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Direct fermentation of *Laminaria japonica* for biohydrogen production by anaerobic mixed cultures

Xueqing Shi [a], *Kyung-Won Jung* [a], *Dong-Hoon Kim* [b], *Yong-Tae Ahn* [c], *Hang-Sik Shin* [a,*]

[a] *Department of Civil and Environmental Engineering, KAIST, 373-1 Guseong-dong, Yuseong-gu, Daejeon 305-701, Republic of Korea*
[b] *Wastes Energy Research Center, Korea Institute of Energy Research, 102 Gajeong-ro, Yuseong-gu, Daejeon 305-343, Republic of Korea*
[c] *Department of Civil and Environmental Engineering, The Pennsylvania State University, University Park, PA 16802, USA*

ARTICLE INFO

Article history:
Received 28 September 2010
Received in revised form
18 January 2011
Accepted 22 January 2011
Available online 31 March 2011

Keywords:
Fermentative hydrogen production
Laminaria japonica
ASBR
Methane

ABSTRACT

A few studies have been made on fermentative hydrogen production from marine algae, despite of their advantages compared with other biomass substrates. In this study, fermentative hydrogen production from *Laminaria japonica* (one brown algae species) was investigated under mesophilic condition ($35 \pm 1\,^{\circ}C$) without any pretreatment method. A feasibility test was first conducted through a series of batch cultivations, and 0.92 mol H_2/mol $hexose_{added}$, or 71.4 ml H_2/g TS of hydrogen yield was achieved at a substrate concentration of 20 g COD/L (based on carbohydrate), initial pH of 7.5, and cultivation pH of 5.5. Continuous operation for a period of 80 days was then carried out using anaerobic sequencing batch reactor (ASBR) with a hydraulic retention time (HRT) of 6 days. After operation for approximately 30 days, a stable hydrogen yield of 0.79 ± 0.03 mol H_2/mol $hexose_{added}$ was obtained. To optimize bioenergy recovery from *L. japonica*, an up-flow anaerobic sludge blanket reactor (UASBr) was applied to treat hydrogen fermentation effluent (HFE) for methane production. A maximum methane yield of 309 ± 12 ml CH_4/g COD was achieved during the 90 days operation period, where the organic loading rate (OLR) was 3.5 g COD/L/d.

1. Introduction

Energy supply and environmental protection are two crucial issues for the sustainable development of global prosperity. Over 80% of the energy consumed today in the world is derived from fossil fuels [1]. However, this current energy system is now facing two fundamental problems: gradual depletion and environmental pollution. This lack of sustainability has led researchers to search for new alternative energy sources [2]. Among various kinds of energy sources, H_2 is regarded as the most promising future energy carrier, because it produces only water upon combustion, generating a higher energy yield (122 kJ/g), which is 2.75 times greater than that of hydrocarbon fuels. In addition, hydrogen can be easily used as an automotive fuel in conventional internal combustion engines, and also can be applied in proton exchange membrane fuel cell vehicles [3].

H_2 is commercially produced by either electrolytic or thermo-chemical processes, both of which are energy intensive [4]. From an environmental engineering point of view, H_2 made from renewable resources seems to be more promising, since it meets the goal of sustainable development. In this regard, fermentative hydrogen production, where carbohydrates are directly fermented into H_2, CO_2, and organic acids/alcohols without any external energy or electron acceptors, is considered a feasible biological process to produce H_2 [5].

One of the main concerns in fermentative hydrogen production (FHP) is the high cost of the feedstock. In efforts to

* *Corresponding author*. Tel.: +82 42 350 3613; fax: +82 42 350 8640.
E-mail address: hs48@kaist.ac.kr (H.-S. Shin).

0360-3199/$ – see front matter
doi:10.1016/j.ijhydene.2011.01.125

resolve this problem, many researchers have recently focused on lignocellulosic materials, which are composed of cellulose, hemicelluloses, and lignin, as new fermentative H_2 production substrates [5–9].

Lignocellulosic biomass in nature is by far the most abundant raw material, originating from hardwood, softwood, grasses, and agricultural residues. The annual yields of lignocellulosic biomass residues worldwide are estimated to exceed 220 billion tons, equivalent to 60–80 billion tons of crude oil [10]. However, yields of H_2 produced by direct fermentation of lignocellulosic biomass are very low, mainly due to the complex structure of these substrates [11]. In order to enhance the digestibility of lignocellulosic material, different pretreatment methods have been applied, such as thermal, mechanical, acid, alkaline pretreatment, etc. However, various kinds of inhibitors are generated during these processes [6].

