Effect of Organic Loading Rate on Biohydrogen Production from Sweet Sorghum Syrup by Anaerobic Mixed Cultures in Anaerobic Sequencing Batch Reactor

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***Abstract*—** This study reported the effect of 4 organic loading rates (OLR) varied from 25-40 g hexose/L-d on bio-hydrogen production from sweet sorghum syrup by anaerobic mixed cultures in anaerobic sequencing batch reactor. The optimum OLR was found to be 30 g hexose/L-d in which a maximum yield, a maximum specific hydrogen production rate and a hydrogen content of 0.53 mol H_2/mol hexose, 32.52 mL/g MLVSS.d and 37.66%, respectively, were achieved. Microbial community analyzed by DGGE at the optimum OLR of 30 g hexose/L-d indicated that the predominance hydrogen producer was *Clostridium acetobutylicum*, *C. proteolyticum* and *Clostridium* sp. A low hydrogen yield obtained might be resulted from the presence of *Lactococcus lactis* and *Lactobacillus* sp. in the fermentation broth.

***Keywords*—** sweet sorghum syrup, organic loading rate, anaerobic sequencing batch reactor, anaerobic mixed cultures

I. Introduction

HYDROGEN is an ideal energy due to its advantages including clean, efficient and nonpolluting characteristics [1]. Biological hydrogen production derived from renewable energy sources is a clean bioenergy replacement for fossil fuels. Biohydrogen production includes direct biophotolysis, indirect biophotolysis, photo fermentation and dark fermentation. Among the biological hydrogen production processes, dark fermentation demonstrates a high potential for practical application [2] due to its lower energy requirements, process simplicity, utilization of low value waste as raw materials and higher rates of hydrogen production [2-4].

Organic loading rate (OLR) is an important parameter for continuously producing hydrogen in the bioreactors. In order to optimize a system for hydrogen production, it is essential to define either a range of the OLR that the system can handle effectively, or optimal OLR for a maximum hydrogen yield. However, from the literature search, there is no clear relationship between the hydrogen yield and the OLR. In some cases high OLR decreased the hydrogen yield [13] whereas in some others high OLR increased the hydrogen yield [14]. For waste activated sludge as a seed material, it appears that increasing the OLR within the ranges of 40–160 g-COD/L-d increased hydrogen yield in which the optimum yield of 1.6 mol H_2/mol glucose was obtained at an OLR of 120 g-COD/L-d [15]. However, the hydrogen yield was found to decrease with an increase in OLR when anaerobically digested sludge [16] and soil microorganisms [13] were used as the inoculums. Although lower molar hydrogen yields at higher OLR have been attributed to the inhibitory effect of higher hydrogen partial pressure in the growth medium [13, 17], variations in the composition of bacterial communities that become established at different OLR [18] may be a major reason for lower yields. Hafez et al. (2010) [19] reported 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 to maintain 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 [13]. For that reason, ASBR process has become the reactor option for producing hydrogen in continuous mode. Advantages of ASBR include high biomass concentration, a high degree of process flexibility, no requirement to apply a separate clarifier [20]. Various kinds of substrates such as dairy wastewater, chemical wastewater and palm oil mill effluent (POME), sucrose, sweet sorghum extract and sweet sorghum syrup have been used to produce hydrogen in ASBR [21-26].

Sweet sorghum (Sorghum bicolor var. Keller) was used to produce hydrogen due to a high sugar yield which mostly contains sucrose, fructose and glucose. 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 [25]. A 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 [27]. In addition, heat treated microflora could generate hydrogen from sweet sorghum syrup supplemented with $FeSO_4$ at the maximum yield of 2.22 mol

H_2/mol hexose [28].

Although a few studies have been conducted on the hydrogen production from sweet sorghum extract and syrup, however, the information on the effect of OLR on hydrogen production from sweet sorghum syrup by mixed cultures in ASBR are still lacking. Therefore, this study aims to investigate the effects of OLR on bio-hydrogen from sweet sorghum syrup by heat treated microflora. The shift of microbial community at each OLR was also investigated. Results from this study would provide the optimum OLR for maximizing hydrogen from sweet sorghum syrup in ASBR.

