by anaerobic microflora, suppression of non hydrogen producing bacteria and maintenance of stable microbial communities are important [Lin et al., 2006].

Biomass from crops and wastes are the most potential and promising ones due to the abundant and low/no cost. Our previous researches have focused on the production of biohydrogen as renewable energy from sweet sorghum (*Sorghum bicolor* var. Keller) as a potential alternative energy (non-food) plant. The hydrogen fermentation was conducted in a sequencing batch reactor (ASBR) with the variation of HRT to obtain the optimal condition for hydrogen production (Saraphirom and Reungsang, 2010b). The hydrogen yield obtained were in the range of 0.43 to 0.63 which were relatively low (Saraphirom and Reungsang, 2010b). Since the microbial community composition in the fermentative bioreactor directly contributes to the hydrogen fermentation efficiency, the investigation of the predominant hydrogen producer and the other microorganisms existing is important.

In the present study, the diversity of microbial communities of the anaerobic sludge from hydrogen fermentation of sweet sorghum syrup in an ASBR at different HRT was analyzed. The anaerobic sludge was collected at different time which represented the different hydrogen production performances. The information regarding microbial community structure was compared with the hydrogen production efficiency to obtain a better understanding and improving the fermentative hydrogen production in ASBR in biological perspective.

4.2 Materials and Methods

4.2.1 Seed sludge

Anaerobic seed sludge was obtained from a full scale anaerobic digester of Up-flow 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 in drying oven (LDO-100E, Daihan Labtech Co., Ltd, Korea) 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 [Lay et al., 1999]. The volatile suspended solid of seed sludge is 5 g VSS/L.

4.2.2 Sweet sorghum syrup

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. Total sugar of sweet sorghum syrup was 75-80 °Brix determined by a hand refractometer (HRB-32 ATC). The syrup was diluted by sterile filtered water to obtain 25 g/L total sugar as initial substrate concentration. After dilution, the syrup composition consisted of, in mg/L, 0.94±0.03 acetone, 406±17.5 ethanol, 3.5±0.9 butanol, 9.58±0.7 acetic acid, 0.08±0.001 propionic acid and 8.90±1.6 butyric acid, respectively.

4.2.3 Reactor configuratuion

The reactor was designed 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 operated at room temperature (35±3 °C) 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). Oxidation reduction potential (ORP) was monitored using the same model of pH meter.

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 (35±3 °C). After 24 hr of reactor operation, 500 mL of the culture medium was replaced by the fresh enrichment medium and the reactor was operated again for 24 hr. The medium replacement was

repeated 5 times in order to obtain a stable microbial community 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 obtained from the batch experiment [Saraphirom and Reungsang, 2010a]. The ASBR was first purged with nitrogen gas for 15 min to create anaerobic condition before operating in a 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 ASBR operational design conditions were tested at various HRTs as shows in Table 1. Constant substrate removal efficiency 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 [Saraphirom and Reungsang, 2010b].

