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Effects of temperature and substrate concentration on biological hydrogen production from starch

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ABSTRACT

This study investigates the effects of temperature and substrate concentration on biological hydrogen production from starch using mixed cultures. In this work, although hydrogen was successfully produced under the thermophilic condition, stable hydrogen production was not observed under the mesophilic condition. In the thermophilic reactor, the maximum hydrogen yield was 2.8 mol H_2/mol glucose at 20 g/l-starch; however, hydrogen yield decreased drastically with the change of by-product distribution when substrate concentration was over 30 g/l-starch. A negative correlation was observed between the hydrogen yield and the total concentration of undissociated acids.

1. Introduction

Global environmental problems and sustainable energy supply are serious concerns today and for future generations. With fossil fuel supplies coming ever closer to being exhausted, there is a very real need to discover an alternative source of energy. Thus, technological developments for the generation of sustainable bio-energy are of significance. A sustainable production system for hydrogen, which is one of the promising alternative energy carriers, is expected to be developed from biomass by fermentation.

The precise role that temperature has on the stability of hydrogen production remains unclear at this stage. There are two temperature phases that have been commonly used for hydrogen fermentation: the mesophilic and thermophilic conditions. The mesophilic condition is preferable to the thermophilic condition from economical and technological viewpoints, providing a high yield of hydrogen could be produced stably under such conditions. Nevertheless, when mesophilic fermentation takes place, the presence of non-hydrogen-producing bacteria presents a critical problem with regard to the sustainability of hydrogen production [1,2]. Some researchers have succeeded in producing a continuous hydrogen production using the acclimated inocula without any pre-treatment [3–5], and others have reported on various physicochemical treatments which can be added to the

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inoculum or culture to enhance hydrogen production and its sustainability by harvesting the spore-forming clostridial bacteria [1,6–9]. To date, the feasibility of mesophilic continuous hydrogen fermentation in the absence of treatment has yet to be clarified. In the case of the thermophilic condition, however, it has been recently demonstrated that stable continuous hydrogen production can be achieved [10–15]. The clarification of the differences between the mechanisms of hydrogen production in the absence of treatment in both the mesophilic and thermophilic conditions is necessary in order to have a better understanding of the hydrogen production process from actual wastewater and waste.

Substrate concentration has recently been recognized to be an important factor for hydrogen production in continuous cultures. A number of investigations have shown that the metabolic pathways and microbial community structures, for example, are affected by it and that hydrogen production is inhibited by the intermediate products [16–18]. These studies, however, were performed mesophilically with thermally pretreated inocula, which suggest that the effects on the mesophilic spore-forming clostridial bacteria were the focus of the studies, whereas the microbial community structure in the actual hydrogenic reactor is diverse due to contamination from influent biomass or wastewater. It should also be noted that, to our knowledge, there have been few studies investigating the effects of substrate concentration on thermophilic continuous hydrogen fermentation.

The objective of this study is to ascertain the stability of hydrogen production in both the mesophilic and thermophilic conditions and to clarify the effects of the substrate concentration on those fermentative characteristics by operating two identical reactors using the same seed sludge, without any treatment of the seed sludge or culture. The results of this study are expected to provide an insight into the actual mechanisms of the hydrogen production process.

2. Materials and methods

2.1. Seed sludge

To investigate the effect of temperature on enrichment of H_2 producing bacteria in both of the mesophilic and thermophilic conditions, the microbial community of the seed sludge was diversified by combining two types of digested sludge (1, 2) and acidogenic sludge (3). They were cultured in different environments (conditions for reactor operation and feeds). The three types of sludge used in this study are as follows:

1) the mesophilically digested sludge treating waste activated sludge;
2) the thermophilically digested sludge treating night soil and kitchen waste;
3) the thermophilic acidogenic sludge treating potato waste [19].

Each was diluted to a total solid (TS) of 1% prior to mixing with the same volume. The seed sludge was not subjected to any pre-treatment.

