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Hydrogen production potentials and fermentative characteristics of various substrates with different heat-pretreated natural microflora

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ABSTRACT

Batch tests were carried out to investigate the effects of heat-pretreated inocula on the fermentative hydrogen production characteristics of various types of substrates. A total of 8 different inocula and 4 different substrates (starch, glycerol, oil and peptone) were used. Heat pretreatment of the inocula was conducted in order to harvest spore-forming clostridial bacteria. Significant hydrogen production potentials were observed from starch (20.5–174.4 ml H_2/g-COD_{starch}) and glycerol (11.5–38.1 ml H_2/g-$COD_{glycerol}$); however, almost no hydrogen was produced from oil and peptone. When starch was used as a substrate, two different fermentation patterns were observed, according to the inocula: butyric acid-type and ethanol-type fermentation. Polymerase chain reaction combined with denaturing gradient gel electrophoresis (PCR-DGGE) analysis was conducted to compare the bacterial structures cultivated on the starch medium. Different species of clostridial bacteria were observed between the butyric acid-type and ethanol-type fermentation cultures. When glycerol was used as a substrate, 1,3-propanediol was the main by-product with each inoculum. The results of the present study suggest that simultaneous production of ethanol or 1,3-propanediol in addition to hydrogen is a more promising strategy than conventional hydrogen production in acidogenesis.

1. Introduction

Biomass is one of the ideal candidate-alternatives to fossil fuels, because it is carbon-neutral and exists in abundance. Biomass can be fermented into various bio-energies, including hydrogen. In future generations, hydrogen will be the main energy carrier and, as such, the demand for it of course will increase. Thus, biological hydrogen production from biomass is promising eco-friendly hydrogen-producing processes.

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In previous studies, the effects on hydrogen production of operational parameters such as pH, hydraulic retention time (HRT) and temperature have been quite thoroughly investigated [1,2]. Generally speaking, successful hydrogen fermentation is attained under short HRT/low pH conditions [1,2]. Even under such conditions, however, hydrogentrophic methanogens would proliferate [3–5]. Some studies have demonstrated that suppression of hydrogen consumption by methanogens under mesophilic conditions is difficult without chemical pretreatment to inocula [4,6]. Besides, bacteriocins excreted by lactic acid bacteria, *Lactobacillus paracasei* and *Enterococcus durans*, inhibit hydrogen production [7]. In order to inactivate those microorganisms and to harvest H_2-producing bacteria, especially the spore-forming clostridial bacteria, heat pretreatment commonly has been used in hydrogen fermentation [7–9].

An H_2-producing consortium can be obtained from various environmental sources [2]. A few studies have investigated the effects of inocula on hydrogen production; however, those studies have not investigated the effects of inocula on fermentation patterns and their bacterial structures [10,11]. Although many kinds of bacteria associated with *Clostridia* have been isolated [12], only a few of them have been studied for hydrogen production; this prompts speculation that heat-pretreated inocula would show different hydrogen fermentation characteristics paralleling the differences in their bacterial structures.

In fact, many studies have investigated hydrogen production from various types of actual biomass such as food waste [13,14], sewage sludge [15,16] and palm oil mill effluent [17]. Determining the applicability of biomass to hydrogen fermentation by investigating the characteristics of hydrogen fermentation of each biomass component as well as those of actual wastes composed of complicated components, would be very much of interest to future studies.

The objectives of this study were to clarify the effects of inocula on the hydrogen production potentials and fermentative characteristics of various types of biomass using heat pretreatment to enrich *Clostridia*. The differences in bacterial structures were analyzed by PCR-DGGE.

2. Materials and methods

2.1. Inocula

A total of 8 different inocula were used in this study: thermophilic acidogenic sludge treating potato waste (TAS), mesophilically digested sludge treating waste-activated sludge (MDS), thermophilically digested sludge treating waste-activated sludge (TDS), soybean meal (SBM), kitchen waste (KW), activated sludge (AS), cattle manure (CM) and soil (SL). These inocula were washed with distilled water to eliminate aqueous inhibitors of hydrogen production. After centrifugal separation (4000 rpm, for 10 min), the settled solid-phase of 2 g was added to 100 ml of distilled water in a vial. The inocula were heat-pretreated at 80 °C for 20 min to inactivate hydrogentrophic methanogens and to harvest the spore-forming clostridial bacteria. The head space in the vial was filled with pure nitrogen gas. After being sealed with butyl rubber stoppers, the vials were incubated in a shaking water bath unit at 35 °C in order to prevent the production of hydrogen. Subsequently, the vials were maintained for 30 min, and finally, the supernatant from the vials was inoculated into the medium.