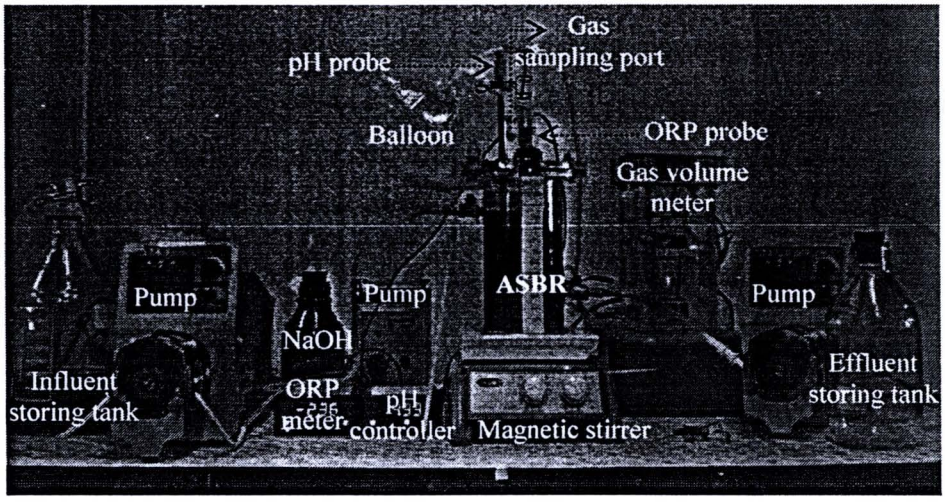

Figure 1 Anaerobic sequencing batch reactor configuration.

4.2.4 Analytical method

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 0.2 μm nylon syringe filter. 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. Lactic acid was analyzed by high performance liquid chromatography (Shimadzu LC-10AD) with a UV detector (210 nm) and Prevail Organic Acid 5μ column (250 mm x 4.6 mm) using 25 mM KH_2PO_4 (pH 2.5) with a flow rate of 0.8 to 1.2 mL/min as the mobile phase.

Table 1 ASBR operation

Parameters	**HRT (hr)**			
	12	**24**	**48**	**96**
Cycle period (hr)	6	12	24	48
Filling, settling and decanting period (min)	20	20	20	20
Reaction period (hr)	5	11	23	47
OLR (g total sugar/L-d)	50	25	12.5	6.25
Filling &Decanting volume (mL)	500	500	500	500

4.2.5 Microbial community analysis

Liquid samples were collected from ASBR under steady state conditions. Total genomic DNA was extracted 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) [Muyzer et al., 1993]. 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 30 s 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 (1000x concentration) for 1 hr and visualized on a UV transilluminator. Most of the bands were excised from the gel and reamplified with the 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 [Altschul et al., 1997]. A Phylogenetic tree was then constructed using the neighbor-joining method [Saito and Nei, 1987] with PHYLIP 3.69 [Felsenstein, 1993]. Bootstrapping analysis [Felsenstein, 1985] for 1,000 replicates was performed to estimate the confidence of tree topologies.

4.3 Results and Discussion

4.3.1 Hydrogen production performance

Hydrogen production was performed under variation of HRT from 96 to 12 hr. Important parameters i.e. pH, ORP, biogas content, hydrogen yield and hydrogen production rate were monitored during the course of continuous hydrogen fermentation (Figure 2). The average range of ORP was 271 to 465 (-mV) which confirmed the anaerobic condition in ASBR (Figure 2b). The biogas production tended to increase when the HRT was shortened (Figure 2b). The biogas produced consisted of carbon dioxide (40-61%) and hydrogen (21-44%) (Figure 2c). Methane could not be detected in the produced biogas which indicated that the methanogens were completely inhibited under the operated condition. The efficiency of hydrogen production was expressed as hydrogen content (Figure 2c), hydrogen yield and hydrogen production rate (Figure 2d). The results suggested that the optimum HRT

for hydrogen production in ASBR was 24 hr giving the maximum hydrogen yield of 0.67 mol H_2/mol hexose and the relatively high hydrogen content and hydrogen production rate of 44.3% and 1.12 L H_2/L-d, respectively.