2.2. Medium composition

One liter of the medium was composed of the following: starch; peptone; K_2HPO_4; yeast extract, 500 mg; $NaHCO_3$, 4000 mg; Na_2CO_3, 2000 mg; $MgSO_4 \cdot 7H_2O$, 120 mg; $FeSO_4 \cdot 7H_2O$, 25 mg; KI, 2.5 mg; $MnSO_4 \cdot 4H_2O$, 2.5 mg; $CoCl_2 \cdot 6H_2O$, 2.5 mg; $ZnSO_4 \cdot 7H_2O$, 0.5 mg; $NiCl_2 \cdot 7H_2O$, 0.5 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.5 mg; H_3BO_3, 0.5 mg. The ratio (w/w) of starch:peptone:-phosphorus was maintained at 100:10:1 during the experiment, in consideration of the fact that the nitrogen concentration of the peptone was $14.6 \pm 4.4\%$ (w/w) ($n = 3$) [20]. The concentration of starch was changed from 15 to 70 g/l in each operational phase. The composition of minerals and trace elements in this study was modified according to Lee et al. [21].

2.3. Experimental apparatus and reactor operation

Two identical completely stirred tank reactors (CSTRs) with a working volume of 1 l were used in this study [15]. Prior to the continuous operation, a batch operation was carried out for each reactor. At the beginning of the batch operation, 300 ml of the seed sludge was mixed with 700 ml of the medium and the headspace of the reactor was replaced with pure nitrogen gas. Continuous operation was started after the exponential growth phase in the batch operation was reached. The hydraulic retention time (HRT) was maintained at 24 h. The temperatures of the two reactors (35 °C and 55 °C) and the substrate tank (4 °C) were controlled with the water jacket externally equipped on the reactor. The produced gas was collected in aluminum gasbags and calibrated to 0 °C and 760 mmHg. The pH of the reactor was not regulated.

2.4. Batch tests to investigate effects of hydrogenotrophic bacteria

The batch tests were carried out to investigate the effects of hydrogenotrophic homoacetogens in both of the mesophilic and thermophilic reactors following the procedures (1)–(8) [22]. A vial with an internal volume of 122 ml was used for the batch tests. The inocula were taken from both reactors on the 140th day (cultivated at 15 g/l-starch). The batch tests were carried out in duplicate.

(1) The medium (35 ml) without any starch was prepared in the vial (the composition of 1 l of this medium was as follows: $FeSO_4 \cdot 7H_2$, 125 mg; $MgSO_4 \cdot 7H_2O$, 120 mg; yeast extract, 200 mg; peptone, 200 mg; NH_4Cl, 4000 mg; the mineral solution, 1 ml; 0.1% rezazurine solution, 2 ml; $NaHCO_3$, 4 g).
(2) The headspace was replaced with the nitrogen gas (purged for 2 min).
(3) This vial was incubated in a shaking water bath at 35 °C.
(4) The culture broth (5 ml) in the reactors was inoculated into the medium using sterilized syringes.
(5) The pHs were adjusted at the same values in the reactors with 1 N of HCl and NaOH.
(6) The headspace of the vial was replaced with a mixed gas of H_2 (80%) and CO_2 (20%).
(7) The pressure of the headspace was adjusted to 1 atm using a glass syringe (Tamano, Japan).

(8) Finally, the vial was incubated in a shaking water bath (BT 200, Yamato Scientific Co., Ltd., Japan) at either 35 °C or 55 °C.

2.5. *Analytical methods*

The proportion of hydrogen in the biogas was determined by a gas chromatograph (Shimadzu 8A) equipped with a thermal conductivity detector (TCD) and a stainless steel column packed with molecular sieve 5A (60/80 3 mm∅). The temperatures of the detector and column were maintained at 100 °C and 60 °C, respectively. In order to determine the proportion of carbon dioxide, nitrogen and methane, the same model of a gas chromatograph (Shimadzu 8A) equipped with a TCD and a stainless steel column packed with Porapak Q was used. The temperatures of the detector and the column were maintained at 100 °C and 70 °C, respectively. The carbohydrate concentration was analyzed by the phenol–sulfuric acid method, using glucose as a standard [15]. The organic acid concentration was analyzed by capillary electrophoresis (I.D., 75 μm; UV detector 220 nm) (Photal CAPI-3200, Ohtsuka, Japan). The solvent concentration was measured by a gas chromatograph (Shimadzu GC-1700) equipped with a flame ionization detector (FID) and a 30 m column (J&W DB-WAX). The volatile suspended solids (VSS) and the chemical oxygen demand (COD) were determined according to the procedures described in the Standard Methods [23]. The compositions of the VSS and peptone were analyzed by an elemental analyzer VARIO EL III CHNSO (Elementar Analysensysteme).

3. Results

3.1. *Effects of temperature on hydrogen production*

The time courses of gas production in the mesophilic (35 °C) and thermophilic (55 °C) reactors are illustrated in Fig. 1. Methane was not detected in either of the reactors during the experiment.