Marine algae is an aquatic group of cellulosic biomass. Although it has not been actively explored as an energy crop, it has many advantages for FHP, including the followings: 1) The main components of marine algae are cellulose and hemicellulose, not lignin, and thus fewer inhibitors will be generated during the pretreatment or fermentation process; 2) It has higher carbohydrate content compared with lignocellulosic biomass; and 3) It is massively abundant and easy to obtain or harvest [12,13]. Among this group, *Laminaria japonica,* a brown algae species, is a potential candidate for H_2 fermentation [14]. In 2006, the production of *L. japonica* in South Korea was estimated at around 0.8 million tons [14]. Like other brown algae, the main carbohydrate constituents of *L. japonica* are mannitol, laminaran, cellulose, and fucoidan and alginic acid, some of which are already known as good substrates for FHP [15,16]. Moreover, Jung et al. [16] reported that among various marine algae candidates, *L. japonica* showed the highest potential for FHP.

The aim of this study was to establish a stable FHP system using *L. japonica* as a feedstock. After a feasibility test under a series of batch cultivations, continuous operation was conducted using an anaerobic sequencing batch reactor (ASBR). To optimize bioenergy recovery, an up-flow anaerobic sludge blanket reactor (UASBr) was applied to treat the hydrogen fermentation effluent (HFE) for methane production.

2. Materials and methods

2.1. Seed sludge and substrate

The seed sludge was taken from an anaerobic digester in a local wastewater treatment plant in South Korea. The pH, alkalinity, and volatile suspended solid (VSS) concentration of the sludge were 7.6, 2.83 g $CaCO_3$/L, and 5.5 g/L, respectively. For screening hydrogen producing bacteria (spore-forming anaerobic bacteria such as *Clostridium sp.*) and inactivating hydrogen consumers, 20 min heating at 90 °C was applied as a pretreatment step.

The feedstock was first dried at room temperature and then ground into 0.5 mm (diameter) particles by a normal blender. There was no external nutrient addition. The composition of *L. japonica* is shown in Table 1.

2.2. Batch test

To investigate the feasibility of utilizing *L. japonica* for biohydrogen production and to determine optimal operation parameters, three batch tests were conducted under mesophilic condition (35 ± 1 °C). Batch reactors with a working volume of 3 L were seeded with heat-pretreated sludge, equivalent to 30% of the working volume, and filled with a specific amount of *L. japonica* particles and tap water. The reactor was purged with N_2 for 5 min to provide an anaerobic condition and agitated at 150 rpm pH was monitored by pH sensors and controlled by the addition of 3N KOH solution. The produced gas was collected by gas collectors and sampled using a 1 ml syringe to analyze H_2 content. Three operation parameters, substrate concentration, initial pH, and cultivation pH (initial pH was controlled at the beginning of experiment, to provide suitable growth environment for H_2 producing microbes, and cultivation pH was controlled as constant during the fermentation process to ensure the microbial metabolic pathway was suitable for H_2 production and also inhibit H_2consuming methanogenic activity), were evaluated in terms of their effect on hydrogen production. In the first batch test, the substrate concentrations were 5, 10, 20, 30, and 40 g Carbo. COD/L (calculation based on carbohydrate content and TCOD/TS ratio of the substrate); in the second batch test, the initial pH values were 7.0, 7.5, 8.0, 8.5, and 9.0; and in the third batch test, the cultivation pH values were 5.0, 5.5, 6.0, and 6.5, respectively. In the first batch test, the initial pH and cultivation pH values were kept at 8.0 and 5.5, while for the second and third batch tests, the operation parameters were selected as the optimal values based on the previous batch results (as shown in the result part, for the second batch test, the substrate concentration was 20 g Carbo. COD/L, and cultivation pH was 5.5; for the third batch test, the substrate concentration was 20 g Carbo. COD/L, and initial pH was 7.5).

To describe the hydrogen production, cumulative H_2 production curves were obtained using the modified Gompertz Eq. (1) [17].

$$H(t) = P \times \exp\left\{ -\exp\left[\frac{R \times e}{P}(\lambda - t) + 1\right]\right\} \quad (1)$$

Table 1 – Composition of *Laminaria japonica*.

