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Technical Communication

Bioaugmented hydrogen production from microcrystalline cellulose using co-culture—*Clostridium acetobutylicum* X_9 and *Ethanoigenens harbinense* B_{49}

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ABSTRACT

Dark fermentation of microcrystalline cellulose to produce biohydrogen using mono-culture or co-culture of isolated strains was studied. A strain (X_9) with high hydrogen yield from microcrystalline cellulose was isolated and identified to be closely affiliated with *Clostridium acetobutylicum*, ATCC 824. At 37 °C and pH 5.0, the mono-culture of X_9 yields hydrogen with a 5-h time lag and end liquid products primarily of acetate and butyrate. The co-culture of X_9 with another strain, *Ethanoigenens harbinense* B_{49}, which can produce hydrogen efficiently from monosaccharides but directly from microcrystalline cellulose, produced more efficiently the biohydrogen via ethanol-type fermentation metabolism compared with mono-culture X_9 test. Bioaugmentation with $X_9 + B_{49}$ improved cellulose hydrolysis and subsequent hydrogen production rates as compared with that of mono-culture bioaugmentation with X_9.

1. Introduction

Bio-conversion of a biomass to produce hydrogen has been demonstrated utilizing anaerobic fermentation of some well-defined compounds in water [1–4]. Only limited data on hydrogen yield from wastewater sludge have been reported [5,6]. Production of biohydrogen from waste materials is considered an attractive future energy source [7]. Cellulose is a predominant constituent of agricultural waste and waste generated by the pulp and paper industry. To generate hydrogen directly from cellulose materials using dark fermentation requires expensive pretreatment processes such as delignification and hydrolysis to dissolve organic matter from a lignocellulose complex [8–10].

Lay [11] investigated the potential of producing hydrogen from microcrystalline cellulose using mesophilic digestion with heat-shocked sludge. With a 4-day lag, a maximum hydrogen yield of 4.36 mg g^{-1} cellulose was produced from suspensions containing 12.5 g cellulose l^{-1}. The metabolites were predominantly alcohols, followed by volatile fatty acids (VFAs).

Liu et al. [12] determined that their mixed culture comprising microbes closely affiliated with the genus *Thermoanaerobacterium* produced hydrogen that peaked at 7.56 mg H_2 g^{-1} cellulose and a maximum rate of 21.2 mg H_2 g^{-1} VSS d^{-1} from a 5 g cellulose l^{-1} suspension maintained at pH 6.5 and 55 °C. The metabolites observed were primarily acetate, butyrate, and ethanol.

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Isolating strains that can effectively utilize cellulose materials to produce hydrogen at room temperature is of great practical interest. This study isolated a strain, a member of clostridia genera (*Clostridium acetobutylicum*, ATCC 824), from a hydrogen-producing reactor, and determined hydrogen production potential of this strain from microcrystalline cellulose suspensions at 37 °C. This work also tested whether this stain can work with another strain, *Ethanoigenens harbinense* B_{49}, which can produce hydrogen efficiently from monosaccharides, for bioaugmented biohydrogen production from microcrystalline cellulose. The maximum hydrogen yield in the bioaugmented co-cultured system was 16.2 mg H_2 g^{-1} cellulose, the highest amongst those reported in literature to produce biohydrogen from cellulose materials.

