JMB
Journal of Microbiology and Biotechnology

Online ISSN : 1738-8872
Pimt ISSN : 1017-7825

JMB Papers in Press. First Published online Dec 7, 2018
DOI: 10.4014/jmb.1808.08023

Manuscript Number: JMB18-08023

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Article Type: Research article

Keywords: Ammonia, salt, bio-hydrogen, dark fermentation

Fermentative bio-hydrogen production of food waste in the presence of different concentration of salt (Na^+) and nitrogen

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Fermentation of food waste in the presence of different concentrations of salt (Na^+) and ammonia was conducted to investigate the interrelation of Na^+ and ammonia contents for bio-hydrogen production. Analysis of the experimental results showed that the peak hydrogen production differed according to the ammonia and Na^+ concentration. The peak hydrogen productions achieved were (97.60, 91.94, and 49.31) mL/g COD at (291.41, 768.75, and 1,037.89) mg-N/L of ammonia and (600, 1,000, and 4,000) mg-Na^+/L of salt concentration, respectively. At peak hydrogen production, the ammonia concentration increased with increasing salt concentration in the medium. This means that for peak hydrogen production, the C/N ratio reduced with increasing salt content in the medium. The butyrate/acetate (B/A) ratio was higher in proportion to the bio-hydrogen production (r-square: 0.71, p-value: 0.0006). Different concentrations of Na^+ and ammonia in the medium produced diverse microbial communities. *Klebsiella* sp., *Enterobacter* sp., and *Clostridium* sp. were predominant with high bio-hydrogen production, while *Lactococcus* sp. was found with low bio-hydrogen production.

Keywords: ammonia, salt, bio-hydrogen, dark fermentation

Introduction

Hydrogen production technologies have gained significant attention due to the increased demand for hydrogen as an energy carrier [1]. The only by-product of combustion produced is hydrogen, making it a pollutant-free energy alternative. Hydrolysis of chemical hydrides or metals has recently received attention as an optimal method for the production of hydrogen.

Running title: Fermentative bio-hydrogen production of food waste.
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[2-4] However, other chemical hydrogen production methods, such as steam reforming of hydrocarbon and partial oxidation of combustible fossil, are energy intensive and require high temperature, resulting in the release of carbon dioxide and other pollutants as by-products [5]. Under these circumstances, biological hydrogen production from biomass appears to be one of the promising ways to replace traditional methods, as it can produce hydrogen competitively under ambient conditions, without causing pollution problems [6-7].

Hydrogen can be generated in a biological production process of direct bio-photolysis, indirect bio-photolysis, photo-fermentation, or dark-fermentation [8]. Compared to the other three processes, the dark-fermentation process has a high production rate, and much simpler operational requirements [9-10]. The dark fermentation process does not need light, and has a very high evolution rate of hydrogen, compared to photosynthetic processes [9]. Valuable by-products and biomass produced in the hydrogen fermentation make dark fermentation more favorable for commercialization [11-12].

Even though dark fermentation of organic waste has high potential to produce bio-hydrogen together with the reduction of waste [13], low bio-hydrogen yield is a bottleneck to its practical application. Further research is needed on optimizing microbial community enrichment or the operational parameters of the reactor, such as pH, salt content, temperature, or H_2 partial pressure to improve bio-hydrogen yield [14]. Na^+ is one of the parameters that affect bio-hydrogen production. It is originally a micronutrient for bacterial growth [15], but it is also related to hydrogen formation based on the Na^+ gradient in cell. The Na^+ gradient produced by high extracellular Na^+ concentration enhances Fd_{ox} reduction by NADH, resulting in a higher concentration of Fd_{red}, which favors the formation of H_2 [16-17]. Nevertheless, high Na^+ concentrations could cause an inhibitory effect on biological hydrogen production, because of changes in the metabolic pathway to favor the formation of other metabolites, rather than hydrogen production. [18-19]. Nitrogen is another affecting factor for hydrogen producers, as it is an important component of living matter [20]. Although ammonia nitrogen (from NH_3 or NH_4^+) is an essential nutrient for bacterial growth, it has been reported to be toxic in dark fermentation processes if the concentration is higher than threshold [21, 22]. When NH_3 penetrates the cell membrane, it is protonized to NH_4^+, which destroys the balance in intracellular pH [23]. But to date, little information is available about variations of bio-hydrogen production caused by the interrelation effect of two or more parameters in the sources.

Food and food processing wastes could be potential feedstock for biological hydrogen production, because of the high content of organic matter and nutrients [24]. Food waste contains large portions of carbohydrate, protein, and fat, which can readily be degraded via dark fermentation. However, the composition of food waste is varied depending on its source. Most food waste may be lacking in nitrogen, but some food wastes contain high salt or nitrogen content [17, 25]. Therefore, in order to utilize food wastes appropriately as a feedstock for dark-fermentation, the effects of salinity or nitrogen contents on the activities and metabolic processes of hydrogen production microbes need to be studied.

This study investigated the interrelation of salt (Na^+) and ammonia contents on the fermentative bio-hydrogen production of food waste. For this purpose, the bio-hydrogen production efficiency of food waste with different Na^+ and ammonia regimes was analyzed by a modified Gompertz equation. Volatile Fatty Acids (VFAs) formations under the different conditions of salt (Na^+) and ammonia contents were also investigated. In addition, analysis of the microbial community involved in the fermentative bio-hydrogen production of the food waste was conducted by denaturing gradient gel electrophoresis (DGGE) of a PCR-amplified v3 region of 16s rDNA.

Materials and Methods

Microorganisms and Culture

The return activated sludge from a wastewater treatment plant was used as seed sludge for this study. The synthetic medium [26] contained 15 g/L of sucrose, 10 mg/L of $CaCl_2 \cdot 2H_2O$, 100 mg/L of $MgSO_4 \cdot 7H_2O$, 10 mg/L of NaCl, 2 g/L of NH_4HCO_3, 1 g/L of KH_2PO_4, 15 mg/L of $MnSO_4 \cdot 7H_2O$, 10 mg/L of $NaMoO_4 \cdot 2H_2O$, and 2.78 mg/L of $FeCl_2$. The 10 mL of seed sludge was added into 90 mL of the synthetic medium under anaerobic conditions [27]. The initial pH of the medium at serum bottles was set to be 7.0. For the acclimation of seed microflora, the cultures were sequentially transferred from the serum bottle into fresh medium.