II. Materials and Methods

A. Seed Preparation

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 ºC for 2 hr to inactivate methanogenic bacteria and then cooled at room temperature in dessicator. For inoculums 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 [29]. The volatile suspended solid of seed sludge is 5 g VSS/L.

B. 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 ºC for 28 min to prevent the contamination. Total sugar of sweet sorghum syrup was 75-80 ºBrix 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±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.

C. Reactor Configuration and Operation

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 Fig. 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.

The reactor was started up by inoculating 100 mL of seed inoculums (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 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 before fed with sweet sorghum syrup containing 1.45 g/L $FeSO_4$ at controlled pH of 4.90 to 5.0 at 24 hr HRT which were the optimum condition from previous study [28]. 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 12 hr reaction period. The experimental design conditions in the ASBR system were tested at various OLRs at 4 levels i.e. 25, 30, 35 and 40 g total sugar/L-d, respectively. Constant substrate consumption and hydrogen production (±5% variation) were considered as indicators for the steady state conditions. The gas produced and liquid samples were collected daily for samples analysis. The biogas volume was measured by water replacement method.

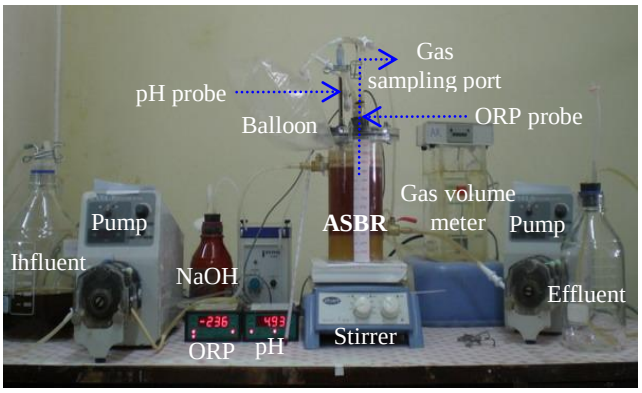

Fig. 1 ASBR configuration

D. 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 ºC, respectively. Helium was used as the carrier

gas at a flow rate of 25 mL/min. For acetic acid, propionic acid, butyric acids, 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 ºC. The initial temperature of column oven was 50 ºC for 2 min followed with a ramp of 15 ºC/min for 12.6 min and to final temperature of 240 ºC 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.