2. Materials and methods

2.1. Strain cultivation

Liquor samples were obtained from a pilot-scale hydrogen fermenter in Harbin, China, which is a continuous flow anaerobic fermentation reactor with working volume of 1.48 m^3 and fed with molasses as the substrate. The operation was performed under organic loading rates of 3.11–85.8 kg COD m^{-3} d^{-1} for over 200 days. The liquid samples were placed in aseptically mixed sterilized tubes containing 50 ml medium. The chemical composition of the medium was (g l^{-1}): microcrystalline cellulose, 10 g; peptone, 4.0 g; beef extract, 2.0 g; yeast extract, 1.0 g; $MgCl_2$, 0.15 g; $FeSO_4$, 0.15 g; NaCl, 1.0 g; K_2HPO_4, 1.5 g; L-cysteine, 0.5 g. An amount of 10 ml l^{-1} medium of both vitamins (per liter: cyanocobalamin 0.01 g, ascorbic acid 0.025 g, riboflavin 0.025 g, citric acid 0.02 g, pyridoxine 0.05 g, folic acid 0.01 g, 4-aminobenzoic acid 0.01 g, and creatine 0.025 g) and micronutrients ($MnSO_4 \cdot 7H_2O$ 0.01 g, $ZnSO_4 \cdot 7H_2O$ 0.05 g, H_3BO_3 0.01 g, $N(CH_2COOH)_3$ 4.5 g, $CaCl_2 \cdot 2H_2O$ 0.01 g, Na_2MoO_4 0.01 g, $CoCl_2 \cdot 6H_2O$ 0.2 g, $AlK(SO_4)_2$ 0.01 g), and 1 ml of resazurin (0.2%) were also added. The microcrystalline cellulose utilized has cellulose content at 97.2% (dry basis) and a water solubility $<0.1\%$.

The mixtures were incubated at 37 °C for 18 h. Suspension samples collected from the incubated tubes were plated on agar plates. Three isolates with maximum potential to produce hydrogen from microcrystalline cellulose were chosen for further study.

2.2. DNA extraction and sequencing

The strain DNA was extracted by enzymatic lysis using extraction buffer (100 mM Tris HCl, pH 8.0; 100 mM EDTA, pH 8.0; 1.5 M NaCl) containing proteinase K (10 mg ml^{-1}). The samples were incubated at 37 °C and shaken at 150 rpm for 30 min. The SDS was added (20%) to samples and incubated at 65 °C for 90 min. Samples were subjected to two cycles of freezing and thawing. Supernatant was obtained after centrifugation at 6000 g for 10 min, and the aqueous phase was extracted with phenol:chloroform:isoamyl alcohol (25:24:1, v:v:v). The DNA was precipitated using isopropanol, pelleted by centrifugation (10 000 g) for 10 min, and resuspended in water. The DNA amplification reaction was conducted with primers BSF8/20—5'-AGAGTTTGATCCTGGCTCAG-3' and PLA—5'-GGTACTTAGATGTTTCAGTTC-3'. The PTC-100 (MJ Research, San Francisco, CA, USA) was utilized for polymerase chain reaction (PCR) multiplication. The reaction system for PCR multiplication was 10 μl buffer (10 μmol), 3 μl primer BSF8/20 (0.6 μmol), 3 μl primer PLA (0.6 μmol), 8 μl dNTP (200 μmol), 0.5 μl Tag enzyme (5U μl^{-1}), 1 μl template (0.05–1.00 μg μl^{-1}), and 74.5 μl ddH_2O. The DNA was amplified using eppendorf mastercycler (eppendorf AG, Germany) by denaturation at 94 °C for 4 min, annealing at 55 °C for 1 min, elongating at 72 °C for 1.5 min for 30 cycles, and elongating at 72 °C for 10 min. The PCR-amplified 16S rRNA was purified by low melting point agarose electrophoresis. The DNA was sequenced using the ABI Prism model 3730 (version 3.2) DNA sequencer.

2.3. Fermenting test

Fermentation tests were performed using the same medium as that in cultivation tests (Section 2.1). Briefly, 50 ml sterilized medium was mixed with 10 ml inoculum (biomass of 0.375 g dry cells) and was kept at 37 °C for 18 h. The isolated strain that had the highest hydrogen potential among those isolates from microcrystalline cellulose suspensions was tested further.

Another strain isolated by [13], B_{49}, AF481148 in NCBI, was utilized to co-work with the isolated strain X_9 in fermentation tests. In the co-culture tests, the two strains were mixed at the same volumes at a fixed total biomass quantity (0.375 g dry cells).