Operation of Batch Reactor

The substrate, a food waste, was obtained from a university cafeteria and was crushed by an electrical blender in the anaerobic glove box filled with nitrogen gas. Table 1 shows the characteristics of the food waste, of which all of the substrates were filtered through a stainless

steel sieve (US Mesh No. 10). NH_4Cl and NaCl were analytical grade (Ducksan Co., Korea) and used as the nitrogen and Na source, respectively. Ammonia and Na^+ concentration of the medium were prepared in the range of (100–5,000) mg-N/L of ammonia and (600–4,000) mg-Na^+/L. Initial COD concentration was adjusted to be 10,000 mg/L. The carbon to nitrogen (C/N) ratio in the feed stock, which ranged (0.75–37.5), was artificially adjusted according to the purpose of the experiment.

Table 1. Characteristics of the collected food waste.

Biological hydrogen production potential (BHP) tests of food waste were conducted in the batch reactor with a 1.5 L working volume. Fig. 1 is a schematic diagram of the batch reactor, which was stirred at 250 rpm using a magnetic stirrer. The headspace was initially maintained in an anaerobic condition using nitrogen gas. After that, 15 mL of seed solution was added, resulting in an initial volatile suspended solids (VSS) concentration of approximately 10 mg/L. The reactor was kept at 30 °C in the dark to avoid the growth of photosynthetic bacteria. The pH of the reactor was kept constant at 5.5 by the addition of KOH solution [27]. The generated biogas was collected by a gas collector of the reactor. At regular intervals, 0.2 mL of headspace gas was withdrawn for analysis.

Fig. 1. A sketch of the batch reactor for bio-hydrogen production.

Analytical Methods

The hydrogen and CO_2 concentration were analyzed using a gas chromatography (GOW-MAC, USA), equipped with thermal conductivity detector (TCD). The applied separation column and carrier gas were a 6'×1/8" stainless steel SS 350A Molsieve 13X and nitrogen gas at a flow rate of 30 mL/min, respectively. The injection port, column, and detector temperature were (80, 100, and 100) ℃, respectively.

VSS for cell concentration, total or soluble chemical oxygen demand (COD), total nitrogen (T-N), and total phosphorus (T-P) were analyzed according to the standard methods [28-29]. Carbohydrate and protein concentrations were measured by the phenol-sulfuric acid and modified Lowry methods, respectively [29-30]. High performance liquid chromatography (KNAUER, Germany) and a UV detector operating at 210 nm were used for VFAs (butyrate, acetate, lactate, and propionate) analysis. The column was a Supelcogel C-610H (130 mm × 7.8 mm ID). Ammonia and sodium concentrations were measured using ion chromatography (IC, Metrohm compact 761, Switzerland).

Model Development

The cumulative bio-hydrogen production of the food waste was analyzed by the modified Gompertz equation (Eq. (1)) [27]

$$M = P \cdot \exp\{-\exp[\frac{R \cdot e}{P}(\lambda - t) + 1]\} \qquad (1)$$

where, M and P are the cumulative hydrogen production (in mL) and the hydrogen production potential (mL), respectively. R is the maximum hydrogen production rate (mL/h), λ is the lag-phase time (h), e is exp(1)=2.718, and t is the incubation time (h).

Microbial Community Analysis

PCR was performed to assess the composition of the microbial community. DNA was extracted from microorganisms using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., USA). The eluted DNA from the microorganisms in the samples was reamplified by PCR with the forward primer 341f (5'-CCT ACG GGA GGC AGC AG-3') and the reverse primer 518r (5'-ATT ACC GCG GCT GCT GG-3') [31]. The quality of the DNA samples was analyzed by DGGE [32]. Sequencing analyses of the bands on the gradient gel were conducted for the closest reference microorganisms in the GenBank database using the BLAST program [33].

Results and Discussion

Bio-hydrogen Production according to Various Salt (Na^+) Concentration

A numbers of studies have reported the negative effect of Na^+ and ammonia on hydrogen production with mixed culture [17, 34-38]. High Na^+ concentrations exceeding the threshold level may result in the formation of other metabolites than hydrogen, which cause the suppression of bio-hydrogen production [19]. The low cell growth caused by this utilization of energy finally results in low hydrogen production [17]. High concentrations of ammonia have also been reported to be toxic on anaerobic digestion processes [37].

Several studies [16, 21, 38] reported that ammonia concentrations resulted in shifts to generate varying concentration of soluble metabolites by altering metabolic pathways of the dark fermentation process. These end-products could also have a suppressive effect on bio-hydrogen production. Such a strategy as dilution of the reactor contents below suppressive threshold concentration, or microbial acclimation to the inhibitors prior to the fermentation process, could be proposed to reduce the inhibition of bio-hydrogen production. To optimize the strategies, it is worthwhile evaluating the interrelation of ammonia and Na^+ concentration on bio-hydrogen production, rather than evaluating the influence of the independent factor.

The effects of Na^+ and ammonia concentration for bio-hydrogen production were investigated with medium containing in the range of (200–5,000) mg-N/L of ammonia and (600–4,000) mg-Na^+/L. The hydrogen production curves over time were analyzed by Eq. (1). Table 2 lists the hydrogen production rates against the Na^+ and ammonia concentrations.

Table 2. Parameter values for bio-hydrogen gas with different salt (Na^+) and ammonia concentrations of the food waste.