In the mesophilic reactor, stable continuous hydrogen production was not attained, although hydrogen was produced for a short time when the substrate concentration was increased. The produced biogas was mostly composed of carbon dioxide during the steady states, since the hydrogen produced was readily consumed by hydrogen consumers such

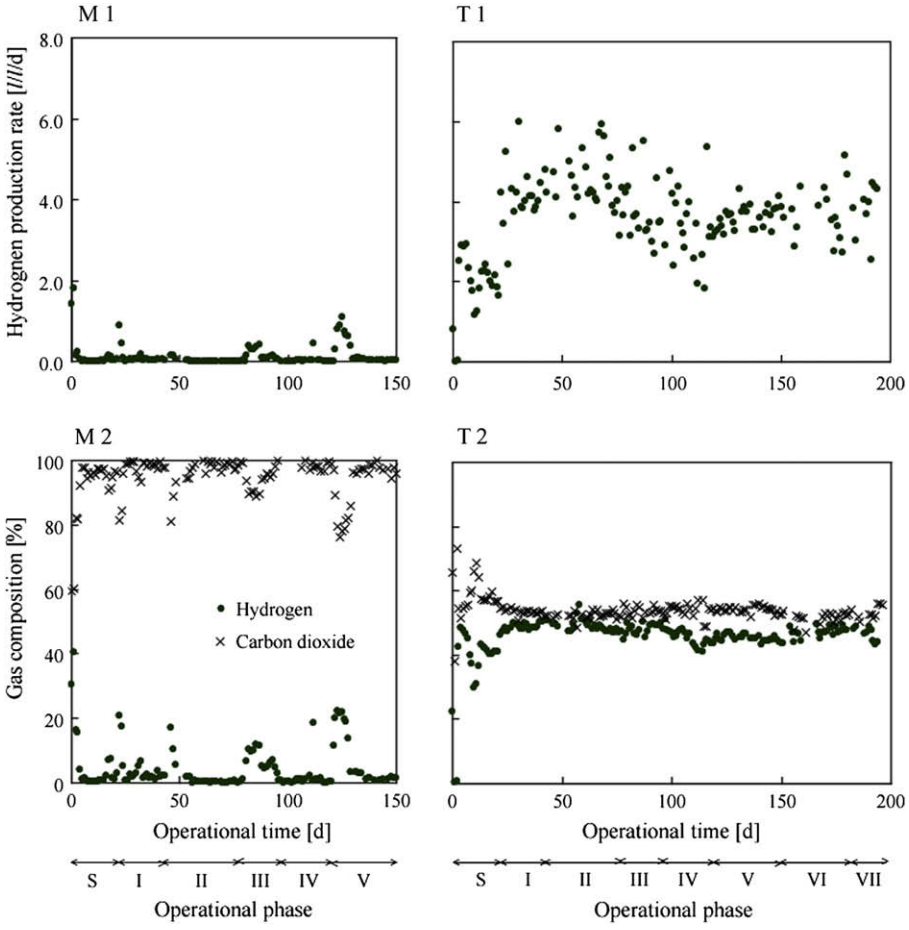

Fig. 1 – The time courses of gas production in the mesophilic and thermophilic reactors. The 'S' represents start-up and I–VII represent each operational phase. M1 and M2 show the hydrogen production rate and biogas composition in the mesophilic reactor, respectively; while T1 and T2 show the hydrogen production rate and biogas composition in the thermophilic reactor, respectively.

as hydrogenotrophic homoacetogens (see below). In the thermophilic reactor, on the other hand, hydrogen was produced stably during the whole experimental period (over 6 months). This study obviously shows that the thermophilic condition is preferable to the mesophilic condition for producing hydrogen stably.

3.2. *A comparison of the influence of hydrogenotrophic homoacetogens between mesophilic and thermophilic conditions*

Fig. 2 illustrates the time courses of hydrogen content in the headspace at 35 °C and 55 °C. Table 1 summarizes the production of volatile fatty acids (VFAs) during the batch tests at 35 and 55 °C. Methane was not detected in either temperatures condition during the batch test. Hydrogen was more rapidly consumed at 35 °C than at 55 °C. The main product was acetic acid under both temperature conditions; however the increase of acetic acid at 35 °C was significantly larger than at 55 °C. The production of VFAs was not significant in the controls, indicating that increased acetate was converted from hydrogen in the headspace. When adjusted for acetate production by the control at 35 °C, the amount of acetate produced accounted for 93% (based on COD) of hydrogen supplied as a substrate in the headspace. The batch test indicates that the influence of hydrogenotrophic homoacetogenesis (equation (1)) was stronger at 35 °C than at 55 °C.