2.4. Analytical methods

A wet gas meter was utilized to measure biogas production volume at room temperature. The VFA and alcohol contents in centrifugated (4000 rpm) fermenting liquor were determined by a gas chromatography (GC112, Perkin Elmer, USA) with nitrogen as the carrier gas (flow rate of 60 ml min^{-1}). The hydrogen flame detector were employed with hydrogen and air flow rates of 50 and 490 ml min^{-1}, respectively, and a stainless steel column (2 m) packed with GDX-103 (60–80 meshes). The column and all cells were kept at 190 °C. Hydrogen content in the gas phase was determined by gas chromatography with nitrogen as the carrier gas (70 ml min^{-1}) and a thermal conductivity detector. The column (2 m) was packed with TDS-01 (60–80 meshes). The column and detector were kept at 150 °C. Based on the measured hydrogen production quantity and cell amount, and maximum hydrogen production rate (Q_{H_2}) was calculated as Q_{H_2} =(maximum hydrogen production rate)×(hydrogen content in gas)/dry cell mass (mmol g^{-1} dry cells). The specific hydrogen yield (Y_{H_2}) was approximated as Y_{H_2} =(accumulated biogas production quantity)×(hydrogen content in gas)/(medium volume).

The contents of reduced sugars were determined using the dinitrosalicylic (DNS) technique. The hydrolysis content in microcrystalline cellulose was estimated using the carbon

balance calculation:

$$\text{Hydrolysis ratio (\%)} = (C(CO_2) + C(EtOH) + C(Ac) + C(Bu) + C(\text{reduced sugar}))/(C(\text{cellulose}))$$

An atomic force microscope and a scanning electron microscope (model S-4500, Hitachi Co., Japan) were utilized to assess the morphology of isolates and stain B_{49}.

3. Results and discussion

3.1. Isolated strains and screening tests

The strains isolated from fermenting liquor were tested with their hydrogen production potentials from microcrystalline cellulose. Three isolated strains, X_9, B_2, and C_3, had high hydrogen yields from microcrystalline cellulose (Table 1). That is, strains X_9, B_2, and C_3 generated hydrogen yields of 755, 68.8, and 267 ml l^{-1} medium, respectively. Furthermore, as expected, strain B_{49} did not produce hydrogen from microcrystalline cellulose (Table 1). The associated metabolites for strain X_9 using microcrystalline cellulose were primarily acetate and butyrate, whereas those for B_2 and C_3 were ethanol and acetate. That is, strain X_9 had typical butyrate-type fermentation metabolism, whereas B_2 and C_3 underwent so-called ethanol-type fermentation metabolism [14].

To determine the maximum hydrogen yield the present isolates could derive from microcrystalline cellulose, strain X_9 is discussed further in subsequent sections.

3.2. Strain X_9 and hydrogen production

The partial genome of 1496 bp from strain X_9 was isolated and aligned using NCBI blast. This strain had 96% sequence similarity with *C. acetobutylicum* ATCC 824. The cells were regular rods ($0.3-0.4 \times 5-8\,\mu m$) without flagellum and occurred singly (Fig. 1).

Physiological tests demonstrated that the optimal temperature and pH for strain X_9 to degrade microcrystalline cellulose

Table 1 – Hydrogen yields and metabolites from microcrystalline cellulose with different inoculum

Innoculum	Y_{H_2} (ml c^{-1} culture)	Gas production (ml)	Final pH	Metabolites (mg c^{-1})		
				Ethanol	Acetate	Butyrate
B_{49}	ND	ND	NA	ND	ND	ND
X_9	755	87	4.21	ND	1700	1920
B_2	68.8	3	4.65	801	740	ND
C_3	267	43	4.32	1100	959	ND
$X_9 + B_{49}$	1810	127	3.79	1780	2060	987

ND: not detectable; NA: not available.