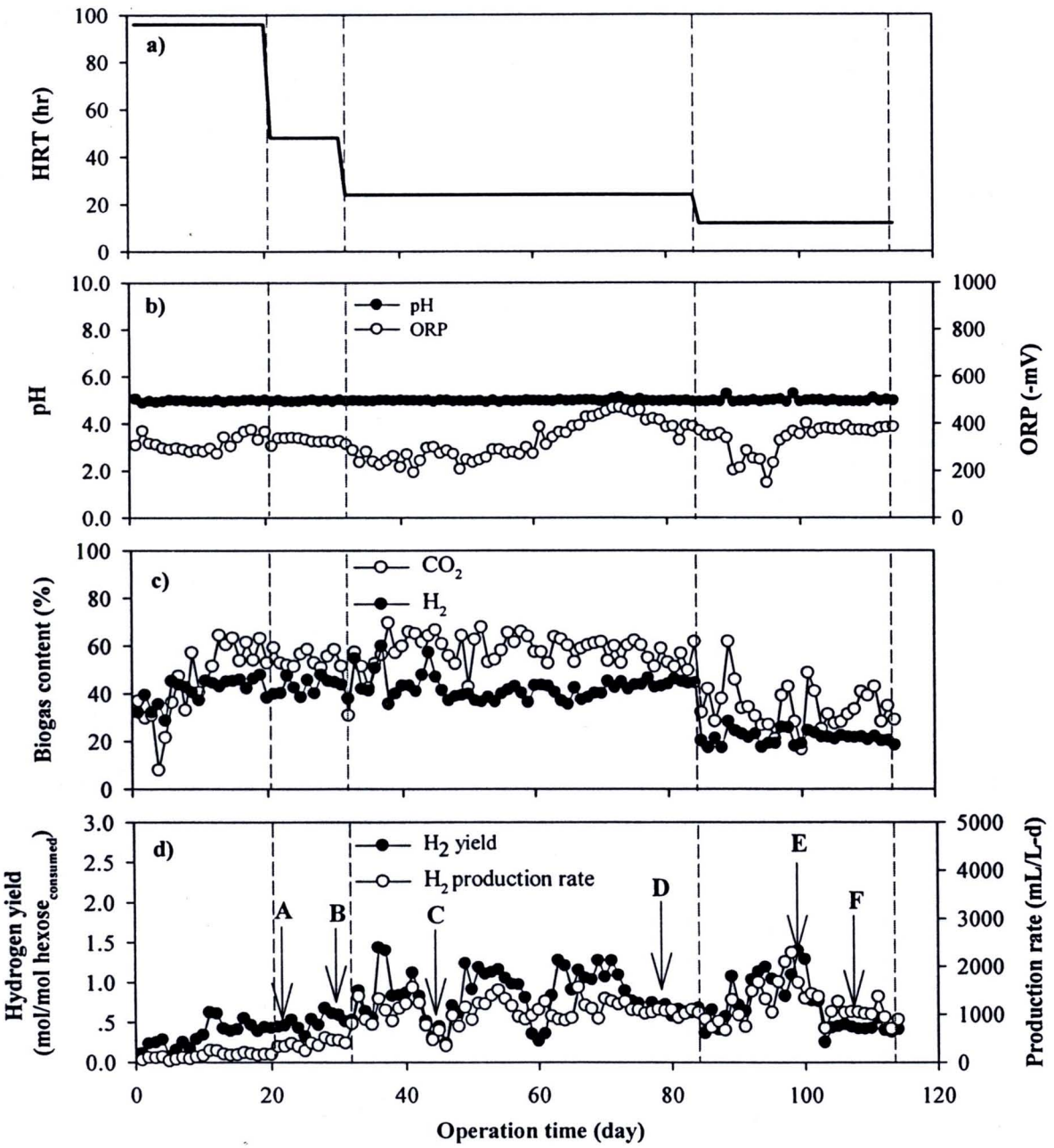

Figure 2 Profiles of a) HRT, b) pH and ORP, c) biogas content, d) H_2 yield and production rate during ASBR operation. A, B, C, D, E and F indicate the sampling times of anaerobic sludge for DGGE analysis.

4.3.2 Change in microbial community during hydrogen production in ASBR

The diversity of microbial communities at unsteady state (time A, C and D; Figure 2d) and steady state (Time B, D and F; Figure 2d) of different HRTs was analyzed and compared using PCR–DGGE techniques. The different hydrogen production performances were represented in each sampling time which time C and E represented the lowest and highest hydrogen production efficiency, respectively (Figure 3). The DGGE profiles of 16S rDNA gene fragments are depicted in Figure 4. Six major bands (band 1 to 6) were detected in DGGE profiles. When considered the intensity of the each band separately, the band 6 was strong at sampling time C which implied that the band 6 was hydrogen stoppage. However, the band of hydrogen producing bacteria could not be clearly defined. Therefore, we further analyzed the relative abundances (%) by calculated from the intensity of the bands and the specific species in the microbial community corresponded to each band. The results are summarized in Table 3.