E. Microbial Community Analysis

Total genomic DNA was extracted from samples collected at the steady state of OLRs using a modified phenol/chloroform method [30]. Briefly, the cell pellets were re-suspended in 560 mL of saline-EDTA (150 mM NaCl, 100 mM EDTA [pH 8.0]). A volume of 7 mL of freshly prepared 50 mg/mL lysozyme was added to the mixture and incubated at 50°C for 1 hr. Then 30 mL of 10% (w/v) sodium dodecyl sulfate and 3 mL of 2% (w/v) proteinase K were added to the mixture and then incubated at 50°C for 1 hr. Nucleic acid, 500 mL, from the aqueous phase was taken out from the top part of the mixture and then the DNA was extracted by adding 800 mL of phenol chloroform isoamyl alcohol (25:24:1) (v/v) and then hand-mixing for 10 min. The top part was transferred to the fresh tube and the DNA was precipitated by adding 50 mL of sterile 3 M sodium acetate and 1 mL of ice-cold 100% ethanol and incubating for 2 hr at -20°C. The DNA pellet was recovered by centrifuging the solution at 12,000 x g for 20 min at 4°C. The pellet was washed by adding 1 mL of 70% ice-cold ethanol and was recovered by centrifuging at 12,000 x g for 10 min at 4°C. The pellet was then air dried, and the nucleic acids were dissolved in 50 mL of sterile milliQ-purified (mQ) water. The DNA was visualized by agarose gel electrophoresis. After DNA extraction, two steps of PCR amplification were employed in this study. For the 16S rDNA analysis, a universal primer set including forward primer PA19-38 (5'-AGAGTTTGATCCTGGCTCA G-3') and reverse primer PH1541-1561 (5'-AAGGAGGTGATCCAGCCGCA-3') was used for amplifying an approximately 1,500 bp fragment of bacterial 16S rDNA. PCR amplification was conducted in an automated thermal cycler using the follow protocol, that is; initial denaturation for 3 min at 95 ₀C, 30 cycles of denaturation for 45 sec at 95 ₀C, annealing for 1 min at 55 ₀ C, extension for 2 min at 72 ₀C, followed by a final extension for 7 min at 72 ₀C. For the DGGE profile analysis, PCR amplification was used on the primer set of 357f with GC clamp (5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCAC GGGGGGCCTACGGGAGGCGCAG-3') and 518 r (5'-ATTACCGCGCTGCTGG-3') [31]. PCR amplification was conducted in a P X 2 thermal cycler (PX2, USA) using the follow protocol, that is; initial denaturation for 3 min at 95 ₀ C, 30 cycles of denaturation for 45 sec at 95 ₀C, annealing for 1 min at 57 ₀C, extension for 2 min at 72 ₀C, followed by a final extension for 7 min at 72 ₀C. The DGGE analysis of PCR products was performed by electrophoresis for 20 min at 20 V and 16 h at 70 V through a 7.5 % polyacrylamide gel containing a linear gradient of denaturant ranging from 30% to 70% in 0.5xTAE buffer at a constant temperature of 60 ₀C. The gel was stained with SYBR-Gold (1,000 ng/mL) for 20 min and visualized on a UV transilluminator. Most of the bands were excised from the gel and re-amplified with the forward primer without a GC clamp and the reverse primer. After re-amplification, PCR products were purified using the QIAquick PCR purification Kit (QIAGEN,USA) and sequenced using primer 518r and 357f 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 [32]. CLUSTAL X was used to align obtained sequences with sequences of reference microorganisms retrieved from GenBank [33], and a Phylogenetic tree was then constructed using the neighbor-joining method [34] with PHYLIP 3.69 [35]. Bootstrapping analysis [36] for 1,000 replicates was performed to estimate the confidence of tree topologies.

III. Results and Discussion

A. Reactor Performance

Hydrogen production was conducted in ASBR in order to evaluate the performance of the reactor, pH, ORP, biogas content, hydrogen yield and hydrogen production rate were monitored during the course of continuous hydrogen fermentation (Fig. 2). The ASBR was operated with different OLRs i.e. 25, 30, 35 and 40 g/L-d for 30, 37, 30 and 31 days, respectively.

Fig. 2a illustrated the average range of ORP was -431 to -346 mV which confirmed the anaerobic condition in ASBR. The pH was controlled at 5.0±0.1 using 2N NaOH solution. Results in Fig. 2b indicated that the variation in OLRs affected hydrogen metabolism which led to distinction in biogas production, hydrogen content, yield, and production rate. At steady state, the biogas at the rate of 1.03 to 2.46 L/d was observed. Biogas produced consisted of 21.5 to 40.8% hydrogen, 60.4 to 78.2% carbon dioxide and no methane produced. Hydrogen content was found to increase with an increase in OLRs. The absence of methanogens in the system

might be a consequence of sludge was pretreated and a low pH operation.

Fig. 2c shows the reactor performance on hexose consumed and hydrogen production rate at steady state with average range of 11.8 to 28.3 g/L and 413.8 to 781.1 mL H_2/d, respectively. Maximum hydrogen production rate (413.8 mL H_2/d) and hydrogen yield (0.53 mol H_2/mol hexose) were observed at 30 g/L-d OLR (Fig. 2c and d). For that reason, 30 g/L-d OLR was regarded as the optimal operating HRT. However, it should be noted that the hydrogen content at optimal OLR 30 g/L-d (37.6%) was lower than at 40 g/L-d (40.8%). A low hydrogen yield in the ranges of 0.15 to 0.53 mol H_2/mol hexose was comparable to hydrogen production from starch by mixed cultures [43].