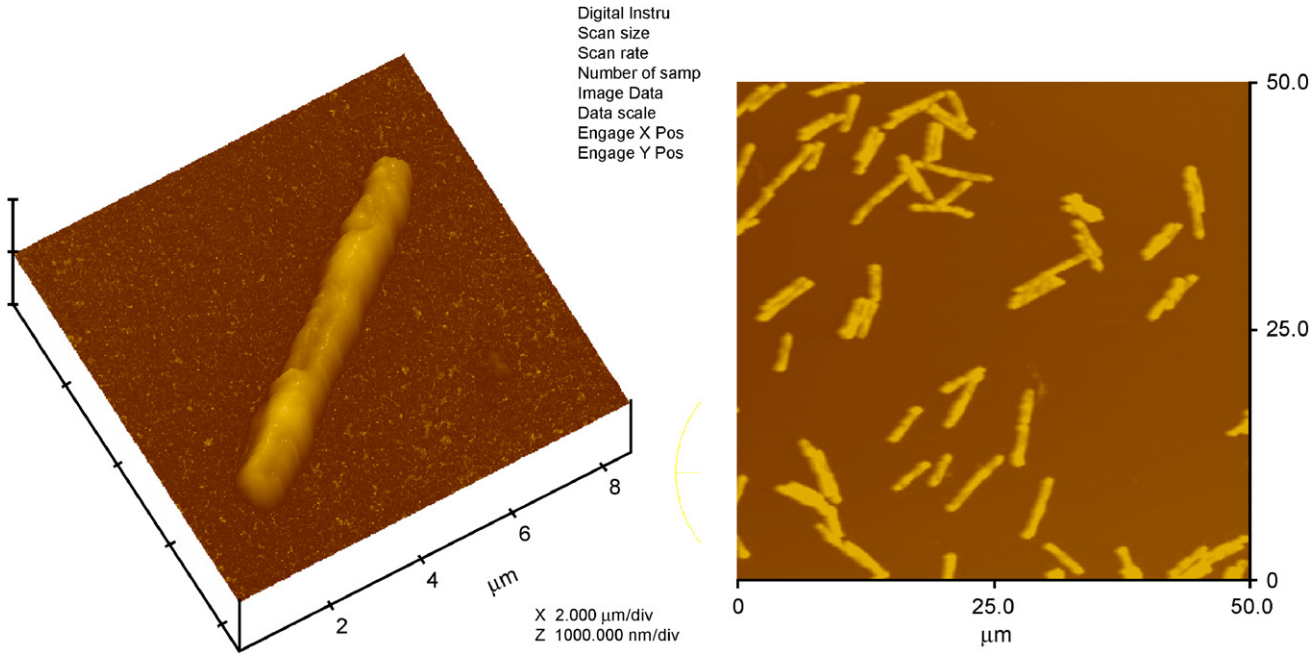

Fig. 1 – The AFM images of strain X_9.

were 40 °C and 5.0, respectively (data not shown). Doubling time of X_9 was 1.6 h.

Fig. 2 presents the hydrogen yield and cellulose hydrolysis ratio for strain X_9 to degrade microcrystalline cellulose at 37 °C and pH 5.0. Initially, approximately 10% of the cellulose was rapidly hydrolyzed. Further cellulose hydrolysis and hydrogen production did not occur during a 5-h lag phase. The cellulose was then promptly hydrolyzed and hydrogen was produced primarily in the following 3–6 h. The corresponding hydrogen yield, maximum hydrogen production rate, and cellulose hydrolysis ratio reached 755 ml l^{-1} medium, 6.4 mmol H_2 h^{-1} g^{-1} dry cell, and 68.3%, respectively. Hence, strain X_9 has a good capability to hydrolyze microcrystalline cellulose and then convert it into hydrogen.