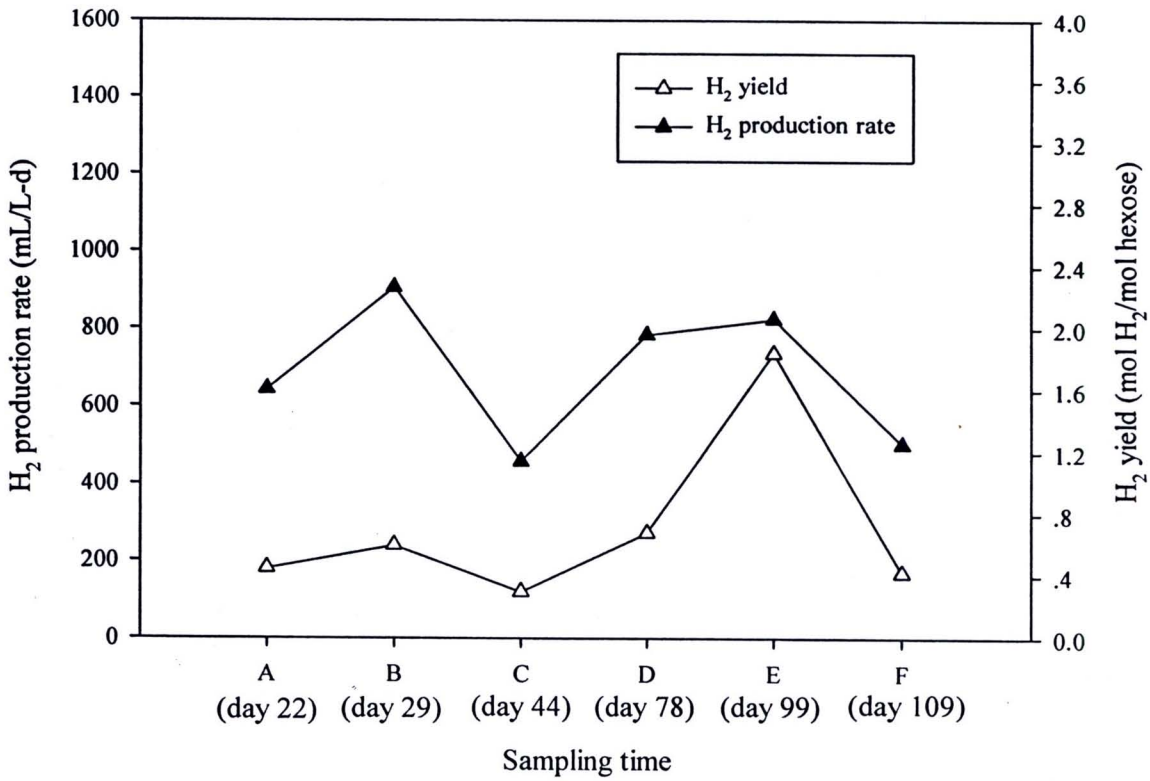

Figure 3 Hydrogen production performance at different sampling times.