Name	Composition (%)							
Laminaria japonica	Carbohydrate					Protein	Lipid	Etc.
	Total	Cellulose	Hemi-cellulose	Lignin	Etc.			
	56.4	16.9	31.0	0	8.5	8.4	1.6	33.6

where $H(t)$ = cumulative H_2 production (L) at cultivation time t (hr); P = ultimate H_2 production (L); R' = H_2 production rate (L/L/hr); λ = lag phase (hr); and e = exp(1) = 2.71828.

2.3. ASBR and UASBr operation

A schematic diagram of ASBR and UASBr is presented in Fig. 1. In this study, an ASBR with 5 L working volume was seeded with 30% of heat-treated sludge and filled with the substrate at 35 ± 1 °C. The anaerobic condition was provided by N_2 purging, the mixing ratio was 150 rpm, and pH was controlled by the addition of 3N KOH solution. The substrate concentration, initial pH, and operational pH were 20 g Carbo. COD/L, 7.5 and 5.5 (determined by batch tests). Continuous operation was delayed until approximately 0.5 mol H_2/mol $hexose_{added}$ of H_2 was produced in the batch operation [18]. For ASBR operation, the hydraulic retention time (HRT) was 6 days, and the batch cycle was operated at 36 h, where 0.5 h was for feeding, 32 h for reaction, 3 h for settling, and 0.5 h for decanting. Once stable H_2 production was achieved after 30 days of continuous operation, the hydrogen fermentation effluent (HFE) was collected and used as a substrate for methane production in an UASBr with a working volume of 3.5 L. The granular sludge was obtained from a brewery wastewater treatment plant. And as substrate, HFE was centrifuged to remove the solid particles. HRT was maintained at 2 days. In order to optimize methane production, the organic loading rate (OLR) was gradually increased from 1 to 5 g COD/L/d. The methane production rate and methane yield were measured daily.

2.4. Analytical methods

H_2 content in biogas was determined by a gas chromatography (GC, Cow Mac series 580) using a thermal conductivity detector and a 1.8 mm × 3.2 mm stainless-steel column packed with molecular sieve 5 A with N_2 as a carrier gas. The contents of CH_4, N_2, and CO_2 were measured using a GC of the same model noted previously with a 1.8 mm × 3.2 mm stainless-steel column packed with Porapak Q (80/100 mesh), using helium as a carrier gas. The temperatures of the injector, detector, and column were kept at 80, 90, and 50 °C, respectively, in both GCs. Volatile fatty acids (VFAs, C2–C6) and lactic acid were analyzed by high performance liquid chromatography (HPLC) (Finnigan Spectra SYSTEM LC, Thermo Electron Co.) with an ultraviolet (210 nm) detector (UV1000, Thermo Electron) and an 100 mm × 7.8 mm Fast Acid Analysis column (Bio-Rad Lab.) using 0.005 M H_2SO_4 as a mobile phase. Carbohydrate was determined using the phenol-sulfuric acid method [19]. COD was measured according to Standard Methods [17]. RNA concentration was determined by the colorimetric method of Liwarska-Bizukoje E. and Ledakowicz [20].

2.5. Microbial analysis

To analyze the microbial communities, DNA in the mixed sample during continuous operation was extracted using an Ultraclean Soil DNA Kit (Cat # 12800-50; Mo Bio Laboratory Inc., USA). The 16S rDNA fragments were amplified by polymerase chain reaction (PCR). The region corresponding to positions 357–518 in the 16S rDNA of *Escherichia coli* was PCR-amplified using the forward primer EUB357f (5′-CCTACGGGAGGCAGCAG-3′) with a GC clamp (5′-CGCCC-GCCGCGCCCCGCGCCCGGCCGCCGCCCCCGCC CC-3′) at the 5′ end to stabilize the melting behavior of the DNA fragments and the reverse primer UNIV518r (5′-ATTACC GCGGCTGCTGG-3′). The procedure for PCR-DGGE was described in a previous study by the authors [18]. After PCR amplification, PCR products were purified with using a Multiscreen Vacuum Manifold (MILLIPORE com., USA). All strands of the purified PCR products were sequenced with the primer EUB357f using an ABIPRISM Big Terminator Cycle Sequencing Kit (Applied Biosystems, USA) in accordance with the manufacturer's instructions. A search of GenBank was conducted using the BLAST program.