2.2. Substrates and batch tests

The medium constituted 5 g-COD/l of substrate along with sufficient mineral and vitamin solutions, including (per liter) NH_4HCO_3, 6000 mg; K_2HPO_4, 250 mg; KH_2PO_4, 250 mg; $MgSO_4 \cdot 7H_2O$, 120 mg; $FeSO_4 \cdot H_2O$, 25 mg; $NaS \cdot 9H_2O$, 0.3 mg; Cystein·HCl, 0.5 mg; 0.1% resazurin solution, 2.0 ml, KI, 2.5 mg; $MnSO_4 \cdot 6H_2O$, 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; biotin, 0.02 mg; folic acid, 0.02 mg, pyridoxine·HCl, 0.1 mg, thiamine·HCl, 0.05 mg; riboflavin, 0.05 mg; nicotinic acid, 0.05 mg; DL-calcium pantothenate, 0.05 mg; vitamin B_{12}, 1 μg; p-aminobenzonic acid, 0.05 mg, and lipoic acid, 0.05 mg. Four substrates (starch, glycerol, peptone and cooking oil) were employed. Starch, peptone and cooking oil were used as representatives of carbohydrate, protein and lipid, respectively. The glycerol was used as a substrate. Glycerol is emitted as a by-product of the bio-diesel and bio-ethanol production processes. It can be anaerobically bio-degradable via pyruvate, suggesting that it would be a good substrate for hydrogen production.

Batch tests were conducted with 120 ml vials. Ten ml of the inoculum obtained by the procedure of 2.1 was added to 50 ml of the medium in the vials. The head space was filled with a mixed gas of nitrogen (80%) and carbon dioxide (20%) for 2 min, and then sealed with a butyl rubber stopper. The initial pHs of the cultures were adjusted to 6.5 ± 0.1 using concentrated HCl solution. These vials were incubated in a water bath unit at 35 °C. The batch tests were conducted multiple times for each inoculum.

2.3. Analytical methods

2.3.1. Analysis of gas and broth

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 mmφ). The temperatures of a detector and a column were maintained at 100 °C and 60 °C, respectively. In the determination of the carbon dioxide, nitrogen and methane, the same model of gas chromatograph (Shimadzu 8A), this one equipped with a TCD and a stainless steel column packed with Porapak T was used. The temperatures of the detector and the column, here, were maintained at 100 °C and 70 °C, respectively. The carbohydrate was analyzed by the phenol–sulfuric acid method, using glucose as a standard. The glycerol was analyzed using an F-kit glycerol (JK international, Japan). The organic acids were analyzed by capillary electrophoresis (I.D., 75 μm; UV detector 220 nm, Photal CAPI-3200, Ohtsuka, Japan). The solvent concentrations were measured using 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 solid (VSS) and chemical oxygen demand (COD)

were measured according to the procedures described in Standard Methods [18].

2.3.2. PCR-DGGE analysis

DNA was extracted from eight different inocula using a Mag Extractor-Genome (Toyobo, Osaka, Japan). The V3 region of the 16S ribosomal RNA gene was amplified using the forward primer EUB341f (5′-CCTACGGGAGGCAGCAG-3′) with a GC clamp (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3′) at the 5′ end and the reverse primer UNI518r (5′-ATTACCGC GGCTGCTGG-3′). The procedure for PCR-DGGE analysis was described in our previous study [19]. 16S rDNA sequences were deciphered by an automated sequence analyzer (CEQ 8000, Beckman coulter) after dye terminator cycle sequencing with a Quick Start kit (Beckman coulter). Comparative analysis of the 16S rRNA gene sequences was carried out by the search program BLAST (http://www.ddbj.nig.jp).