Fig. 2 plots the hydrogen production potential rate (P_h) against the ammonia concentration in the presence of Na^+, and the relationship was obtained using a Weibull, 5-parameter Equation (r^2 = 0.97, Sigma Plot 2006 Systat Software Inc., USA). Analysis of the data showed that the peak hydrogen production differed according to the ammonia and Na^+ concentrations in the medium. The peak hydrogen productions achieved were (97.60, 91.94, and 49.31) mL/g COD at (291.41, 768.75, and 1,037.89) mg-N/L of ammonia and (600, 1,000, and 4,000) mg-Na^+/L of salt concentration, respectively. Since initial COD value was 10,000 mg/L, C/N ratios at peak hydrogen production were 12.87, 4.88, and 3.61, respectively. This means that the presence of higher salt (Na^+) contents reduced the optimum C/N ratio for bio-hydrogen

production. An earlier study [25] reported that peak hydrogen productivity reached a C/N-ratio of 47, but this experimental result implies that to maximize the production of bio-hydrogen, inhibition factors must be considered, because the C/N ratio could change according to the Na^+ concentration, as shown above.

Hydrogen production and substrate consumption could be inhibited in the presence of toxic substances while different degree of inhibition would be shown, depending on the microbial species [15, 39, 40]. Our experimental results show increasing peak hydrogen production with lower sodium concentration. A previous study [41] reported that NADH-ferredoxin oxidoreductase activity could be enhanced in the presence of the proper concentration of Na^+ for hydrogen production. This result suggests that bio-hydrogen production using food waste as substrate could be more efficient when Na^+ and ammonia concentration are properly adjusted. It is also noteworthy that at the high strength of sodium (Na^+) content in Fig. 2, the slope of cumulative hydrogen production against ammonia concentration in the medium is much more gentle than a lower strength. It is believed the bio-hydrogen generation considering the complex correlation of nitrogen and salt (Na^+) concentration, has not been published previously.

Fig. 2. Bio-hydrogen production profile in the various concentrations of ammonia and sodium (Na^+). Symbols on the graph, represent average values of three experimental data. Error bar of experimental data, was conducted using Sigma Plot software.

Variations of individual VFAs concentration and B/A ratio

Acid production is usually accompanied by hydrogen production, which in turn is coupled with solvent production, such as ethanol, methanol, butanol, propanol, and acetone. Thus, monitoring of these intermediates reflects metabolic pathways of microorganisms. Table 3 shows VFAs distribution and COD balance in the reactor. Carbon content in biomass was assumed at $C_5H_7O_2N$. Others (%) in Table 3 mean the soluble COD minus the sum of VFAs and soluble carbohydrate concentration divided by the initial COD concentration, referring to solvent concentrations of products that were not detected by the UV detector on HPLC at 210 nm after reaction [27, 42]. Initial COD of 10,000 mg / L was converted to 100%. Values of other compounds are also converted into COD value, and divided by initial COD for recovery calculation. At various concentrations of Na^+ and ammonia, acetate, butyrate, and propionate were the abundant species. As hydrogen production increased, concentrations of acetate and

butyrate increased; in contrast, reduction of hydrogen production resulted in high propionate concentration.

Table 3. Product distribution and recovery ratio on COD basis as a function of the Na^+ and ammonia concentrations (unit: %).

Fig. 3 shows the B/A ratio plotted and analyzed, with hydrogen production. The B/A ratio was higher in proportion to bio-hydrogen production (B/A ratio = 0.3242×Hydrogen production (%) - 0.4297, r-square: 0.71, p-value: 0.0006). Previous studies [27, 43] have reported that higher ratio of B/A, could be more preferable for hydrogen production. The B/A ratio change implied a microbial community shift, due to changes of ammonia or Na^+ concentrations, resulting in lower production of hydrogen. This finding agreed with previous results, that in the presence of high Na^+ concentration, hydrogen-producing bacteria displaced the fermentation process towards the acetate synthesis pathway, instead of the butyrate pathway [17].

Fig. 3. Changes of B/A ratio against the bio-hydrogen production. Hydrogen production (%) represents the produced bio-hydrogen gas from the initial substrate concentration (as COD). B/A ratio = 0.3242×Hydrogen production (%) - 0.4297, r-square: 0.71, and p-value: 0.0006

PCR-DGGE Profile Analysis of the Microbial Community

The experimental results suggested that the microbial community changes in accord with the Na^+ and ammonia concentrations leads the changes of hydrogen production activities and acid production. The effect of Na^+ and ammonia on the microbial community responsible for bio-hydrogen production was investigated and confirmed by PCR-DGGE analysis on 16s rDNA. The relative diversity of the microbial community and the degree of abundance of each microbial group are closely related with the number of bands and the band intensity in the gel, respectively [44]. Fig. 4 shows the PCR-DGGE profiles of each sample, which revealed band patterns in the presence of Na^+ and ammonia.

Fig. 4. PCR-DGGE profile of the cultures at various concentrations of Na^+ and ammonia. The numbers above the arrow on each lane represent the concentrations of Na^+ and ammonia, and the B/A ratio, respectively.

Based on the band intensities, in 600 mg-Na^+/L sodium concentration, bands (B, D, F, H, I, J, K, M, Q, S, and V) are present. In 1,000 mg-Na^+/L sodium concentration, bands (A, B, C, E, N, P, Q, R, U, X, Y, Z, AA, and AB) are detected, indicating the existence of various microbial species. In 4,000 mg-Na^+/L sodium concentration, bands (B and C) are clearly evident, while bands (G, L, N, O, T, W, and AA) are dimly visible. In 600 mg-Na^+/L of sodium concentration, (F, H, I, J, K, M, Q, S, and V) are major bands with relatively high B/A ratio, representing high production of bio-hydrogen. In 1,000 mg-Na^+/L, bands (C, P, U, X, and Y) are predominant, with high B/A ratio. In 4,000 mg-Na^+/L, bands (N and R) are distinguishable with high B/A ratio. Taken together, under different Na^+ concentration, distinct band patterns with similar B/A ratio are evident. This represents that each sample has microbial diversity according to the Na^+ and ammonia profiles, even though it has a similar hydrogen production rate. Bands of interest were sequenced to investigate the microbial diversity among samples. The NCBI BLAST program was used to identify microbes and accession numbers from the 16s rDNA. Table 4 shows the closet affiliation and similarity of the bands on the gradient gel. Three major bacterial taxa were identified, with nine sequences related to the class γ-Proteobacteria, five affiliated with Bacteria, and fourteen related to the phylum Firmicutes.