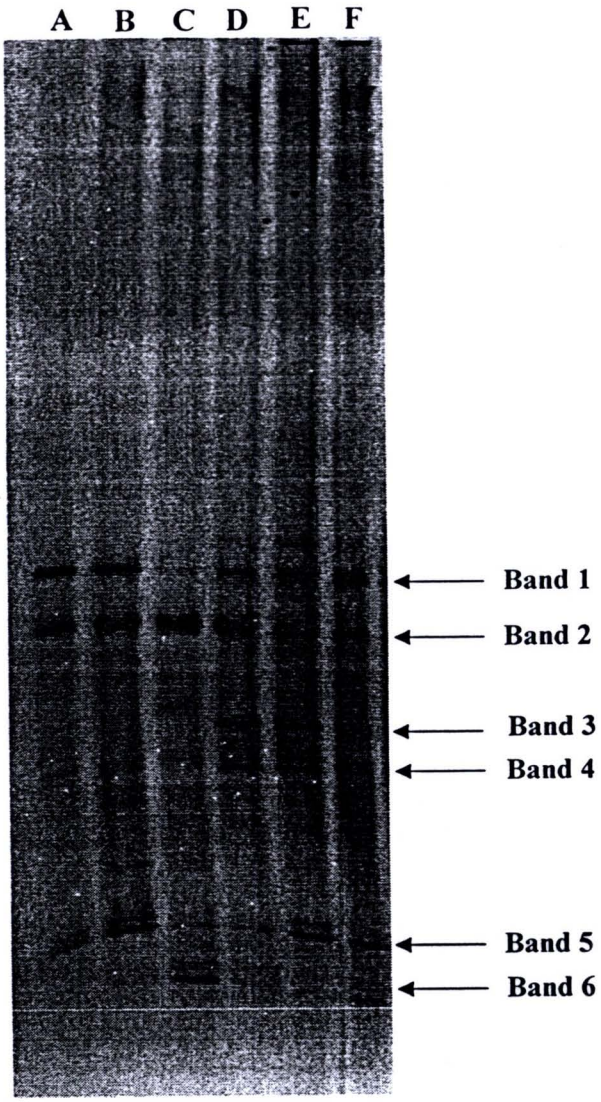

Figure 4 DGGE profiles of 16s rRNA gene fragments from sludge sampled under various ASBR performances. Lenes: A, sludge taken from unsteady state of 48 hr HRT; B, sludge taken from steady state of 48 hr HRT; C, sludge taken from unsteady state of 24 hr HRT; D: sludge taken from steady state of 24 hr HRT; E: sludge taken from unsteady state of 12 hr HRT; F, sludge taken from steady state of 12 hr HRT.

The phylogenetic tree showing the relationship between the microorganisms detected and the close relative reference species is depicted in Figure 5. Four species of microorganism i.e., *Sporolalactobacillus* sp. (band 1), *Clostridium* sp., (bands 2 and 3), *Prochlorococcus* sp. (band 5) and *Bacillus* sp. (band 6) were

detected in the anaerobic sludge. Clostridia sp. has been reported as classical acid producers and usually ferments glucose to butyrate acetate, carbon dioxide, and molecular hydrogen [Chen et al., 2005]. *Sporolalactobacillus* sp. and *Bacillus* sp. have been reported as the lactic acid bacteria (LAB) which might cause a reduction in hydrogen production efficiency in the hydrogen fermentation system. *Prochlorococcus* sp. is the cyanobacterium which has not been found in the dark fermentation system in any published research. We speculated that this cyanobacterium might present in the connecting or tubing system of sampling and did not affect the hydrogen production efficiency. Therefore, our discussion will focus on two groups of bacteria which are hydrogen producing bacteria (*Clostridium* sp.) and lactic acid producing bacteria (*Sporolalactobacillus* sp. and *Bacillus* sp) relating to the hydrogen production efficiency.