2.4. Data analysis

Based on the results obtained in the batch tests, the hydrogen production potentials, maximum hydrogen production rates and lag-phase time were determined, using Gompertz equation (1), in order to describe the effects of the inocula and substrates on the hydrogen fermentation characteristics [8]:

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

where H is the cumulative hydrogen (ml/l), λ is the lag-phase time (h), H_p is the hydrogen production potential, R_m is the hydrogen production rate (ml/l/h), and $e = \exp(1) = 2.718$. Fig. 1 illustrates the time course of cumulative hydrogen production from the starch medium when MDS was inoculated. The Figure also shows the definitions of the parameters in equation (1). These parameters were determined by the least squares method, using the ''solver'' function in the ''tools'' menu in Microsoft Excel 2002.

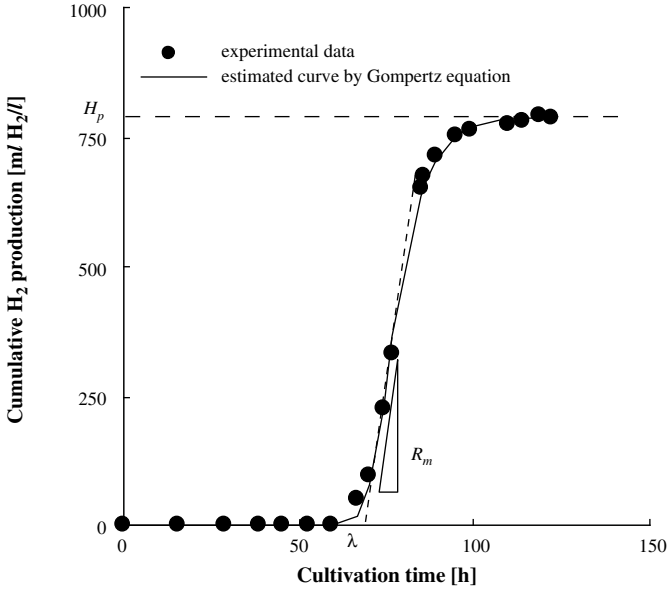


Fig. 1 – The definitions of the parameters (H_p, R_m, λ) in equation (1). The data in this figure was taken when the mesophilically digested sludge was inoculated into the starch medium.

3. Results

3.1. Hydrogen production potentials

Table 1 lists each parameter obtained by Gompertz equation (1), the final VSS concentrations and the final pHs. When the R^2 values were larger than 0.95, the data analysis with equation (1) was determined to be valid; otherwise, the actual hydrogen production potentials experimentally obtained were used to calculate the representative values. The content of the methane was below the detection limit.

Starch and glycerol were found to be suitable substrates for hydrogen production. The hydrogen production potentials from the starch and glycerol media were 83.3–785.5 ml H_2/l and 58.2–195.8 ml H_2/l, respectively; from the peptone and oil media, significant hydrogen production was not observed. The effects of the inocula on the hydrogen production potentials were significant in the case of the starch medium, but insignificant in the case of the glycerol medium.

3.2. Effects of inoculum on fermentation patterns based on COD mass balance

Table 2 summarizes the COD mass balance, which describe the effects of the inocula on the hydrogen production and fermentation patterns from the starch and glycerol media. The VSS formula was assumed to be $C_5H_7NO_2$ [20]. The COD mass balance was calculated using the samples that produced the largest amount of hydrogen in each inoculum.

3.2.1. Fermentation patterns from starch medium

The fermentation patterns from the starch medium were divided into butyric acid-type fermentation (MDS, KW, SBM and CM), which showed a butyric acid yield of 27–47% based on the COD, and ethanol-type fermentation (AS, SL, TDS and TAS), which showed an ethanol yield of 31–51% based on the COD. Butyric acid-type fermentation tended to show higher hydrogen production potentials; by contrast, ethanol-type fermentation showed lower hydrogen production potentials. However, it is noteworthy that the activated sludge (AS) produced as high yields of hydrogen in ethanol-type fermentation as the inocula which showed butyric acid-type fermentation, including mesophilic digested sludge (MDS), kitchen waste (KW), soybean meal (SBM) and cattle manure (CM).