$$4H_2 + CO_2 \rightarrow CH_3COOH + 2H_2O \ (\Delta G^0 = -105 \text{ kJ/reaction}) \quad (1)$$

3.3. *Effects of substrate concentration on hydrogen production in the thermophilic reactor*

Table 2 summarizes the hydrogen production rate, the specific hydrogen production rate, the hydrogen yield and pH in the thermophilic reactor. The content of hydrogen in the biogas was in the range of 45–50%. The pH of the thermophilic reactor was in the range of 4.8–5.3, which indicates the pH in the thermophilic reactor was kept suitable for hydrogen fermentation [24–26].

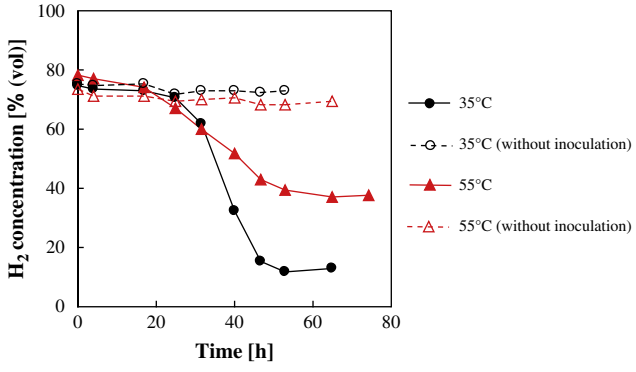

Fig. 2 – Comparison of hydrogen consumption by homoacetogens between mesophilic and thermophilic conditions.

Table 1 – Production of volatile fatty acids in the batch tests.

Experimental condition	Initial/final	Acetic acid [mM]	Propionic acid [mM]	Butyric acid [mM]	Caproic acid [mM]
Temperature H_2 as a substrate					
35° C Supplied	Initial	13.2	0.4	3.3	0.3
	Final	29.0	0.6	3.6	0.2
Not supplied	Initial	0.8	0.0	0.4	0.2
	Final	0.7	0.0	0.3	0.1
55° C Supplied	Initial	8.7	0.1	3.1	1.0
	Final	19.4	0.1	3.6	1.1
Not supplied	Initial	1.0	0.0	0.4	0.2
	Final	0.7	0.0	0.3	0.2

The starch concentration was stepwise increased from 20 to 70 g/l between the operational phases I and IV. The hydrogen yield peaked at the operational phase I (20 g/l-starch), and decreased with increasing starch concentration. To investigate the optimum substrate concentration, the starch concentration was decreased to 15 g/l in the operational phase V. The hydrogen yield was 2.31 mol/mol glucose, which was 18% lower than that at 20 g/l-starch. Subsequently, the starch concentration was again increased to 50 g/l and 70 g/l in the operational phases VI and VII respectively, in order to confirm reappearance of the phenomena observed at the operational phases III and IV. It was observed that the trends of hydrogen production in the phases VI and VII were almost the same as those in the operational phases III and IV, respectively.

The maximum hydrogen production rate (4.37 l/l/d), the maximum specific hydrogen production rate (4.03 l H_2/g-VSS/d) and the maximum hydrogen yield (2.82 mol H_2/mol glucose) in the thermophilic reactor were obtained at the starch concentrations of 20 g/l-starch or 30 g/l-starch.

3.4. *Material balance based on COD and by-products distribution*

The COD mass balance in the thermophilic reactor is shown in Table 3. It was calculated with hydrogen, the concentration of aqueous by-products, the VSS and the residues of starch and peptone in the reactor effluents. The formula for the VSS was determined as $C_5H_{9.59}N_{1.12}O_3$ in this study ($n = 5$). The COD conversion factor of the VSS in this study was calculated as 1.215, according to the stoichiometric equation of $C_5H_{9.59}N_{1.12}O_3 + 5.06O_2 \rightarrow 5CO_2 + 3.12H_2O + 1.12NH_3$. The data analysis was conducted using the average values during the stable states. The COD recovery ranged from 95 to 109%, which means most of the COD in the influent was recovered.