3.3. *Co-culture of strains* X_9 *and* B_{49}

Strain B_{49}, identified as a novel *Ethanoigenens* strain, is gram-positive, no-spore-forming, no encapsulate, strict anaerobe [13]. The cells of B_{49} were straight rods (0.3−0.4 × 1−5.5 μm) without flagella and occurred singly (Fig. 3). The optimal temperature and pH for B_{49} to grow and produce hydrogen from glucose was 37 °C and 4.8, respectively. Doubling time was 7.2 h. Strain B_{49} can utilize glucose, wheat, soybean, corn, potato starch, and molasses in wastewater, but cannot utilize microcrystalline cellulose. This strain produced hydrogen from glucose with maximum hydrogen production rate of 25 mmol H_2 h^{-1} g^{-1} dry cells and a hydrogen yield of 1810 ml l^{-1} medium [13].

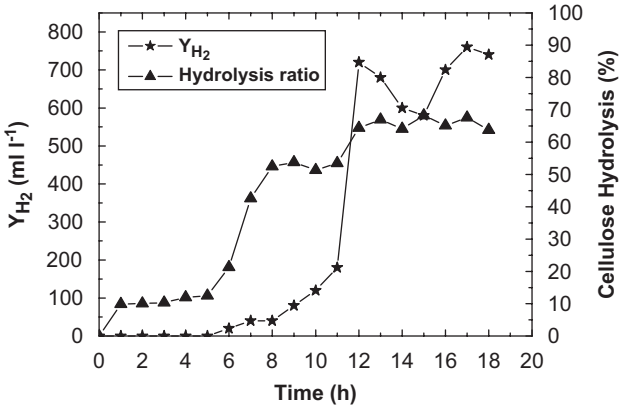

Fig. 2 – Hydrogen yield and cellulose hydrolysis ratio for strain X_9 at 37 °C and pH 7.0. Microcrystalline cellulose content 25 g l^{-1} medium; 50 ml medium. Total cell dry mass 0.375 g.

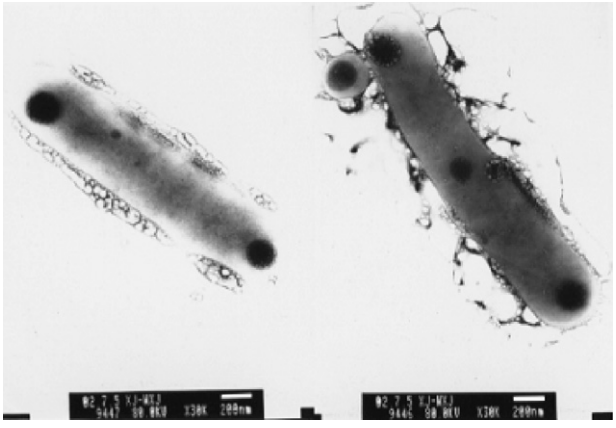

Fig. 3 – The SEM images of strain B_{49}.

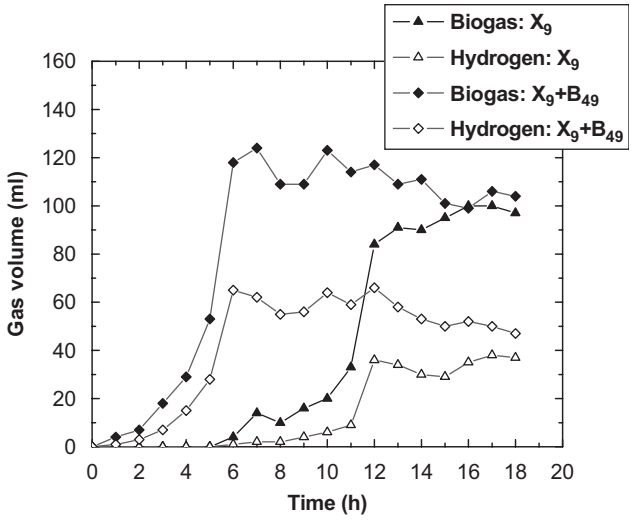

Fig. 4 – Biogas production quantity and hydrogen yield for cocultured strains $X_9 + B_{49}$ at 37 °C and pH 7.0. Microcrystalline cellulose content 25 g l^{-1} medium; 50 ml medium. Total cell dry mass 0.375 g.