3.2.2. Fermentation patterns from glycerol

In the batch tests, almost all of the glycerol in the glycerol medium was degraded with each inoculum. The glycerol medium's fermentation patterns were similar in all of the inocula. 1,3-propandiol in the yield of 50–70%, based on the COD, was produced as the main by-product. Acetic acid in the yield of 15–20%, based on the COD, was produced as the second major by-product. Almost no butyric acid was produced from the glycerol medium.

Table 1 – The each parameter in Gompertz equation (1), VSS concentrations and final pHs.

Inoculum	Starch					Glycerol					Oil[a]	Peptone[a]
	H_p [ml H_2/l]	R_m [ml H_2/l/h]	λ [h]	Final VSS[b] [mg/l]	Final pH[b]	H_p [ml H_2/l]	R_m [ml H_2/l/h]	λ [h]	Final VSS[b] [mg/l]	Final pH[b]	H_p [ml H_2/l]	H_p [ml H_2/l]
MDS	785.5	128.6	31.7	681.7	5.33	58.2	0.8	73.0	225	6.02	14.4	8.37
	(14.6)	(6.28)	(0.1)	(47.8)	(0.01)	(2.98)	(0.04)	(1.55)	(5.0)	(0.02)	(7.31)	(5.28)
SBM	720.7	53.2	77.3	603.3	5.38	155.9	6.5	73.0	185	6.13	24.1	9.24
	(49.0)	(25.3)	(6.8)	(61.3)	(0.31)	(2.09)	(0.7)	(14.6)	(32.4)	(0.07)	(1.40)	(2.65)
KW	655.1	102.2	69.5	633.3	4.87	195.8	13.6	54.7	263	6.08	17.7	13.2
	(58.9)	(16.5)	(2.6)	(18.4)	(0.05)	(22.0)	(8.03)	(3.4)	(29.0)	(0.38)	(6.58)	(2.22)
CM	590.0	17.9	80.3	158.3	6.17	169.8	11.1	63.0	182	6.20	6.93	2.50
	(87.7)	(7.01)	(7.3)	(24.9)	(0.05)	(13.8)	(2.38)	(9.35)	(2.36)	(0.00)	(5.42)	(2.12)
AS	451.9[a]	22.9[a]	92.3	527.5	5.14	141.5	4.0	99.4	165	6.35	12.1	6.65
	(74.2)	(9.86)	(6.9)	(122.5)	(0.09)	(1.20)	(1.45)	(12.8)	(10.8)	(0.11)	(7.10)	(1.13)
TDS	238.3	2.05	63.7	286.7	5.77	60.2[a]	0.70[a]	n.d.	177	6.40	10.7	1.86
	(22.2)	(0.56)	(9.2)	(79.6)	(0.27)	(8.25)	(0.13)		(51.2)	(0.22)	(3.27)	(0.78)
SL	257.9[a]	3.87[a]	78.3	242	5.55	142.0[a]	24.7[a]	65.1	183	6.50	5.05	0.65
	(100.7)	(2.69)	(11.8)	(118.4)	(0.28)	(15.8)	(16.0)	(24.5)	(2.5)	(0.03)	(4.09)	(0.62)
TAS	83.3	0.94	59.6	558.3	5.43	111.3	4.1	66.4	205	6.21	20.8	6.05
	(9.90)	(0.27)	(18.7)	(149.7)	(0.12)	(19.2)	(0.68)	(4.08)	(10.8)	(0.02)	(4.12)	(4.33)

The values in parentheses are standard deviations of each parameter.
n.d.: not determined.
The number of batch tests was 2–4.
a The representative values are mean values of actual data in this experiment.
b The values measured after the batch experiment.

3.3. *Microbial community structure analysis by PCR-DGGE method*

Fig. 2 shows the band profile of the PCR-DGGE analysis results, and Table 3 lists each band's closest relative. In butyric acid-type fermentation (MDS, SBM, KW, CM), the close relatives of *Clostridium acetobutylicum*, *Clostridium butyricum* and *Clostridium paraputrificum* were observed, whereas in ethanol-type fermentation, (AS) the close relatives of *Clostridium pasteurianum* were observed. Those clostridial species are known to be typical hydrogen-producing bacteria [21–25].

The close relatives of *Citrobacter* and *Bacteroides*, which are non-spore-forming bacteria, also were observed in this study, though heat pretreatment was conducted. This is attributable to the influence of the initial bacterial structures prior to the batch tests.