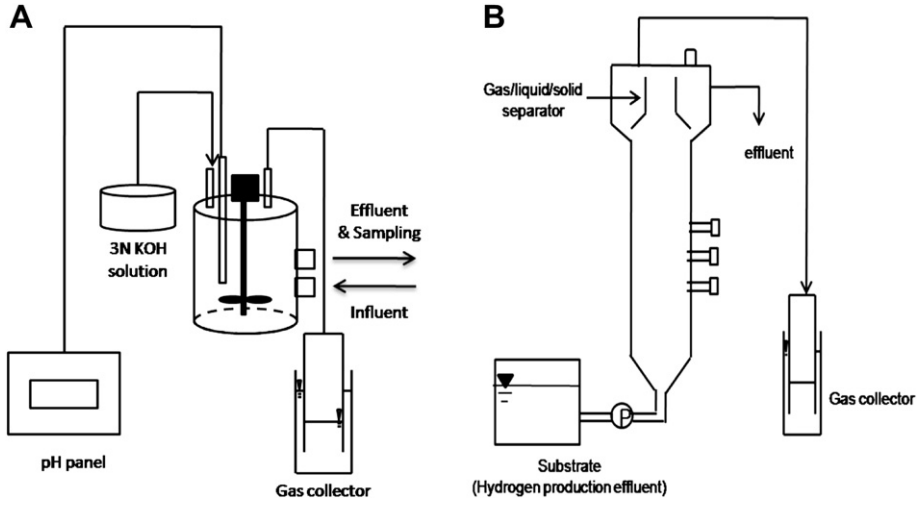

Fig. 1 – Schematic diagrams of (A) ASBR and (B) UASBr.

Table 2 – H_2 production and effluent compositions.

Feasibility test		H_2 Production (L)	H_2 yield (mol H_2/mol $hexose_{added}$)	H_2 production rate (L/L/h)	RNA conc.[a] (mg/L)	Organic acid (g COD/L)			
						Total[b]	HAc[c]	HPr[d]	HBu[e]
Substrate	5	1.4	0.55	0.06	110	4.8	3.6	0	1.2
Conc. (g Carbo.	10	3.5	0.69	0.10	124	8.4	5.3	0	3.1
COD/L)	20	9.4	0.92	0.26	138	19.1	13.6	1.6	3.9
	30	12.6	0.83	0.24	126	26.6	15.9	3.6	7.1
	40	11.6	0.58	0.16	116	26.7	4.7	12.7	20.7
Initial pH	7.0	8.8	0.86	0.15	137	13.2	9.1	11.7	3.0
	7.5	9.6	0.95	0.23	143	18.4	12.3	1.4	4.7
	8.0	9.2	0.91	0.21	142	17.4	10.6	1.5	5.4
	8.5	8.9	0.88	0.22	140	15.4	9.9	2.1	3.4
	9.0	7.8	0.77	0.14	129	12.1	5.7	1.6	4.8
Cultivation pH	5.0	8.7	0.86	0.14	131	15.7	6.3	1.1	8.3
	5.5	9.3	0.92	0.25	141	17.8	14.5	0.6	2.3
	6.0	9.3	0.91	0.23	139	17.7	14.2	1.1	2.4
	6.5	7.3	0.72	0.13	116	10.4	4.6	0	5.8

a RNA conc. = RNA concentration when H_2 production finished.
b Total = sum of acetate, propionate, butyrate.
c HAc = acetate.
d HPr = propionate.
e HBu = butyrate.

3. Results and discussion

3.1. *Feasibility test*

H_2 yield is generally a good indicator of the effectiveness of H_2 production. H_2 yield results obtained through three batch tests are shown in Table 2, and the maximum H_2 yield of 0.92 mol H_2/mol $hexose_{added}$, or 71.4 ml H_2/g TS in Table 3, was achieved under a substrate concentration of 20 g Carbo. COD/L, initial pH of 7.5, and cultivation pH of 5.5. Compared with results from previous studies on H_2 production from raw lignocellulosic biomass, this value is much higher, as shown in Table 3. Some studies have noted that laminarin, the second most abundant compound in brown algae, can be easily degraded by microbes, as laminarase is found in various kinds of microorganisms [21,22]. Thus, owing to high carbohydrate content, an absence of lignin, and high biodegradability, *L. japonica* is a highly feasible feedstock for FHP.