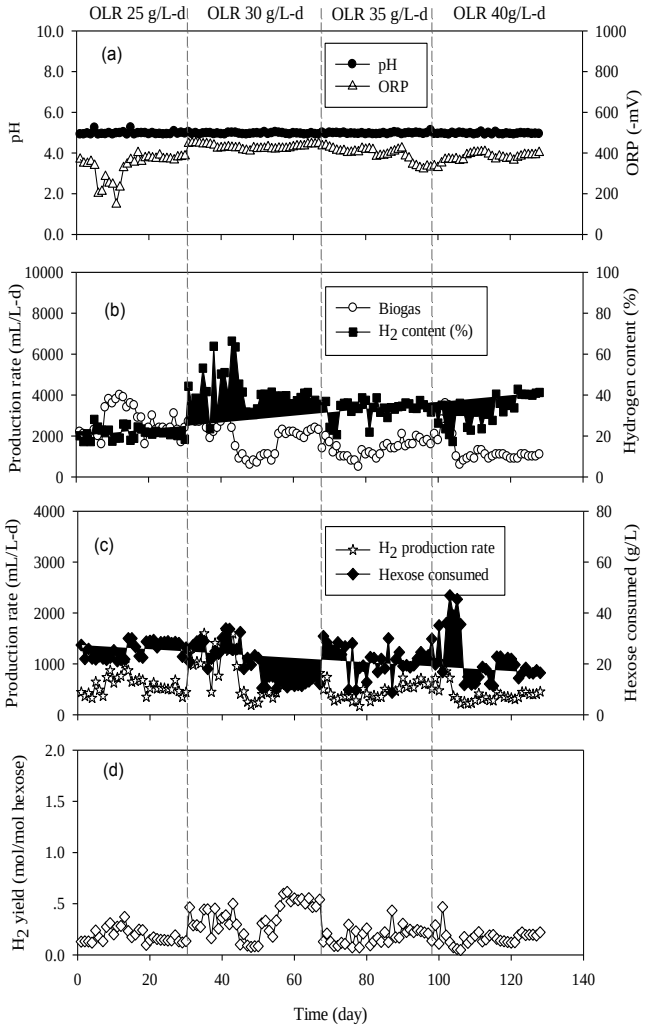

Fig.2 Performance of ASBR (a) pH and ORP, (b) biogas and hydrogen content, (c) hydrogen production rate and hexose consumed and (d) hydrogen yield.

However, the hydrogen production rate and hydrogen yield obtained in this study were lower when compared to the other ASBR systems including hydrogen production from food waste which obtained 0.22-2.69 mol H_2/L-d and 0.18-1.12 mol H_2/mol hexose of the hydrogen production rate and hydrogen yield, respectively [40]. The difference might be the results of the present of lactic acid bacteria present in the fermentation broth.

Table 1 indicated that biomass concentration increased with an increase in OLRs. In contrast, the substrate removal efficiency was found to decrease when OLRs increased. The specific hydrogen production rate of 32.5 mL H_2/g MLVSS was found at the optimum OLR 30 g/L-d. A decrease in hydrogen production rate and hydrogen yield at higher HRT than 30 g/L-d might be due to the competing reactions in hydrogen fermentation pathway in which the substrate as used to produce the other products such as volatile fatty acids and ethanol [25]. In addition, a high concentration of ethanol at approximately 75% of soluble metabolite products in sweet sorghum syrup composition might cause low hydrogen yield and production rate observed in this study.