Table 4. The affiliation of DGGE bands as determined by the rDNA sequences.

Clostridia bacterium, *Clostridium saccharobutylicum*, *Clostridium algidicarnis*, *Clostridium butyricum*, and *Clostridium* sp. were detected as part of the *Clostridium* genus. *Hydrogenoanaerobacterium* sp., *Enterococcus* sp., *Lactococcus lactis*, and *Lactococcus lactis subsp. lactis* were also identified. The *Clostridium* genus of phylum Firmicute showed comparatively high dominance, as shown in Fig. 4 by bands (E, I, M, N, P, V, X, Y, and AB), and had been reported to generate hydrogen gas under the fermentation process [33, 45-48].

The genus in the class γ-proteobacteria were the *Klebsiella* and *Enterobacter* genus, which had also been known as hydrogen-production bacteria [29]. The *Klebsiella* genus was shown in Fig. 4 in bands (H, Q, T, and W). The *Enterobacter* genus was also present in bands (J, K, L, U, and Z). They have been reported for hydrogen production [49–54]. Although *Enterococcus* sp. or *Hydrogenoanaerobacterium* sp. have been reported as hydrogen production microorganism [55-56], *Lactococcus* sp., designated as bands (D and G), is known as an Inhibitor of hydrogen fermentation [57]. It is known that most of the microbes identified in this study are associated with hydrogen production, except for *Lactococcus* sp. This study conclusively shows that the interrelations of the ammonia and Na^+ concentration change the microbial community in the medium, and consequently show different hydrogen production rates and B/A ratios. The analysis of microbial communities with different concentrations of Na^+ and ammonia shows various diversities for each condition. At 300 mg-N/L of ammonia and 600 mg-Na^+/L sodium concentration, *Klebsiella* sp., *Clostridium* sp., and *Enterobacter* sp. were predominant. At 800 mg-N/L of ammonia and 1,000 mg-Na^+/L sodium concentration, *Clostridium* sp., and *Enterobacter* sp. were the most abundant hydrogen-producing bacteria. At 1,500 mg-N/L of ammonia and 4,000 mg-Na^+/L sodium concentration, *Clostridium* sp. and *Hydrogenoanaerobacterium* sp. were the microbes that were most responsible for hydrogen production.

Acknowledgements

This work is financially supported by the Korea Ministry of Environment (MOE) as a Knowledge-based environmental service waste to energy recycling human resource development project.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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Table 1. Characteristics of the collected food waste.

Parameter	Concentration (mg/L)
Total COD	321,788 ± 80,448
Soluble COD	201,117 ± 62,570
Total Nitrogen(T-N)	6,491 ± 292
Total Phosphorus(T-P)	397 ± 9
Total Carbohydrate	263,300 ± 1,571
Protein	9,447 ± 1,158
Na^+	19,200 ± 546

Table 2. Parameter values for bio-hydrogen gas with different salt (Na^+) and ammonia concentrations of the food waste.

Sodium concentration (mg-Na^+/L)	ammonia concentration (mg-N/L)	Production potential		H_2 yield (mL H_2/g COD)	Production rate		r^2
		P_h(mL)	p-value		R_h (mL/g dry wt biomass/hr)	p-value	
600	100	226.86	<0.0001	14.38	13.83	0.0017	0.98
	150	344.64	<0.0001	26.37	15.68	0.0009	0.98
	200	1268.67	<0.0001	102.00	83.43	<0.0001	0.99
	350	1416.06	<0.0001	93.33	94.29	0.0107	0.97
	600	848.27	<0.0001	59.64	18.14	<0.0001	0.98
	1500	379.89	<0.0001	19.64	21.83	0.0011	0.98
	3000	269.21	<0.0001	12.14	13.95	0.0265	0.96
	5000	203.88	<0.0001	13.41	25.52	0.0061	0.98
1000	200	775.20	<0.0001	51.97	36.37	0.0018	0.98
	600	1309.50	<0.0001	77.11	55.60	0.0769	0.92
	1500	579.57	<0.0001	37.44	41.11	<0.0001	0.99
	3000	291.53	<0.0001	16.86	20.74	0.0193	0.97
4000	100	514.28	<0.0001	41.85	19.12	<0.0001	0.99
	200	495.29	<0.0001	39.47	30.91	0.0767	0.98
	600	673.73	<0.0001	65.14	22.87	0.0148	0.97
	1500	661.55	<0.0001	40.45	27.19	0.0091	0.98
	3000	340.08	<0.0001	26.91	4.23	0.0007	0.96
	5000	241.90	<0.0001	18.09	14.50	0.0259	0.96

Table 3. Product distribution and recovery ratio on COD basis as a function of the Na^+ and ammonia concentrations (unit: %).

Sodium (mg-Na^+/L)	Ammonia (mg-N/L)	Initial COD	Fatty acid					Biomass	Soluble COD	remain of carbohydrate	Others	H_2	Recovery	B/A ratio
			Acetate	Pro pionate	Formate	Butyrate	Lactate							
600	150	100	0.32	N.D.	0.06	0.09	0.42	7.17	90.01	9.84	79.28	2.03	99.21	0.28
	200	100	1.11	N.D.	N.D	1.37	0.00	14.54	77.78	3.81	71.49	6.66	98.99	1.23
	350	100	0.88	N.D	N.D	2.53	1.57	16.02	75.76	6.63	64.16	8.53	100.30	2.88
	600	100	0.76	1.30	N.D	1.19	0.00	13.44	81.79	2.03	76.52	4.48	99.71	1.57
1000	200	100	3.09	1.93	0.34	1.66	N.D	15.43	88.01	2.54	78.45	4.45	107.89	0.54
	600	100	1.45	1.75	N.D	1.98	0.00	15.93	85.02	3.98	75.86	6.13	107.08	1.37
	1500	100	2.61	2.46	0.29	2.15	0.00	15.49	85.21	6.47	71.23	3.23	103.93	0.82
	3000	100	3.28	3.34	N.D	2.45	N.D	15.55	91.41	2.77	79.57	2.79	109.76	0.75
4000	100	100	2.34	1.17	0.79	1.21	0.00	19.24	81.50	9.21	66.78	3.48	104.22	0.52
	200	100	3.79	2.06	N.D	1.58	N.D	18.84	76.92	9.03	60.46	3.59	99.35	0.42
	600	100	4.10	2.11	0.00	2.90	0.00	17.48	84.04	9.91	66.60	4.60	106.11	0.71
	1500	100	3.56	2.61	N.D	3.80	2.08	14.31	84.97	5.47	67.44	3.45	102.73	1.07

Recovery: (soluble COD + H_2 + Biomass)/ initial COD×100 (%).