Table 3 – Comparison of H_2 production obtained in this study using *Laminaria japonica* with previous study using raw lignocellulosic biomass.

Substrate	Temperature	Maximum hydrogen yield achieved	Reference
Grass silage	70 °C	16 ml H_2/g TVS	[9]
Cornstalk	36 °C	3.16 ml H_2/g TVS	[10]
Cornstalk	36 °C	23.3 ml H_2/g TVS	[24]
Beer lees	36 °C	6.8 ml H_2/g TVS	[25]
Beer lees	35 °C	3.79 ml H_2/g TS	[26]
Laminaria japonica	35 °C	71.4 ml H_2/g TS	In this study

In terms of organic acid production during H_2 fermentation, acetate and butyrate are accompanied by H_2 production, while propionate is mainly produced in H_2 consuming reactions, while lactate is known as a byproduct that is not related to H_2 production. As provided in Table 2, lactate was not detected in any of the samples, while propionate was found in some of them, which implies that the heat treatment can effectively inactivate non-spore-forming and non-H_2-producing bacteria such as lactic acid bacteria (LAB). However, the propionic acid bacteria (PAB) were inhibited by heat-shock but not totally exterminated [18]. In previous studies, the butyrate/acetate (B/A) ratio has been used as an indicator for evaluating the effectiveness of biohydrogen production. Furthermore, Arooj et al. [23] reported that the relationship between B/A ratio and H_2 yield was linear. In contrast, in this study, there was no linear relationship between B/A ratio and

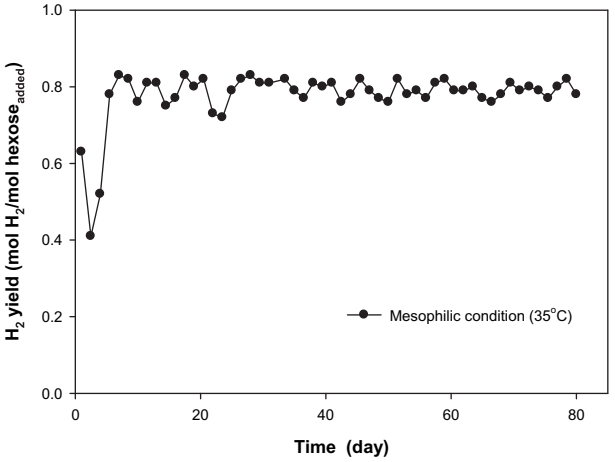

Fig. 2 – H_2 yield during ASBR operation.

Table 4 – H_2 yield and effluent compositions at steady state.

Reactor	H_2 yield (mol H_2/mol hexose$_{added}$)	Carbohydrate removal (%)	Organic acid (g/L)			
			Total[a]	HAc[b]	HPr[c]	HBu[d]
ASBR	0.79 ± 0.03	82.9 ± 1.7	13.9 ± 0.4	9.3 ± 0.3	1.0 ± 0.1	3.6 ± 0.3

a Total = sum of acetate, propionate, butyrate.
b HAc = acetate.
c HPr = propionate.
d HBu = butyrate.

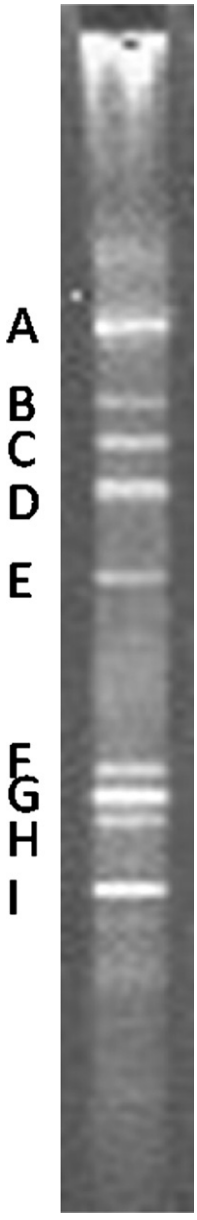

Fig. 3 – DGGE profile for bacterial diversity from mixed sample.

H_2 yield, which indicates that the substrate type can strongly affect the microbial metabolic pathway.