Table 4 summarizes the by-product distribution at each substrate concentration in the thermophilic reactor. Acetic and butyric acids were the dominant by-products in each operational phase; however, the production of formic acid, lactic acid and ethanol was significantly affected by the

Table 2 – Gas production and pHs in the steady state of each operational phase in the thermophilic reactor.

starch concentration [g/l]	Operational phase	HPR[a] [l/l/d]	SHPR[b] [l/g VSS/d]	HY[c] [mol H_2/mol glucose]	Gas composition [%]		pH
					H_2	CO_2	
15	V	3.72 ± 0.28 (18)	2.49	2.31	45.0 ± 0.9 (19)	54.0 ± 1.2 (19)	5.25 ± 0.07 (15)
20	I	4.20 ± 0.57 (13)	4.03	2.82	48.9 ± 0.7 (13)	53.3 ± 1.0 (13)	5.19 ± 0.16 (7)
30	II	4.43 ± 0.48 (11)	3.05	2.66	49.5 ± 1.4 (13)	52.3 ± 1.4 (13)	5.10 ± 0.10 (8)
50	III	3.63 ± 0.75 (11)	2.97	1.97	48.2 ± 1.5 (11)	53.8 ± 1.2 (11)	4.83 ± 0.08 (9)
50	VI	3.99 ± 1.00 (15)	2.38	2.10	47.8 ± 1.2 (17)	52.0 ± 1.2 (17)	4.76 ± 0.07 (9)
70	IV	3.62 ± 0.45 (8)	1.85	1.84	45.0 ± 2.7 (7)	54.3 ± 1.9 (7)	4.87 ± 0.09 (7)
70	VII	4.15 ± 0.27 (6)	2.21	2.14	46.0 ± 2.0 (7)	53.8 ± 1.9 (7)	4.84 ± 0.10 (4)

Numbers in parentheses correspond to the number of measurements used for determination of the mean values and standard deviations.
a Hydrogen production rate.
b Specific hydrogen production rate.
c Hydrogen yield.

substrate concentration. The amounts of these by-products tended to increase when the starch concentration was 50 g/l-starch or higher.

4. Discussion

4.1. *Effects of temperature on stability of hydrogen production*

Stable hydrogen production was not attained under the mesophilic condition in this study; on the other hand, it was stably attained for more than 6 months under the thermophilic condition. Our results show that operational temperature plays a significant role in sustainable hydrogen production. It may well be that, under thermophilic conditions, the hydrogen-producing bacteria are more readily harvested (especially the *Thermoanaerobacterium* [13,15]). The results obtained at 35 °C in this study differed significantly from some previous studies in which it was demonstrated that hydrogen could be produced continuously under mesophilic conditions even if the seed sludge was not subjected to any pre-treatment [3–5]. This difference is probably due to the seed sludge, since the seed sludge has been shown to be an important factor in the microbial community structure in hydrogen fermentation [15]. The seed sludge used in this study was presumably dominated by mesophilic non-hydrogen-producing bacteria which included hydrogenotrophic homoacetogens.

Table 5 compares the hydrogen yield in this study with previous studies using mixed cultures. The hydrogen yields were in the range from 0.8 to 2.8 mol H_2/mol glucose for the mesophilic fermentation, but were from 2.0 to 2.59 mol H_2/mol glucose for the thermophilic fermentation. The maximum hydrogen yield of 2.82 mol H_2/mol glucose in this study is quite high, suggesting that the substrate concentration is an important factor to enhance hydrogen yield.

4.2. *Effects of substrate concentration on hydrogen production*

The maximum hydrogen yield in this study was obtained at a starch concentration of 20 g/l. This optimum substrate concentration is likely to differ according to other factors such as the reactor configuration, the parameters for reactor operation, and the microbial community structure. The reasons for the deterioration of hydrogen yield in this study are discussed below with the by-product distribution (Section 4.2.1) and the inhibition by undissociated organic acids (Section 4.2.2) taken into consideration.

Table 3 – The COD mass balance in the thermophilic reactor.