494
495
496 Table 4. The affiliation of DGGE bands as determined by the rDNA sequences.

Band name	Gen Band Search Result: Phylogenetically Closet Relative	Accession No.	Similarity	Taxonomic Description (Class)
A	*Enterococcus* sp. HGB0104	JX519903.1	99 %	Firmicutes
B	*Bacterium* NLAE-zl-C199	JQ607927.1	95 %	Bacteria
C	Uncultured *bacterium* clone OC2_N15	KC860573.1	98 %	Bacteria
D	*Lactococcus lactis* isolate TIL8	JX893570.1	91 %	Firmicute
E	*Clostridia bacterium* S130(2)-2	GU136556.1	92 %	Firmicutes
F	Uncultured *bacterium* clone 70-1-2	DQ842542.1	100 %	Bacteria
G	*Lactococcus lactis subsp. lactis* strain NM151-4	HM218597.1	82 %	Firmicute
H	*Klebsiella pneumonia* strain ND6	JF927782.1	95 %	γ-proteobacteria
I	*Clostridium saccharobutylicum* DSM 13864	CP006721.1	90 %	Firmicute
J	*Enterobacter hormaechei* strain LAB 229	KJ156326.1	97 %	γ-proteobacteria
K	*Enterobacter* sp. NCCP-231	AB610883.1	94 %	γ-proteobacteria
L	Uncultured *Enterobacteriaceae bacterium* clone CSC13DXRNA 24	KJ624484.1	100 %	γ-proteobacteria
M	*Clostridium algidicarnis* strain E107	JX267120.1	86 %	Firmicute
N	*Clostridium butyricum* strain W5	DQ831124.1	100 %	Firmicute
O	*Hydrogenoanaerobacterium* sp. YN3	AB537982.1	98 %	Firmicute
P	Uncultured Clostridium sp. clone 8426	KF508692.1	90 %	Firmicutes
Q	*Klebsiella* sp. d252	FJ950657.1	86 %	γ-proteobacteria
R	*Hydrogenoanaerobacterium* sp. YN3	AB537982.1	98 %	Firmicutes
S	Uncultured *bacterium* clone B.11	KF512526.1	95 %	Bacteria
T	*Klebsiella pneumonia* strain FY2	KJ599860.1	94 %	γ-proteobacteria
U	*Enterobacter* sp. 2356	JX174233.1	98 %	γ-proteobacteria
V	*Clostridium* sp. R6	EF174500.1	91 %	Firmicute
W	*Klebsiella* sp. CCFM8383	KJ803940.1	95 %	γ-proteobacteria
X	*Clostridium* sp. MCF105	KC155326.1	98 %	Firmicutes
Y	Uncultured *Clostridium* sp. clone b2-73	JX575825.1	98 %	Firmicutes
Z	*Enterobacter* sp. 2358	JX174235.1	99 %	γ-proteobacteria
AA	Uncultured *bacterium* clone GDIC2IK01AKQ69	JF670002.1	85 %	Bacteria
AB	Uncultured *Clostridium* sp. clone T12	DQ168179.1	89 %	Firmicutes

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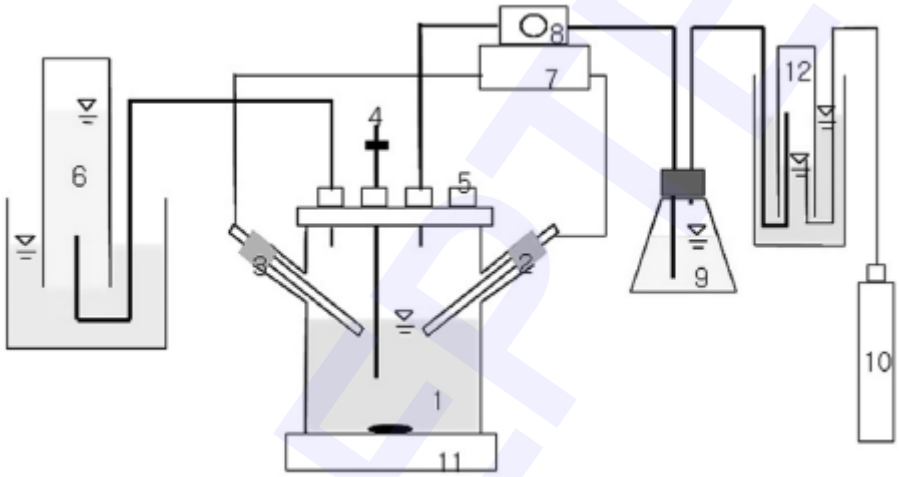
Fig. 1. A sketch of the batch reactor for bio-hydrogen production.



1. Culture medium (1.5L) 2. pH sensor 3. Temperature sensor
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Fig. 2. Bio-hydrogen production profile in the various concentrations of ammonia and sodium (Na^+). The initial concentration of COD at each mode was 10,000 mg/L. Symbols on the graph represent average values, of three experimental data. Error bar of experimental data was conducted, using Sigma Plot software.