VSS generally indicate the presence of microorganisms; however, VSS cannot differentiate organic solids and microorganisms. On the other hand, RNA, result from microbial metabolism, can reflect the microbial growth condition. Thus, in this study, RNA concentration from HFE was measured to show the active microbial concentration and thereby determine the effect of each operation parameter on H_2 production. From the results it was found that RNA concentration accorded with total organic acid production and also H_2 yield, as shown in Table 2. Both H_2 and organic acids are metabolic byproducts from the feedstock, generated through the growth of hydrogen producing microbes. It is therefore reasonable that the optimal growth condition would result in the highest microbial growth (shown as highest RNA concentration) and maximum generation of metabolic products. This is the first report describing the application of RNA concentration along with H_2 yield to suggest the optimal operation conditions for FHP.

3.2. *Continuous operation*

Fig. 2 shows the H_2 yield during ASBR operation. Initially, a drastic decrease of H_2 production was observed upon changing the operation mode from batch to continuous operation. Kim et al. [18] noted that this phenomenon might be due to the regrowth of PAB, which is inhibited by heat-shock but not totally exterminated. After continuous operation for about 30 days, a stable H_2 yield of 0.79 ± 0.03 mol H_2/mol hexose$_{added}$ was obtained. In about 50 days, a steady state was achieved, as listed in Table 4. Acetate and butyrate were

Table 5 – Affiliation of DGGE fragments determined by their 16S rDNA and isolated microorganisms.

Band	Affiliation	Similarity (%)	NCBI GenBank No.
A	*Clostridium sp.*	92	DQ677024.1
B	*Ruminococcus sp.*	85	AY669260.1
C	*Clostridium polysaccharolyticum*	92	X77839.1
D	*Clostridium sp.*	94	AY827856.1
E	*Eubacterium limosum*	89	U67159.1
F	*Clostridium sp.*	96	DQ677005.1
G	*Uncultured Clostridiales bacterium*	97	FR695912.1
H	*Uncultured Clostridiales bacterium*	96	HQ103933.1
I	*Uncultured Bacteroides sp.*	88	HM754520.1

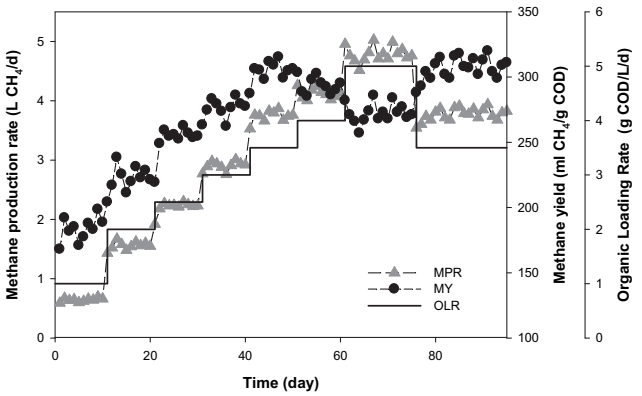

Fig. 4 – CH_4 production from HFE.

the main VFA components while propionate was kept at low concentration and lactate was not detected. These results show that heat treatment of the inoculum was an effective means of inhibiting LAB and H_2 consumers, and that the operation conditions and feedstock were favorable for H_2 production.

In order to detect dominant microorganisms, a mixed sample was taken from ASBR and the bacterial diversity was monitored by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). From the DGGE profile (Fig. 3), a total of 9 DNA bands were detected, where each band represents one microbial species. The results of 16S rDNA sequences shown in Table 5 reveal that, among the total DNA bands, 6 matched well with *Clostridium sp.* (band A, C, D, F, G, H), while the remaining bands were matched to *Ruminococcus sp.*, *Eubacterium limosum*, and *Bacteroides sp.*, respectively. All of these detected bacteria are well known as H_2 producing bacteria and also cellulolytic bacteria [27–30]. Accordingly, high H_2 yield and high degradation efficiency (indicated by the carbohydrate removal rate) from this cellulosic biomass feedstock could be achieved even though no pretreatment method was applied. This is the first report documenting the existence of hydrogen producing bacteria in FHP from *L. japonica*.

Table 6 – UASBr performance for CH_4 production.