Table 2 – The COD mass balance of hydrogen fermentation from the starch and glycerol mediums.

Inoculum	Substrate	Product [%]									VSS[a] [%]	Residue [%]	Recovery [%]
		H_2	FA	SA	AA	LA	PA	BA	Eol	1,3-Pol			
MDS	Starch	10.7	1.5	N.D.	9.0	N.D.	N.D.	46.8	1.4	N.D.	18.6	9.8	97.9
	Glycerol	0.8	3.8	3.3	15.8	0.7	N.D.	N.D.	4.2	56.0	4.0	2.0	94.9
SBM	Starch	10.5	1.3	N.D.	18.7	0.2	N.D.	27.0	21.0	N.D.	13.9	9.1	101.7
	Glycerol	2.1	0.7	4.4	15.5	0.4	1.8	0.0	0.6	64.4	2.9	1.6	98.6
KW	Starch	9.6	3.8	0.7	13.3	N.D.	N.D.	46.2	1.2	N.D.	17.4	14.5	106.8
	Glycerol	3.0	0.7	3.9	16.7	0.5	N.D.	N.D.	1.0	71.0	4.5	1.5	107.1
CM	Starch	7.2	4.6	N.D.	15.1	N.D.	N.D.	32.7	14.6	N.D.	16.2	12.3	102.7
	Glycerol	2.4	0.9	4.1	17.1	0.1	N.D.	0.0	0.7	62.6	3.4	2.0	97.3
AS	Starch	9.1	1.5	N.D.	20.3	N.D.	1.4	3.0	30.9	N.D.	4.9	12.3	83.3
	Glycerol	2.0	1.0	4.8	17.6	2.5	N.D.	1.0	1.5	66.5	4.0	0.2	100.9
TDS	Starch	3.0	N.D.	0.2	26.1	0.0	N.D.	4.4	31.5	N.D.	7.5	10.0	82.7
	Glycerol	1.0	3.3	4.1	17.7	2.4	N.D.	1.0	14.1	64.9	7.0	0.3	115.7
SL	Starch	5.7	1.3	2.8	19.6	0.0	6.4	1.3	32.4	N.D.	10.8	4.3	84.8
	Glycerol	2.3	1.0	5.3	19.7	2.3	N.D.	1.0	0.8	67.4	4.9	0.1	104.7
TAS	Starch	1.2	2.9	5.4	16.6	0.9	N.D.	N.D.	50.8	N.D.	18.4	4.7	100.9
	Glycerol	1.8	0.7	3.7	15.7	0.8	N.D.	N.D.	1.3	52.0	4.1	3.4	83.5

FA, Formic acid; SA, Succinoic acid; AA, Acetic acid; LA, Lactic acid; Propionic acid; BA, Butyric acid; Eol, Ethanol; 1,3-Pol, 1,3-propanediol.
N.D.: not detectable.
a VSS formula was assumed to be $C_5H_7NO_2$.

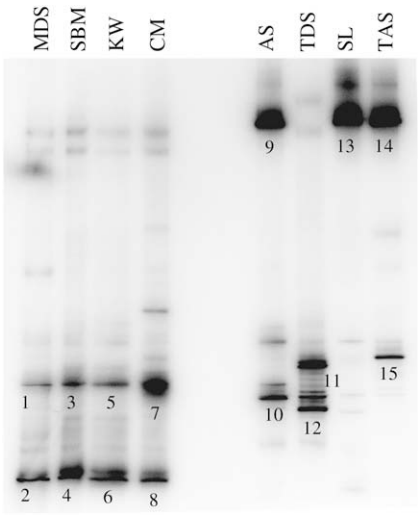


Fig. 2 – The DGGE band profile obtained by using extracted DNA from each reactor.