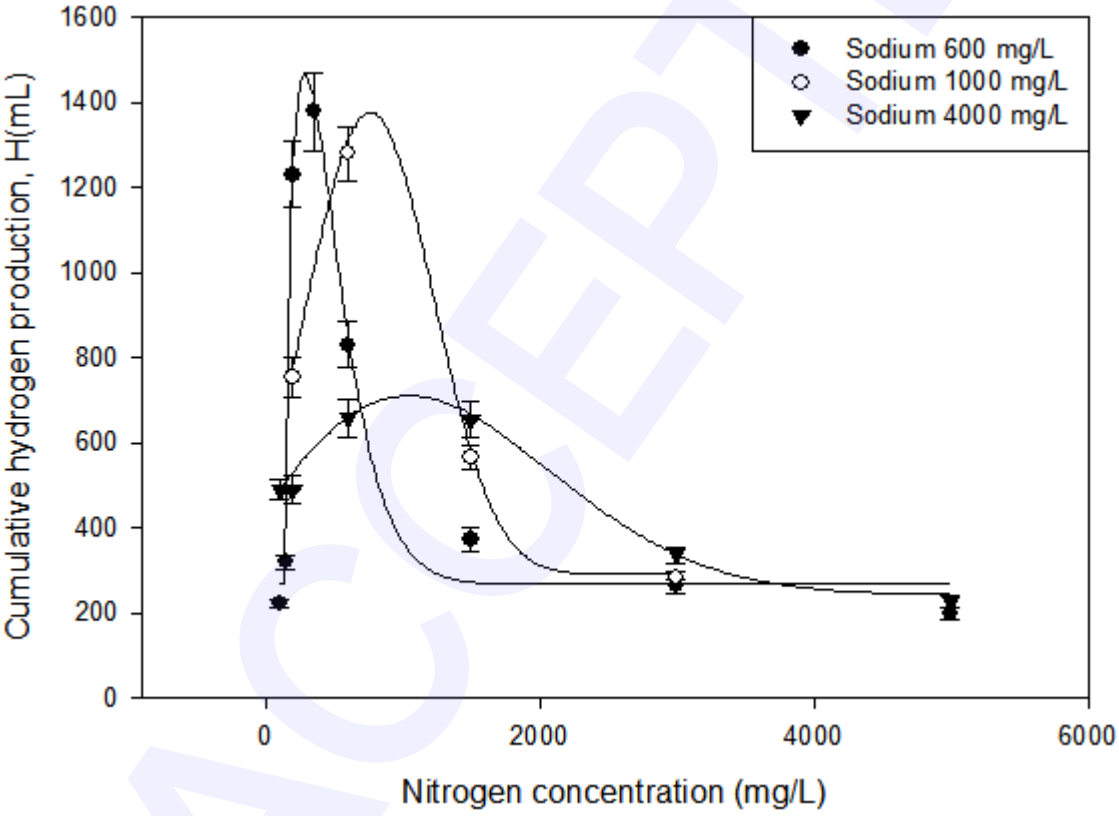


Fig. 3. Changes of B/A ratio against the bio-hydrogen production. Hydrogen production (%) represents the produced bio-hydrogen gas from the initial substrate concentration (as COD). Regression of experimental data was conducted using Sigma Plot software. B/A ratio = 0.3242 × Hydrogen production (%) – 0.4297. (r-square: 0.71, and p-value: 0.0006 Straight line: regression fit, Dotted line: 95% confidence intervals)

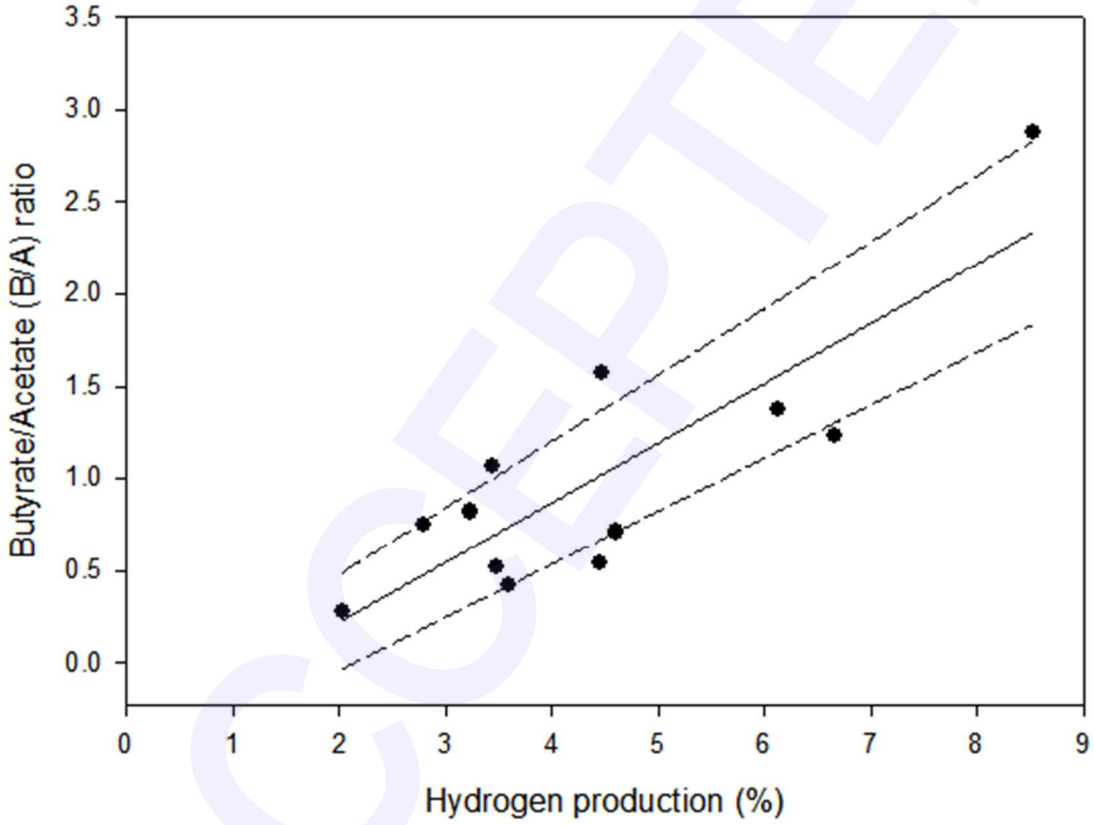
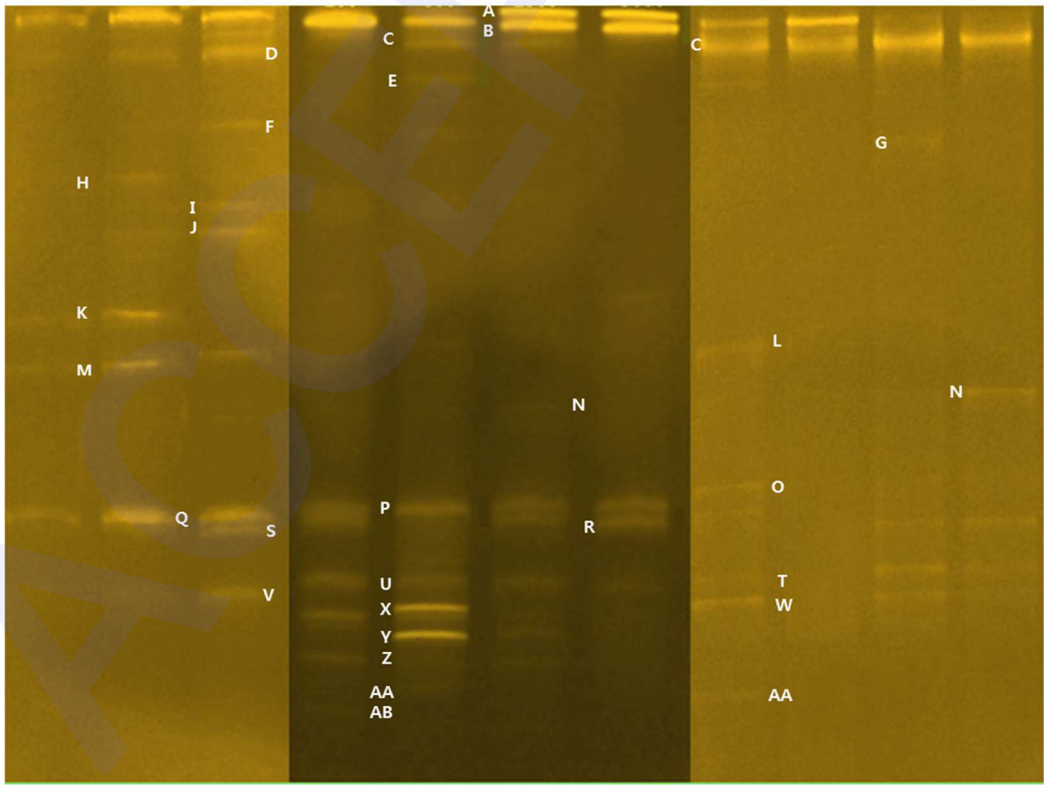