OLR (g-COD/L/d)	Methane yield (mL CH_4/g COD)	Methane production rate (L CH_4/d)	COD removal (%)
1	185 ± 8	0.65 ± 0.03	95
2	223 ± 11	1.56 ± 0.08	94
2.5	254 ± 9	2.23 ± 0.08	94
3	279 ± 10	2.93 ± 0.11	93
3.5	309 ± 12	3.80 ± 0.15	94
4	293 ± 14	4.12 ± 0.20	90
5	273 ± 16	4.80 ± 0.28	86

3.3. *CH_4-UASBr performance*

Fig. 4 shows the results of UASBr operation for 90 days. Methane content in the biogas was around 70–74% during the whole operation period. From day 1 to day 50, as the OLR increased, both methane yield and methane production rate increased simultaneously. When the OLR was 3.5 g COD/L/d, maximum methane yield of 309 ± 12 ml CH_4/g COD was achieved, which was 88.3% of the theoretically achievable CH_4 yield. Further increase of the OLR caused a decrease of methane yield and COD removal efficiency, respectively, thus indicating that an OLR above 3.5 g COD/L/d was beyond the COD degradation capacity of the microorganisms in UASBr.

Table 6 shows the UASBr performance under different OLR. COD removal effective during the whole continuous operation period, indicating that the HFE is a favorable feedstock for methane production.

3.4. *Bioenergy recovery efficiency and COD balance from organic solid particles*

Bioenergy recovery and COD removal efficiency of the two-stage fermentation system treating organic solids were evaluated if which the results were presented in Fig. 5. Optimal

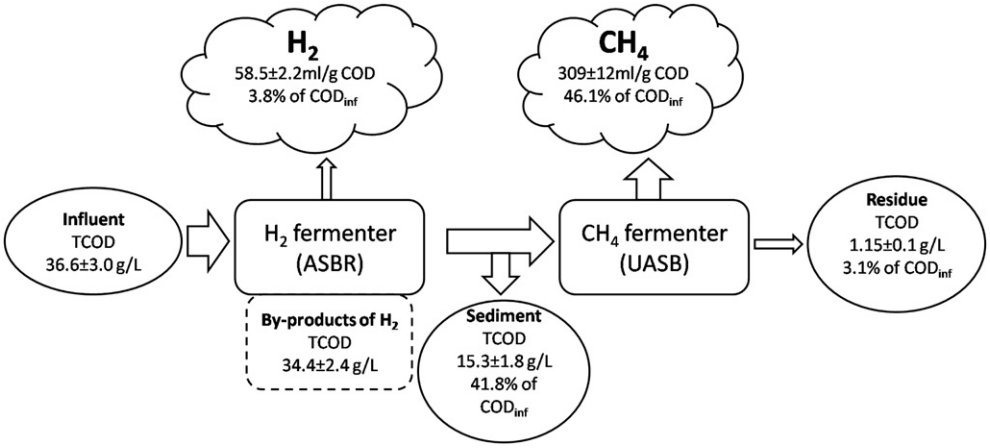

Fig. 5 – Bioenergy recovery and COD removal efficiency of the two-stage fermentation system.

ASBR and UASBr performance (with highest H_2 and CH_4 yield) in this study were used in this evaluation.

It was found that 49.9% biogas conversion (H_2 3.8%, CH_4 46.1%) and 55.1% COD removal were achieved in this system. The overall performance was limited by UASBr, given that 41.8% COD remained as sediment by centrifugation. However, it was difficult to conclude whether UASBr was economically suitable for this two-stage system, because OLR and CH_4 production rates were higher, and HRT was lower than that of other reactor types including CSTR and ASBR [31,32].

In addition to the change of reactor type for CH_4 fermentation, further additional treatment on the sediment, such as post-treatment or anaerobic dry digestion process could be adopted to obtain more CH_4 from the waste portion of the total bioenergy.

4. Conclusions

Direct fermentation of *L. japonica* for biohydrogen production was attempted through batch tests and continuous operation under mesophilic condition. The following conclusions have been drawn from this study:

1. In the feasibility test, high H_2 yield of 0.92 mol H_2/mol $hexose_{added}$, or 71.4 ml H_2/g TS, was obtained, indicating the strong potential of *L. japonica* for fermentative hydrogen production.
2. Stable H_2 production was achieved in the continuous operation for 80 days using ASBR, with a H_2 yield of 0.79 ± 0.03 mol H_2/mol $hexose_{added}$. A microbial diversity analysis indicated that *Clostridium sp*. was the predominant bacterial group in this mixed cultures system.
3. UASBr showed high performance to treat HFE. However, the total bioenergy recovery through this two-stage fermentation system was limited due to particulate solid portion that could not be fed to UASBr.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. 2010-0026904).

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