4. Discussion

4.1. *Characteristics of hydrogen production from each substrate and effects of inocula*

4.1.1. *Hydrogen production from carbohydrate*

Hydrogen production form carbohydrate occurs via two fermentation patterns, the acetic acid pattern and the butyric acid pattern. When one mole of glucose is converted into two moles of ethanol, hydrogen production is neutral; nevertheless, interestingly, when in the present study AS, which produces ethanol as the major by-product, was used as the inoculum, the hydrogen yield in acetic acid-type fermentation was as high as that in butyric acid-type fermentation. This result is similar to that of a previous study reported by Ren et al. [26], who demonstrated stable hydrogen production with a high yield of ethanol using hydrogen-producing microflora enriched from activated sludge. Accordingly, the following stoichiometric equation (2) is suggested as the dominant reaction in hydrogen and ethanol fermentation:

$$C_6H_{12}O_6 + H_2O \rightarrow 2H_2 + 2CO_2 + C_2H_5OH + CH_3COOH\ . \quad (2)$$

4.1.2. *Hydrogen production from glycerol*

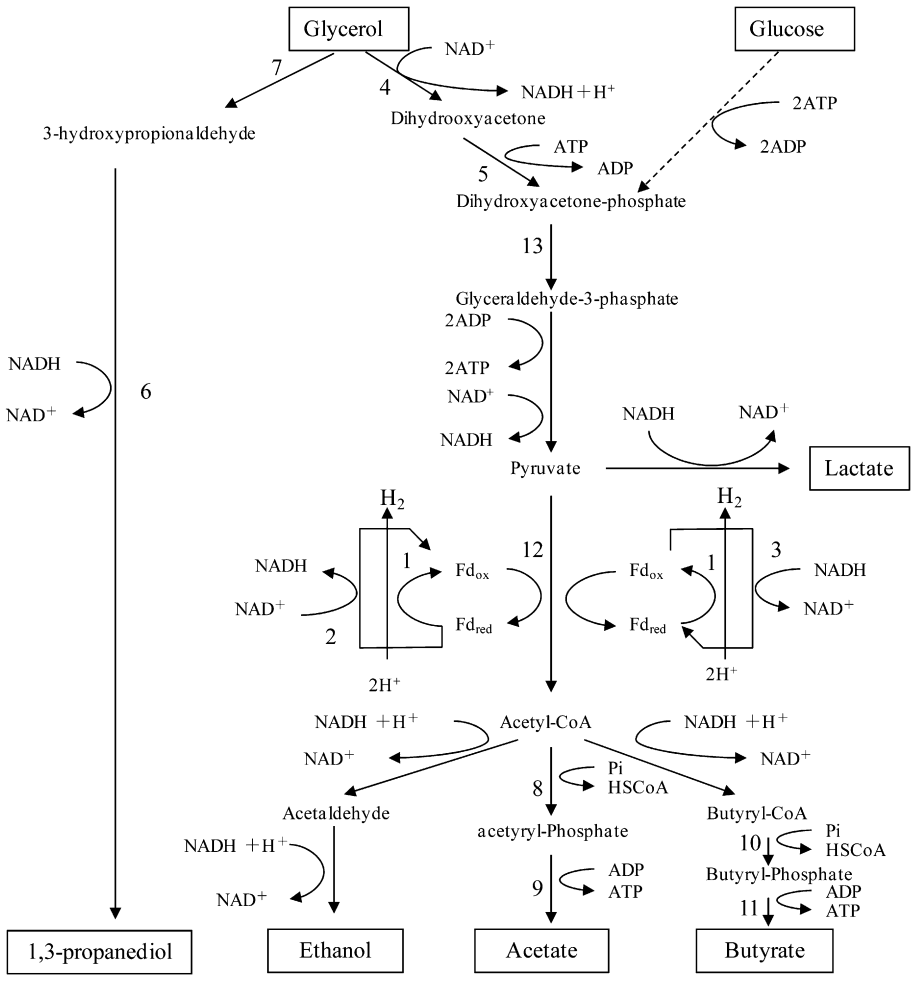
Hydrogen production from glycerol by a mixed culture, to our knowledge, has not yet been demonstrated, though hydrogen production by a pure *Enterobacter* culture has been investigated [27,28]. Glycerol theoretically can be converted into 3 moles of hydrogen and 1 mole of acetic acid by equation (3) [29]:

$$C_3H_8O_3 + 2H_2O \rightarrow CH_3COO^- + HCO_3^- + 2H^+ + 3H_2 \quad (\Delta G^{o'} = -73\ \text{kJ/reaction})\ . \quad (3)$$