Starch concentration [g/l]	Operational phase	H_2 [%]	HAc [%]	HPr [%]	HBu [%]	HCa [%]	HFo [%]	HSu [%]	HLa [%]	Eol. [%]	Carbo [%]	Peptone [%]*	VSS [%]**	Recovery [%]
15	V	15.1	18.3	N.D.	23.7	12.3	0.8	0.6	1.9	3.1	5.9	9.8	10.1	101.4
20	I	13.3	14.3	N.D.	30.4	4.2	0.4	0.0	0.1	1.1	29.2	5.4	5.3	103.7
30	II	8.9	10.1	N.D.	16.0	4.7	0.4	0.2	1.1	1.1	43.0	7.6	5.0	98.2
50	III	4.4	5.5	N.D.	9.1	3.2	1.5	0.7	7.4	10.15	6.7	8.2	2.5	109.3
50	VI	4.8	11.0	N.D.	13.0	0.2	0.6	0.1	1.7	2.1	55.8	8.8	3.3	101.5
70	IV	3.1	5.5	N.D.	5.7	2.4	0.1	0.1	0.6	1.7	63.2	9.5	2.9	94.7
70	VII	3.6	8.0	N.D.	3.9	0.5	0.5	0.1	2.7	4.3	63.5	8.3	2.4	97.8

HAc: acetic acid; HPr: propionic acid; HBu: butyric acid; HCa: caproic acid; HFo: formic acid; HSu: succinic acid; HLa: lactatic acid; Eol.: ethanol; carbo: carbohydrate; N.D.: not detectable.
* Conversion factor of peptone was determined as 1.12 in this experiment.
** Chemical formula of VSS was determined as $C_5H_{9.59}N_{1.12}O_3S_{0.05}$ in this study.

Table 4 – Distribution of the intermediate by-product in the thermophilic reactor.

Starch concentration [g/l]	Operational phase	HAc [mM]		HBu [mM]		HPr [mM]		Ha [mM]		HFo [mM]		HSu [mM]		HLa [mM]		Eol. [mM]	
		M.V.	S.D.	M.V.	S.D.	M.V.	S.D.	M.V.	S.D.	M.V.	S.D.	M.V.	S.D.	M.V.	S.D.	M.V.	S.D.
15	V	51.5	4.1	27.4	2.7	N.D.	–	8.7	1.2	8.9	2.0	0.8	0.4	3.5	4.5	5.8	3.8
20	I	53.5	7.6	45.5	3.0	N.D.	–	3.9	1.2	6.0	2.9	0.0	0.0	0.2	0.5	2.8	0.4
30	II	56.3	9.4	35.7	5.9	N.D.	–	6.5	2.6	9.6	2.1	0.6	0.3	4.2	4.4	4.2	1.4
50	III	50.6	1.0	33.8	1.6	N.D.	–	7.5	0.5	56.3	6.8	2.9	1.0	45.7	3.9	62.0	8.1
50	VI	101.5	12.0	50.9	11.1	N.D.	–	0.5	0.9	22.5	6.0	0.3	0.1	10.6	7.2	13.0	14.3
70	IV	70.3	5.7	29.3	3.3	N.D.	–	7.6	1.1	5.1	0.7	0.3	0.1	4.9	2.6	14.7	4.3
70	VII	103.2	20.7	20.2	3.7	N.D.	–	1.6	1.5	24.1	8.1	0.6	0.1	23.2	8.8	37.0	8.0

HAc, acetic acid; HBu, butyric acid; HPr, propionic acid; HCa, caproic acid; HFo, formic acid; HSu, succinic acid; HLa, lactic acid; Eol., ethanol; M.V.: mean value; S.D.: standard deviation; N.D.: not detectable.

4.2.1. *Relationship between hydrogen yield and by-product distribution*

The hydrogen production is explained by two stoichiometric equations (2) and (3); thus, acetic and butyric acids are key intermediate products.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CO_2 + 2CH_3COOH \ (\Delta G^0 = -206 \text{ kJ/reaction}) \quad (2)$$

$$C_6H_{12}O_6 \rightarrow 2H_2 + 2CO_2 + C_3H_7COOH \ (\Delta G^0 = -254 \text{ kJ/reaction}) \quad (3)$$

The by-product distribution differed significantly depending on the starch concentration, with the most significant change at less than 50 g/l-starch. Acetic, formic and lactic acids and ethanol increased at 50 g/l-starch and higher. The production of hydrogen is not concomitant with the production of formic and lactic acids or ethanol, as is shown in (4)–(6) respectively. In fact, the hydrogen yield deteriorated when they are produced.

$$H_2 + HCO_3^- \rightarrow HCOO^- + H_2O \ (\Delta G^{0\prime} = -1.3 \text{ kJ/reaction}) \quad (4)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3C\,(OH)\,HCOOH \ (\Delta G^{0\prime} = -225 \text{ kJ/reaction}) \quad (5)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \ (\Delta G^{0\prime} = -198 \text{ kJ/reaction}) \quad (6)$$

The facultative anaerobes, such as the *Enterobacter*, *Klebsiella* and *Bacillus*, produce formic acid via pyruvate; subsequently

Table 5 – Comparison of the hydrogen yield obtained in this study with previous studies.