Fig. 4. PCR-DGGE profile of the cultures at various concentrations of Na^+ and ammonia. The numbers above the arrow on each lane represent the concentrations of Na^+ and ammonia, and the B/A ratio, respectively.

Na^+ (mg/L)	600			1,000				4,000			
Ammonia (mg-N/L)	150	200	600	200	600	1,500	3,000	100	200	600	1,500
B/A ratio	0.28	1.23	1.27	0.54	1.37	0.82	0.75	0.52	0.42	0.71	1.07



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	600	100	4.10	2.11	0.00	2.90	0.00	17.48	84.04	9.91	66.60	4.60	106.11	0.71
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X	*Clostridium* sp. MCF105	KC155326.1	98 %	Firmicutes
Y	Uncultured *Clostridium* sp. clone b2-73	JX575825.1	98 %	Firmicutes
Z	*Enterobacter* sp. 2358	JX174235.1	99 %	γ-proteobacteria
AA	Uncultured *bacterium* clone GDIC2IK01AKQ69	JF670002.1	85 %	Bacteria
AB	Uncultured *Clostridium* sp. clone T12	DQ168179.1	89 %	Firmicutes

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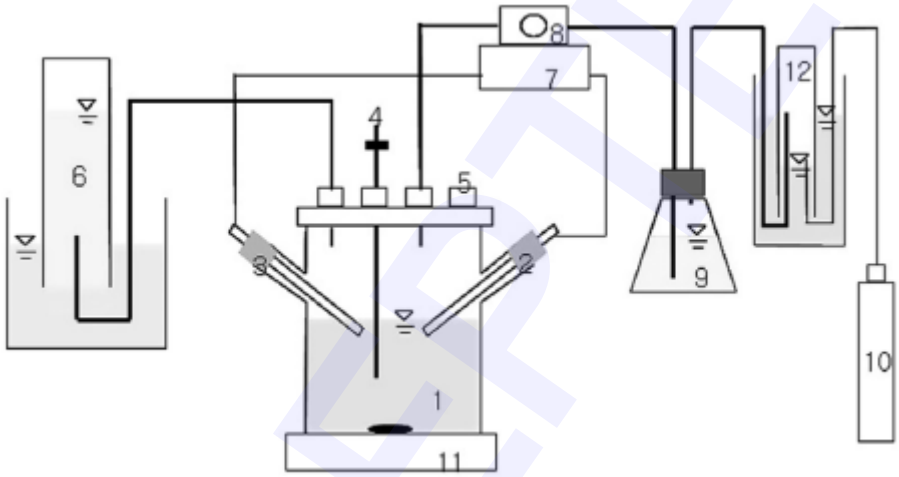