It could be seen in Table 2 that the microbial community and the population in term of relative abundance at each sampling time are different and correlated with hydrogen production efficiency. The larger abundance of hydrogen producing bacteria and smaller abundance of LAB was existed at the sampling time which the higher hydrogen fermentation efficiency was obtained. At time E which the highest hydrogen production efficiency was achieved, the greatest abundance of hydrogen producing bacteria accounting for 59.27% was obtained with the smallest abundance of LAB (18.35%) (Table 2). The large abundance of LAB of 38.5% with the relatively small abundance of hydrogen producing bacteria (41.66%) was obtained at time C which might be the reason for low hydrogen production efficiency obtained in comparison to the other treatments. The relatively low hydrogen production efficiency of was obtained at time B though the large abundance of hydrogen producing bacteria (52.20%) with small LAB abundance or 24.31 were observed. The results indicated that the Clostridia species detected at sampling time B might not play a role in hydrogen production. At the sampling time B, the ASBR was operated at the long HRT of 48 hr in which the amount of VFAs accumulated might be increased to the inhibition level and could cause the metabolic shift of the microorganism presented. The analysis of soluble metabolites production might give a good explanation on this phenomenon.

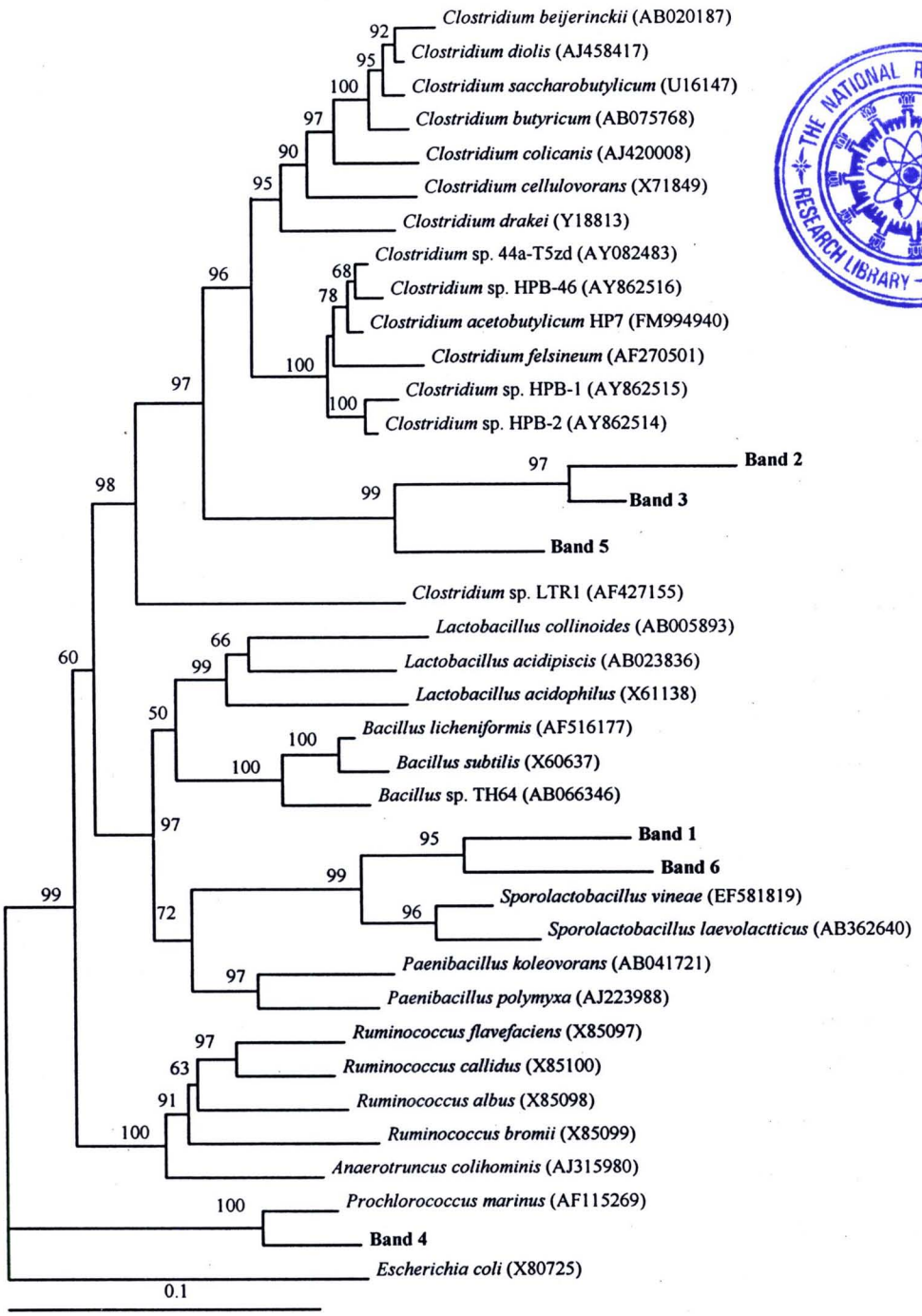

Figure 5 Phylogenetic tree showing the relationship between DGGE bands detected in this study and references sequences based on a comparison of 16S rRNA sequences. *Escherichia coli* was used as the out group. The bar corresponds to a 10% difference in nucleotide sequence. The numbers shown next to the nodes indicate percent bootstrap values from 1000 iterations.

Table 2 Affiliation of denaturing gradient gel electrophoresis (DGGE) fragments determined by their 16S rRNA sequences.

DGGE Band	Abundance (%)						Accession #	Family	Closet relative
	A[a]	B[b]	C[c]	D[d]	E[e]	F[f]			
1	28.36	24.31	17.47	16.87	18.35	17.70	EF581819	Bacillaceae	*Sporolactobacillus vineae*
2	31.02	24.07	24.30	20.05	20.36	18.99	AY862514	Clostridiaceae	*Clostridium* sp.
3	-	-	-	18.37	21.89	16.60	AY862515	Clostridiaceae	*Clostridium* sp.
4	23.04	23.48	19.79	18.46	22.37	19.26	AF115269	Prochlorococcaceae	*Prochlorococcus marinus*
5	17.58	28.13	17.36	13.49	17.02	14.26	AB075768	Clostridiaceae	*C. butyricum*