Seed sludge	Pre-treatment	Reactor type	Influent		Reactor condition			Hydrogen yield [mol H_2/mol glucose]	Reference
			Substrate	Conc. [g/l]	Temp. [C]	pH	HRT [h]		
Agricultural soil	Heat	CSTR	Glucose	5	30	5.5	10	2.5	[36]
Agricultural soil	Heat	CSTR	Glucose	2.34	30	5.5	10	2.8	[18]
Mixed culture	No	CSTR	Glucose	7	36	5.5	6	2.1	[4]
Sewage sludge	Heat	CSABR[a]	Glucose	26.7-35.7	40	6.6	0.5	1.75	[37]
Soybean meal	No	CSTR	Glucose	10	35	6	8.5	0.85	[3]
Digested sludge	Heat	CSTR	Sucrose	26.7	35	5.4	12	1.23	[17]
Digested sludge	Heat	CSTR	Sucrose	10	35	5.2	12	1.65	[16]
Mixed culture	No	UASB	Glucose	5	70	4.8	26.7	2.47	[24]
Digested sludge	No	CSTR	Food waste	25[b]	55	5.5	120	2.2	[13]
Sludge compost	Aeration	CSTR	Sugary wastewater	9.85[b]	60	6.8	12	2.59	[10]
Mixed culture	No	CSTR	Starch	20	55	5.1	24	2.82	This study

a Completely stirred anaerobic bioreactor using silicone-immobilized sludge.
b Carbohydrate concentration in the influent.

converting it into hydrogen and carbon dioxide by the formate hydrogenlyase [27,28]. Nevertheless, the conversion of formic acid into hydrogen is thermodynamically unfavorable [29].

To our knowledge, ours is the first investigation in which a significant amount of formic acid was produced at high substrate concentrations in mesophilic hydrogen fermentation [16–18]. It has been also demonstrated that the production of formic acid is likely to occur around the neutral pH [30,31]. It is suggested that the production of formic acid at higher substrate concentrations is one of the specific characteristics of thermophilic hydrogen fermentation.

Some previous studies indicate that the organic loading rate affects the production of lactic acid and ethanol. Ko et al. [32] reported that an increase of the substrate concentration prompted the production of lactic acid in the mesophilic continuous hydrogen fermentation. Zoetemeyer et al. [30], on the other hand, demonstrated that increasing the organic acid concentration stimulated the production of lactic acid by adding butyric acid into the medium. Moreover, the production of a solvent is likely to be the result of the accumulation of the undissociated organic acids [18,33]. Ethanol production at higher substrate concentrations in this study also could be attributed to the accumulation of the undissociated organic acids. The production of lactic acid and ethanol at higher substrate concentrations in this study is presumed to be consistent with the findings of those previous studies.

4.2.2. *Relationship between hydrogen yield and undissociated organic acids*

The accumulation of undissociated organic acids is another probable reason for the deterioration of the hydrogen yield at higher substrate concentrations. The undissociated organic acids permeate the cell membrane and decrease the intracellular pH. Subsequently, bacterial growth is inhibited because the energy is utilized for decreasing the proton concentration [30,33]. Ginkel and Logan [18] demonstrated that the hydrogen yield deteriorated when the concentration of undissociated organic acid in the mesophilic hydrogen fermentation was at 19 mM and higher. Furthermore, they also reported that when the concentration of undissociated organic acids was increased to 60 mM by adding acetic or butyric acids into the medium, the hydrogen yield decreased by over 93%.

The concentration of undissociated organic acids can be calculated by equation (7).

$$\frac{HA}{HA + A^-} = \frac{10^{(pK_a - pH)}}{1 + 10^{(pK_a - pH)}} \quad (7)$$

where HA: the concentration of undissociated organic acid, A^-: the concentration of dissociated organic acid, K_a: the dissociation constant.

The pK_a is a temperature dependent constant and follows the Van't Hoff equation in equation (8) [34].

$$\frac{d \ln K_a}{dT} = \frac{\Delta H^0}{RT^2} \quad (8)$$

where R and T represent the gas constant (8.314 kJ/mol) and the absolute temperature [K], respectively. ΔH^0 represents the delta enthalpy of dissociation at 25 °C.