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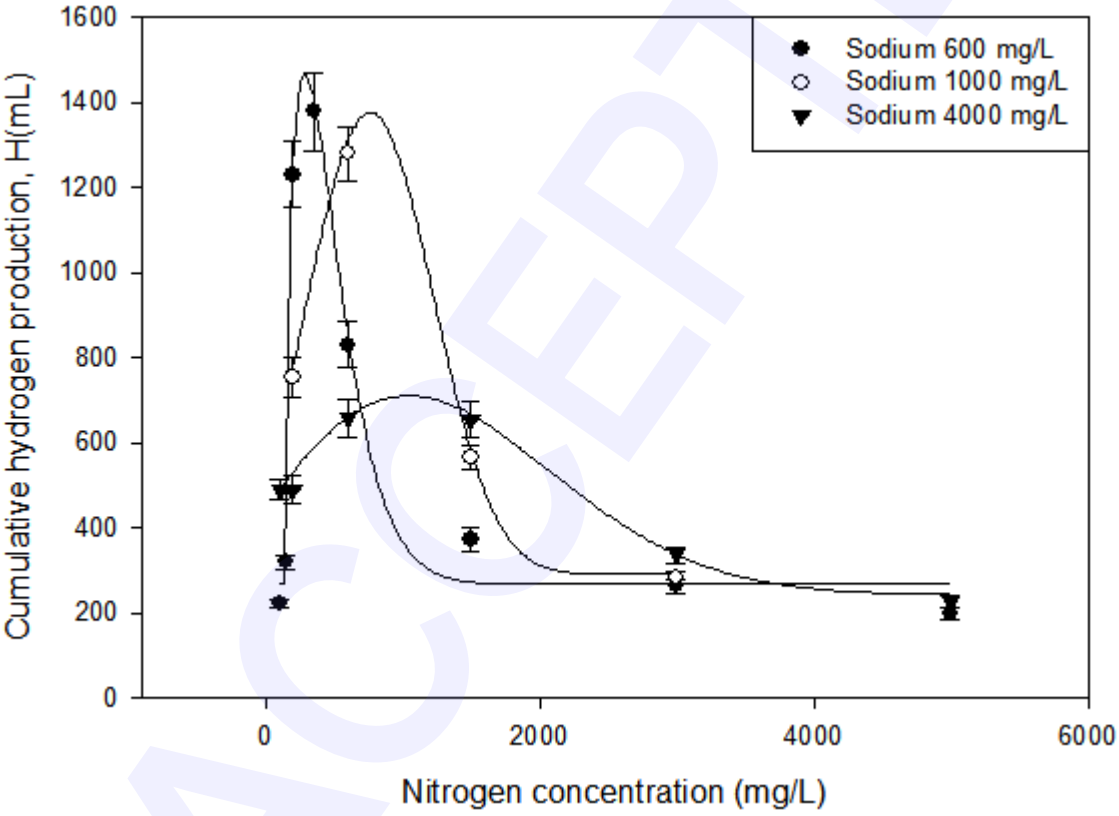
Fig. 2. Bio-hydrogen production profile in the various concentrations of ammonia and sodium (Na^+). The initial concentration of COD at each mode was 10,000 mg/L. Symbols on the graph represent average values, of three experimental data. Error bar of experimental data was conducted, using Sigma Plot software.



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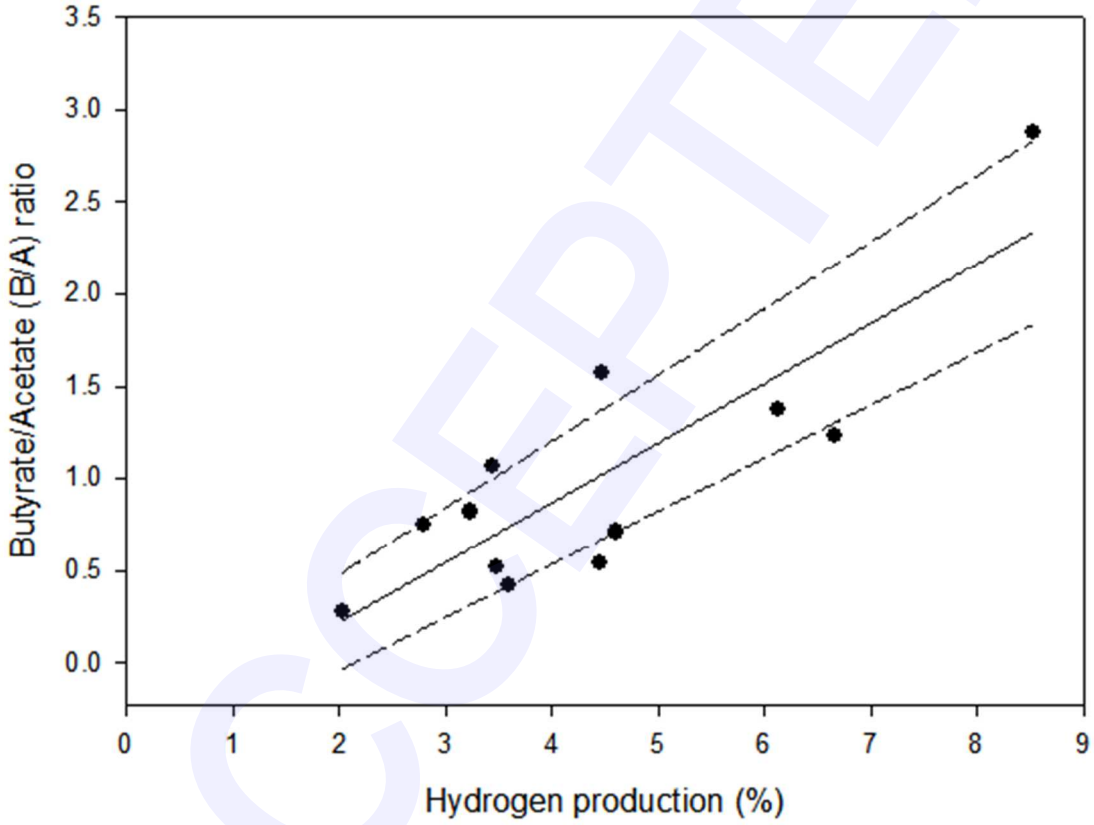

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Na^+ (mg/L)	600			1,000				4,000			
Ammonia (mg-N/L)	150	200	600	200	600	1,500	3,000	100	200	600	1,500
B/A ratio	0.28	1.23	1.27	0.54	1.37	0.82	0.75	0.52	0.42	0.71	1.07