When the two strains were co-cultured in a medium, hydrogen production was initiated immediately following initiation of fermentation (Fig. 4). The entire fermentation process was completed in 6 h, generating a high hydrogen yield, maximum hydrogen production rate, and a cellulose hydrolysis ratio of 1810 ml l^{-1} medium, 55.4 mmol H_2 h^{-1} g^{-1} dry cell, and 77.6%, respectively. The hydrogen yield by strains $X_9 + B_{49}$ was roughly 2.4 times that for the monoculture test with only X_9 (Fig. 2), and was close to the yield from glucose with B_{49} alone. Since strain B_{49} alone could not utilize microcrystalline cellulose (Table 1); hence, simultaneous bioaugmentation with the two strains in the fermenting liquor significantly improved cellulose hydrolysis and subsequent hydrogen production rates as compared with that of monoculture bioaugmentation.

3.4. *Metabolites*

In a mono-cultured test with X_9, the suspension pH dropped from 6.7 gradually during the initial 5 h to approximately 4.2–4.5, as shown in Fig. 5. This pH drop corresponded to the lag phase for hydrogen production noted in Fig. 2. The content of reduced sugar peaked at 0.10−0.13 g l^{-1} (Fig. 6). Following this lag phase, cellulose was rapidly hydrolyzed and hydrogen began to be produced, whereas the corresponding suspension pH was retained near 4.2, and the content of reduced sugar rapidly dropped to very low value at 7 h and after.

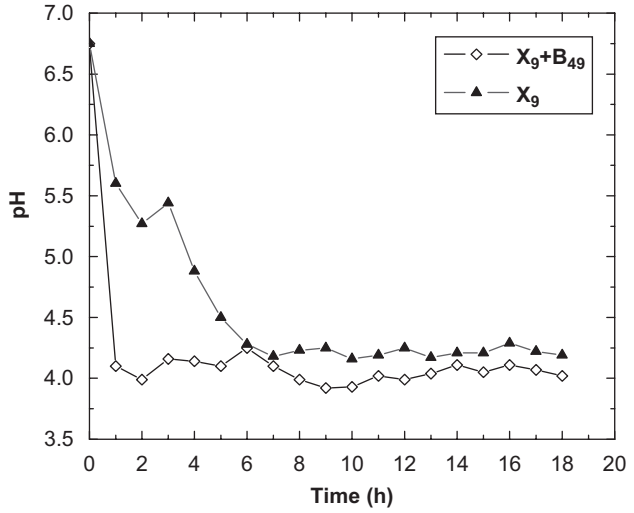

Fig. 5 – Time course of suspension pH in mono-cultured and co-cultured tests. Experimental conditions corresponding to those in Fig. 4.

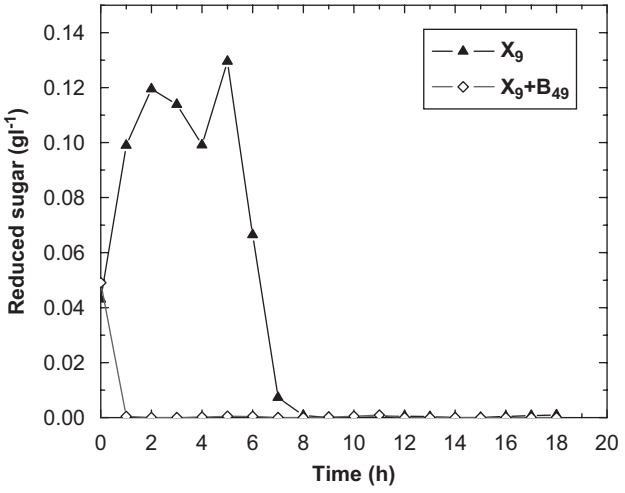

Fig. 6 – Time course of contents of reduced sugar in mono-cultured and co-cultured tests. Experimental conditions corresponding to those in Fig. 4.