B. Microbial Community

Different shifts in the microbial population could be observed at 4 levels of OLRs including 40 g/L-d (lane A), 35 g/L-d (lane B), 30 g/L-d (lane C) and 20 g/L-d (lane D) (Fig. 3). Results suggested that the OLRs variation caused a change in the microbial community composition in the reactor. Phylogenetic tree showing the relationship between DGGE bands detected in this study and references sequences based on a comparison of 16S rRNA (V3-V4) sequences was depicted in Fig. 4. Results revealed that bands 1, 2, 4, 5, 6, 7, 8, 9 and 10 were affiliated with *Clostridium* sp. while bands 3 and 4 were similar to *Lactococcus* sp. and *Lactobacillus* sp., respectively. Most of dominant bands clearly showed a high sequence similarity to *Closrtidium* sp. which identified as the major species for evolving hydrogen during dark fermentation [41]. *Clostridia* species have been reported as classical acid producers and usually ferment glucose to butyrate acetate, carbon dioxide, and molecular hydrogen [42]. However, the presence of lactate producing bacteria i.e. *Lactococus* sp. and *Lactobacillus* sp. might be responsible for the relatively low hydrogen yield obtained from our ASBR system. Lactate producing 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 [43]. In addition, lactate producing bacteria could compete with hydrogen producing *Clostridium* sp. due to sugar was degraded to lactate did not lead to the production of hydrogen [12]. Table 2 contains sequence affiliations of the major bands and soluble metabolite production for hydrogen production at different OLRs. Results indicated lane A, B, C and D were well matched to 16S rDNA fragments of the gram-positive strains related to genus *Clostridia* such as *C. acetobutylicum* and *Clostridium* sp. [43]. The *Clostridia* group is known to produce acetic acid and butyric acid as well as hydrogen through dark fermentation [44]. *Lactococus lactis* was observed at every OLRs while *Lactobacillus* sp.

was observed only at 25 and 30 g/L OLR. These microorganisms are capable of producing lactic acid as major soluble metabolite which coincided with the present of lactic acid in the fermentation broth (Table 2).

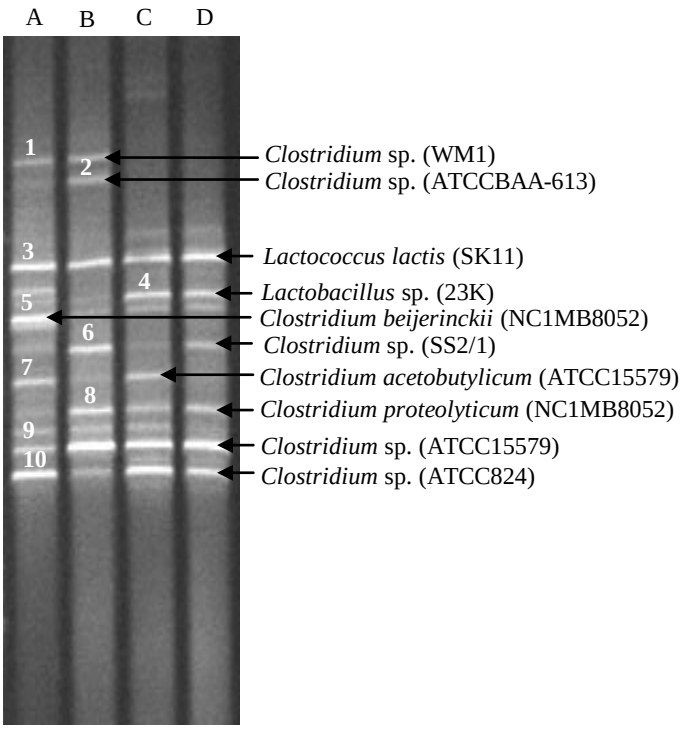

Fig. 3 DGGE profiles of 16s rRNA gene fragments from sludge sampled at steady state under various OLRs. Lenes: A: 40 g/L-d; B: 35 g/L-d; C: 30 g/L-d, D: 25 g/L-d.