The productivity of hydrogen from glycerol differs according to the microorganism. Fig. 3 shows the metabolic pathway of *C. butyricum*, a modified version of that of Saint-Amans et al. [30]. *Clostridia* degrade glycerol into 1,3-propanediol or a variety of by-products via pyruvate. According to previous studies using *Clostridia*, when glycerol is employed for a substrate, intracellular concentrations of ATP and NADH increase, leading to the production of solvents such as 1,3-propanediol, whereas production of organic acids and hydrogen decreases [31–33]. Saint-Amans et al. [30] demonstrated by using *C. butyricum* that most of the reduced ferredoxin, produced by pyruvate-ferredoxin oxidoreductase (Fig. 3, No.12) during anaerobic degradation of glycerol, is used for production of NADH, which results in deterioration of hydrogen production. Girbal et al. [34] demonstrated that the ΔpH (intracellular pH–extracellular pH) was positive when glucose was used as a substrate, but negative when a mixture of glucose and glycerol was used. Girbal et al. [34] demonstrated that this is caused by deterioration of the hydrogenase activity. Hydrogenase catalyzes hydrogen generation from the reaction between protons and reduced ferredoxins. Hence, deterioration of the hydrogenase activity leads to a decrease of hydrogen production [31,35].

Table 3 – The closest relatives in each batch culture identified by PCR-DGGE analysis.

Inoculum	Band no.	Closest relative	Accession no.	Length [bp]	Identity [%]
MDS	1	*C. acetobutylicum*	X78073	138	97
	2	*C. butyricum*	AY442812	142	97
SBM	3	*C. acetobutylicum*	X78073	162	99
	4	*C. Butyricum*	AY442812	161	99
KW	5	*C. paraputrificum*	X75907	137	97
	6	*Citrobacter freundii*	DQ010114	160	98
CM	7	*C. acetobutylicum*	X78073	141	96
	8	*C. butyricum*	AY442812	163	98
AS	9	*Bacteroides eggerthii*	AB050107	157	96
	10	*C. pasteurianum*	M23930	162	99
TDS	11	*Clostridium* sp. FA3/2	AY188848	132	98
	12	*Bacillus* sp.	AY785775	80	96
SL	13	*Bacteroides eggerthii*	L16485	142	96
TAS	14	*Bacteroides* sp.	AB064915	144	95
	15	*C. aminovalericum*	M23929	163	98



1, Hydrogenase; 2, Ferredoxin-NAD(P)$^+$-reductase; 3, NAD(P)H-ferredoxin reductase; 4, glycerol dehydrogenase; 5, DHA kinase; 6, 1,3-propanediol dehydrogenase; 7, glycerol dehydratase; 8, phosphotransacetylase; 9, acetate linase; 10, phosphotransbutylase; 11, butyrate kinase; 12, pyruvate-ferredoxin oxidoreductase; 13, glyceraldehyde-3-phosphate dehydrogenase

Fig. 3 – The metabolic pathway of *Clostridium butyricum* modified from Saint-Amans et al. [30].

Enterobacter aerogenes, unlike *Clostridia*, can produce higher yields of hydrogen from glycerol than from sugars [27]. Nakashimada et al. [27] used *E. aerogenes* in a batch culture on various kinds of substrates, including gluconate, sugars (glucose, fructose, galactose, sorbitol, mannitol) and glycerol. They reported that *E. aerogenes* produced the highest amount of hydrogen when the substrate was glycerol, and suggested that a high intracellular redox state (a high NADH/NAD ratio) accelerates hydrogen production. Their report is very noteworthy, since it shows that the effect of the redox state on hydrogen productivity significantly differs between two typical hydrogen producers. Hence, it can be posited that the fact that all of the inocula tested in this present study produced lower yields of hydrogen from glycerol than from starch is due to the *Clostridia*-enriched microbial structures produced by heat pretreatment.

4.1.3. *Hydrogen production from protein and cooking oil*

The peptone and oil media free of inocula produced almost no hydrogen in this study. This result is consistent with those of previous studies using actual biomass, either including egg and lean meat as protein-rich biomass or chicken skin and fat meat as lipid-rich biomass [36,37]. The reasons that hardly any hydrogen is produced from proteins and lipids, as treated in the literature [36,37], are as follows: amino acids, which are produced via hydrolysis of protein, have only a low hydrogen production potential; the hydrogen produced might be combined with nitrogen as ammonium; β-oxidation (the degradation of long-chain fatty acids, the main constituent of lipids) cannot generate hydrogen. The results of the present study clearly show that hydrogen fermentation should be applicable to a biomass that is composed mainly of carbohydrate or glycerol.