Each pK_a value at 55 °C was calculated by substituting the ΔH^0 value of each organic acid [35] (Table 6).

Table 6 – The values of ΔH^0 and the values of K_a and pK_a in the thermophilic reactor [35].

Organic acid	ΔH^0 [kJ/mol]	K_a (55° C)	pK_a (55° C)
Acetic acid	−0.08	1.71×10^{-5}	4.77
Propionic acid	−0.59	1.34×10^{-5}	4.87
Butyric acid	−2.68	1.51×10^{-5}	4.82
Caproic acid	−2.93	1.38×10^{-5}	4.86
Formic acid	0.04	1.77×10^{-5}	3.75
Succinic acid	3.35	6.21×10^{-5}	4.21
Lactic acid	−0.71	1.38×10^{-5}	3.86

Fig. 3A illustrates the relationship between the hydrogen yield and the total concentration of undissociated organic acids. According to Fig. 3A, hydrogen production decreased when the total concentration of undissociated organic acids was higher than 30 mM. This suggests that hydrogen production under the thermophilic condition might be more tolerant to inhibition by undissociated organic acid than under the mesophilic condition [17].

Fig. 3B illustrates the relationship between the yield of bacterial growth ($VSS/COD_{degradated}$) and the total concentration of undissociated organic acid. ATP molecules generated can be utilized in (1) biosynthesis for the cell growth and (2) membrane energization to generate a pH and electrical

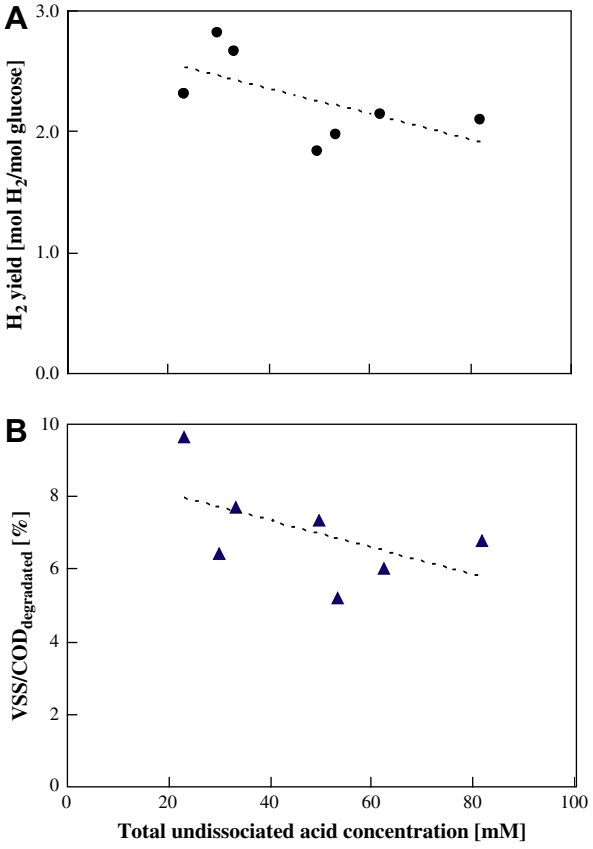

Fig. 3 – The influence of the concentration of total undissociated organic acids on (A) the hydrogen yield and (B) bacterial growth yield ($VSS/COD_{degradated}$ [%]) in the thermophilic reactor.

gradient across the cell membrane. However, the accumulation of organic acids causes the bacteria to expend more energy to maintain the internal pH above the critical level, which results in less ATPs being generated [30,33]. That is why the yield of bacterial growth tended to decrease with an increase in the total concentration of undissociated organic acids as is shown in Fig. 3B.

5. Conclusions

The results and findings of this study can be summarized as follows. Temperature was shown to be an important factor for stability in hydrogen production. Stable hydrogen production was attained for over 6 months at 55 °C; on the other hand, hydrogen was not produced continuously but intermittently at 35 °C. This is thought to be mainly due to the presence of hydrogenotrophic homoacetogens, which were more active at 35 °C than at 55 °C.

Substrate concentration also had a strong influence on continuous hydrogen production. A maximum hydrogen yield of 2.8 mol H_2/mol glucose was obtained at 20 g/l-starch. At higher substrate concentrations (50 g/l-starch or higher), the amount of formic and lactic acids produced increased. This was also true of ethanol. An adverse correlation between the total concentration of undissociated organic acids and the yields of hydrogen and bacterial growth was observed.

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