V. Conclusion

Results indicated that OLRs affected hydrogen production, biomass concentration, substrate removal efficiency and microbial community. Biomass concentration increased with an increase in OLRs which is in contrast with substrate removal efficiency. The optimum OLR was found to be 30 g hexose/L-d in which a maximum yield, a maximum specific hydrogen production rate and a hydrogen content of 0.53 mol H_2/mol hexose, 32.52 mL/g MLVSS-d and 37.66%, respectively, were achieved. Microbial community analyzed by PCR-DGGE at the optimum OLR of 30 g hexose/L.d indicated that the dominant hydrogen producers were *C. acetobutylicum*, *C. proteolyticum* and *Clostridium* sp. A low hydrogen yield obtained in this study might be resulted from the present of *Lactococcus lactis* and *Lactobacillus* sp. in the fermentation.

Acknowledgments

The authors would like to thank the Office of the Higher Education Commission, Thailand for supporting the Ph.D. scholarship to P.S. by grant funded under the program Strategic Scholarships for Frontier Research Network for the Join Ph.D. Program Thai Doctoral degree. The research fund from the Research Group for Development of Hydrogen Production Process from Biomass by Microorganisms is much appreciated.

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Table 1 Summary of hydrogen production at steady state of different OLRs

OLR (g hexose/L-d)	25	30	35	40
Operational mode				
MLVSS (g/L)[a]	23.1±0.73	24.6±1.10	27.3±1.12	35.03±0.25
Substrate removal efficiency (%)[b]	76.09	46.64	24.91	15.65
Biogas production (mL/d)	2466.7±265.8	2080±130.4	1728.6±179.9	1028.6±75.6
Hydrogen content (%)	21.5±0.6	37.6±2.2	35.0±1.4	40.8±0.9
Hydrogen production (mL/d)	529.7±49.2	781.0±3.9	605.8±64.2	413.8±38.4
Hydrogen yield (mol/mol hexose)	0.15±0.01	0.53±0.02	0.22±0.01	0.20±0.01
SHPR (mL H_2/g MLVSS)[c]	22.0±0.75	32.5±2.64	22.5±0.97	12.3±0.73

a Mixed liquor volatile suspended solid
b Substrate removal efficiency = ((Initial hexose-Residual hexose)/Initial hexose) x 100
c Specific hydrogen production rate

Table 2 Affiliation of DGGE analysis for microbial community and soluble metabolite products from different OLRs

Lane	OLR (g/L-d)	Closest relative of sequenced band		Soluble metabolite products (mg/L)				
		DGGE band	Affiliation	Ethanol	Lactic acid	Acetic acid	Propionic acid	Butyric acid
A	40	A1	*Clostridium* sp.	638±93.8	1117±30.3	21.9±3.09	3.6±1.4	15.6±1.8
		A3	*Lactococcus lactis*					
		A5	*Clostridium beijerinckii*					
		A7	*Clostridium*					
		A9	*acetobutylicum*					
		A10	*Clostridium* sp.					
B	35	B1	*Clostridium* sp.	1900±33.5	1031±37.3	13.3±0.82	1.8±0.22	6.5±1.4
		B2	*Clostridium* sp.					
		B3	*Clostridium* sp.					
		B6	*Lactococcus lactis*					
		B8	*Clostridium* sp.					
		B9	*Clostridium proteolyticum*					
		B10	*Clostridium* sp.					
C	30	C3	*Clostridium* sp.	449±26.5	2029±82.3	8.6±0.98	0.9±0.15	8.3±0.69
		C4	*Lactococcus lactis*					
		C7	*Lactobacillus* sp.					
		C8	*Clostridium*					
		C9	*acetobutylicum*					
		C10	*Clostridium proteolyticum*					
D	25	D3	*Clostridium* sp.	190±9.8	1310±55.8	4.2±0.82	3.2±1.79	2.5±0.04
		D4	*Clostridium* sp.					
		D6	*Lactococcus lactis*					
		D8	*Lactobacillus* sp.					
		D9	*Clostridium* sp.					
		D10	*Clostridium proteolyticum*					
			Clostridium sp.					
			Clostridium sp.					