4.2. *Simultaneous production of hydrogen and 1,3-propanediol or ethanol*

It is significant to investigate simultaneous production of ethanol or 1,3-propanediol in hydrogen production from

organic waste, instead of conventional hydrogen production in acidogenesis.

The concept of simultaneous production of hydrogen and 1,3-propanediol from glycerol by a mixed culture has not been investigated up to now. Temudo et al. [38] investigated glycerol fermentation at an alkaline pH by a mixed culture not subjected to any pretreatment. Aiming to evaluate its potential application to the production of bulk chemicals, they reported that the dominant fermentation patterns were ethanol–formic acid and 1,3-propanediol–acetic acid [38]. In the present study, all of the inocula fermented glycerol into 1,3-propanediol (50–70%, based on the COD) as the main product. Biological production of 1,3-propanediol recently has been of interest as a source of polymeric materials for chemical industries [39]. Up to the present time, various types of isolates, including *Clostridia*, have been investigated for 1,3-propanediol production. For example, *C. acetobutylicum* and *C. butyricum* are reported to produce 0.54–0.62 and 0.63–0.68 mole of 1,3-propanediol from one mole of glycerol, respectively [40,41]. The yields of 1,3-propanediol obtained in the present study (0.46–0.62 mol/mol glycerol) are comparable with those values, suggesting that heat-pretreated inocula could be instrumental to simultaneous biological production of 1,3-propanediol and hydrogen from glycerol.

These results of this study demonstrate the importance of inocula to fermentation patterns as well as substrates in hydrogen production. Hydrogen production from starch occurred via two different fermentation patterns: butyric acid-type and ethanol-type fermentations. Hydrogen production by ethanol-type hydrogen fermentation recently has been gaining attention [42–44]. Ren et al. [42], using an inoculum obtained from the bed mud of a domestic wastewater discharge channel, found that the hydrogen production rate in ethanol-type fermentation was higher than that in butyric acid-type fermentation. Ren et al. in another study [43] isolated an ethanol-based H_2-producing bacterium *Ethanoligenens harbinense* from an ethanol-type hydrogenic bioreactor, controlled under 35 °C and a pH value of 4.0–4.5, in which the microbial community structure was formed from the settled solids of domestic sewage. According to their phylogenic analysis, *E. harbinense* is very closely related to *C. cellulosi* [43]. Lin and Hung [44] have reported that thermophilic mixed natural microflora enriched from cow dung produced simultaneously high yields of hydrogen and ethanol from cellulose and xylose. In the present study, a close relative to *C. pasteurianum* was observed from AS, which showed ethanol-type fermentation, indicating that there are some clostridial species that could simultaneously produce high yields of hydrogen and ethanol.

5. Conclusions

This study investigated the effects of heat-pretreated inocula on the characteristics of fermentative hydrogen production from 4 different substrates: starch, glycerol, cooking oil and peptone. Significant hydrogen production potentials were observed with the starch medium (20.5–174.4 ml H_2/g-COD_{starch}) and the glycerol medium (11.5–38.1 ml H_2/g-$COD_{glycerol}$). By contrast, hardly any hydrogen was produced from the cooking oil and peptone mediums. The inocula had significant influences on the hydrogen production potentials and fermentation patterns for the starch medium, but not for the glycerol medium.

There were two distinct fermentation patterns from the starch medium, according to the inoculum: butyric acid-type fermentation and ethanol-type fermentation. It is noteworthy that the activated sludge produced high yields of hydrogen and ethanol simultaneously. According to the PCR-DGGE analysis conducted after the cultivation using starch medium, the dominant clostridial bacteria differed between the butyric acid-type fermentation and ethanol-type fermentation patterns.

However, glycerol was converted mainly into 1,3-propanediol in all of the inocula, to the yield of 50–70%, based on the COD. Hydrogen production concomitant with 1,3-propanediol from glycerol is also interesting.

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