6	-	-	21.06	12.75	-	11.32	AB066346	Bacillaceae	*Bacillus* sp.
H_2 producer abundance[g]	48.60	52.20	41.66	51.91	59.27	49.85			
LAB abundance[h]	28.36	24.31	38.53	29.62	18.35	29.02			

a Sampling time at unsteady state of 48 hr HRT; b Sampling time at steady state of 48 hr HRT
c Sampling time at unsteady state of 24 hr HRT; d Sampling time at steady state of 24 hr HRT
e Sampling time at unsteady state of 12 hr HRT; f Sampling time at steady state of 12 hr HRT
g Summation of Clostridia at each sampling time; h Summation of Bacilli at each sampling time

4.3.3 Microbial community responsible for soluble metabolites production production from sweet sorghum syrup by mixed cultures

The soluble end products in the ASBR effluent collected at steady state of each HRT mainly comprised of lactic acid and ethanol followed by butanol, butyric acid, acetic acid and propionic acid (Table 3). This soluble metabolite composition indicated that the ASBR operated conditions were unfavorable to hydrogen fermentation, because in most efficient hydrogen producing system, butyric or acetic acid was the predominant product [Wu et al., 2006; Wu et al., 2003].

Lactic acid was the dominant metabolite found at the short HRT of 12 and 24 hr. Lactic acid formation has been reported in some hydrogen production system [Oh et al., 2004]. It has also been detected as the major metabolite from hydrogen production under the over load conditions and subsequently caused a reduction in hydrogen production rate in the study of Liu et al. [2008]. In addition the formation of lactic acid could be initiated by many adverse environmental conditions

such as nutrient limitation [Dabrock et al., 1992] and inhibition by organic acid [Ko et al., 2004]. The DGGE analysis results indicated that the total abundance of lactic acid bacteria (*Sporolactobacillus vineae* plus *Bacillus sp.*) accounting for approximately 30% at the short HRT of 12 and 24. This could be responsible for the amount of lactic acid formed during hydrogen fermentation. Lactic acid bacteria could produce lactate as a major metabolic end product of carbohydrate fermentation [Lee et al., 2006]. Additionally, heterofermentative bacteria, *Lactobacillus* sp. could ferment 1 mol of glucose to 1 mol of lactic acid, 1 mol of ethanol, and 1 mol of carbon dioxide [Madigan et al., 2000]. Levin et al., (2004) suggested that the high concentration of lactic acid is associated with low hydrogen yield.