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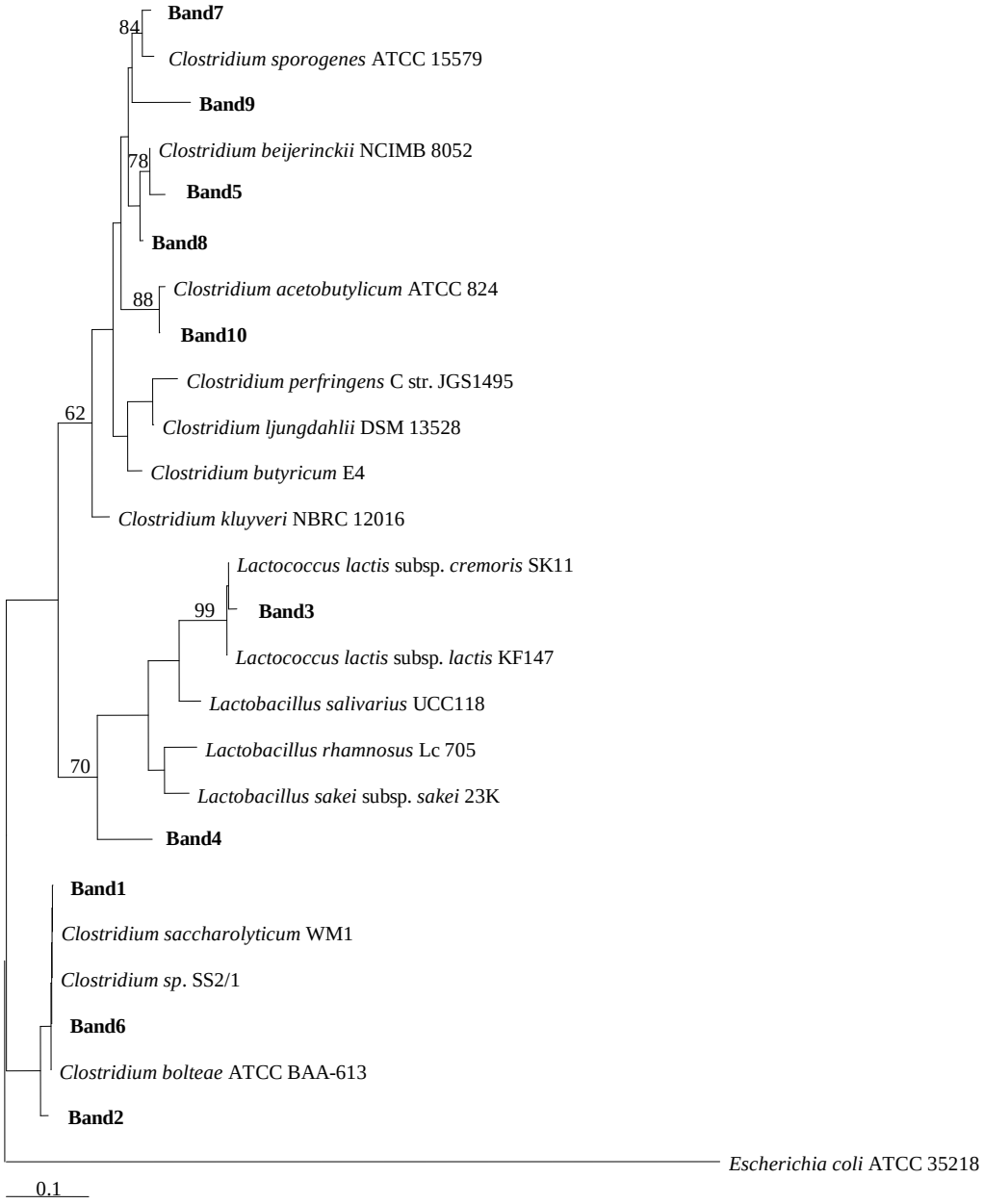

Fig. 4 – Phylogenetic tree showing the relationship between DGGE bands detected in this study and references sequences based on a comparison of 16S rRNA (V3-V4) sequences. *Escherichia coli* was used as the out group. The bar corresponds to a 10% difference in nucleotide sequence. The numbers shown next to the nodes indicate percent bootstrap values from 1000 iterations.

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