Table 3 Summary of ASBR performance for hydrogen fermentation and soluble metabolite production under the steady state of HRT.

HRT (hr)	**12**	**24**	**48**	**96**
Operational mode				
Substrate removal efficiency (%)[a]	75±4.7	75±2.4	95±3.8	93±1.5
Acetone (mg/L)	0	0	0	0
Butanol (mg/L)	0	0	6±1.0	68±15.2
Ethanol (mg/L)	509±30.4	524±36.3	1005±130.8	758±129.4
Acetic acid (mg/L)	12±3.4	12±1.6	11±1.5	15±3.8
Propionic acid (mg/L)	0	0.05±0.02	0.6±0.3	0.2±0.1
Butyric acid (mg/L)	6±1.0	9±1.4	4±0.9	39±4.6
Lactic acid (mg/L)	1111±24.2	1075±30.4	577±19.7	377±0.9
B/A ratio[b]	0.50	0.76	0.34	2.64
TVFA (mg/L)[c]	1129±3.4	1097±2.6	593±2.4	431±6.8
SMP (mg/L)[d]	1638±9.4	1520±5.6	1604±5.9	1258±3.3

a Substrate removal efficiency = ((Initial hexose-Residual hexose)/Initial hexose) x 100

b Butyric/acetic ratio

c Total volatile fatty acid = acetic acid+propionic acid+butyric acid+lactic acid

d Soluble metabolite products = acetone+ethanol+butanol+ acetic acid+propionic acid+butyric acid+lactic acid

Ethanol was the main metabolite found at the long HRT of 48 and 96 hr. When the ASBR was operated at long HRT, amount of VFAs accumulated would

be increased resulting in the drop of pH which could cause the inhibitory effects to the microorganisms. Under this condition the VFAs would be converted to neutral metabolites such as alcohol by the microorganism to reduce the inhibitory effect of VFAs. This kind of metabolic shift has been found in various hydrogen producing bacteria especially *Clostridium* sp. [Okamoto et al., 2004]. The results are coincided with the microbial community analysis results in which at the long HRT of 48 hr, *Clostridium* sp. were dominant at steady state of ASBR operation which accounting for 52.20%. The inhibition of hydrogen producing bacteria by the accumulated VFAs might be the reason for relatively low hydrogen production efficiency obtained at the steady state of 48 hr HRT though the *Clostridium* sp. was dominant.

4.4 Conclusions

The diversity of microbial communities of the anaerobic sludge from hydrogen fermentation of sweet sorghum syrup in an ASBR at different HRT was analyzed. The information regarding microbial community structure and hydrogen production efficiency at different sampling time were compared. The groups of hydrogen producing bacteria (*Clostridium* sp.) and LAB (*Sporolalactobacillus* sp. and *Bacillus* sp.) were detected during hydrogen fermentation in ASBR. The microbial communities in term of relative abundance at each sampling time are different and correlated with hydrogen production efficiency in which the larger abundance of hydrogen producing bacteria and lower abundance of LAB existed at the sampling time that gave the higher hydrogen fermentation efficiency. The soluble end products in the ASBR effluent mainly comprised of lactic acid and ethanol. LAB accounting for approximately 30% could be responsible for the large amount of lactic acid produced at the short HRT of 12 and 24 hr. Ethanol was the main metabolite found at the long HRT of 48 and 96 hr which might be due to the bacterial metabolic shift to reduce the inhibitory effect of VFAs by converted them to neutral soluble metabolite i.e., ethanol.

4.5 References

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