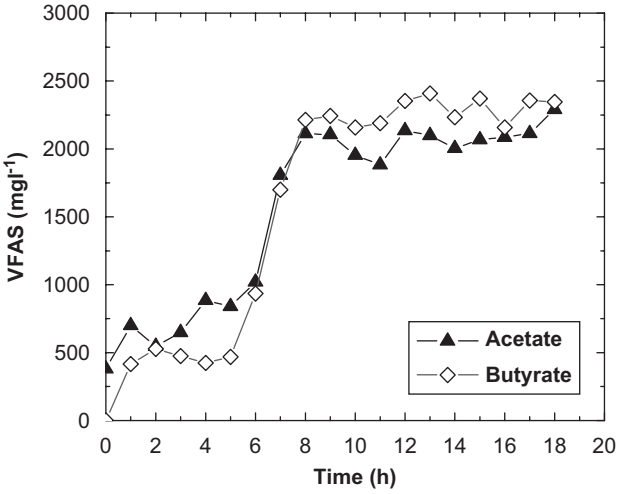

Fig. 7 – Time course of VFA in mono-cultured tests with strain X_9. Experimental conditions corresponding to those in Fig. 2.

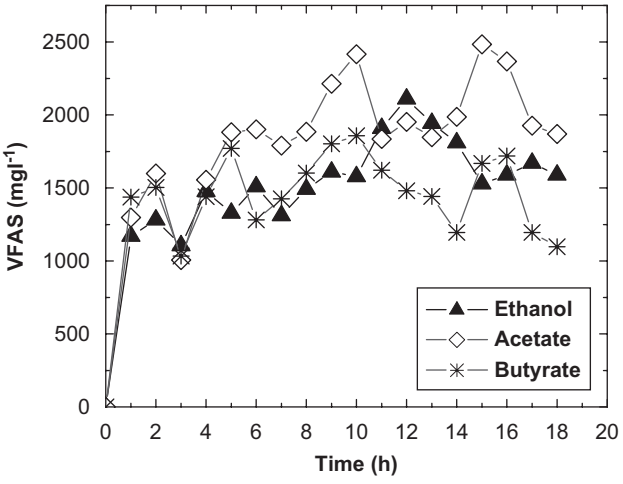

Fig. 8 – Time course of VFA in co-cultured tests with strains X_9 and B_{49}. Experimental conditions corresponding to those in Fig. 4.

In the co-culture test, cellulose hydrolysis and metabolite formation occurred right after fermentation started. The pH dropped to near 4.1 in 1 h of fermentation (Fig. 5). The reduced sugar did not accumulate in the fermenting liquor to a detectable quantity.

The end liquid products of mono-culture test were primarily acetate and butyrate (Table 1 and Fig. 7), whose molar ratio (acetate/butyrate) was near 1.3. When both X_9 and B_{49} were co-cultured in the fermenting liquor, the end liquid products were acetate, ethanol, and butyrate (Fig. 8), with a molar ratio (acetate/ethanol/butyrate) of nearly 2.5:2.5:1.0. In other words, co-cultured strains underwent the ethanol-type fermentation discussed by Ren et al. [14]. This occurrence was attributable to the fact that strain B_{49} degrades glucose via an ethanol-type fermentation pathway [13]. The 5-h phase lag for the mono-culture test with X_9 alone for cellulose hydrolysis and hydrogen fermentation demonstrates that particular metabolites generated in the initial phase of fermentation inhibited X_9 activity. Strain X_9 needed 5 h to adapt to the environment to begin hydrolyzing cellulose. Rapid removal of reduced sugar formed by strain B_{49} may help remove the phase lag from possible inhibition effects on X_9 by hydrolyzed reduced sugars. Additionally, as X_9 hydrolyzed more cellulose in the presence of B_{49} as compared with the mono-culture test, acetate and butyrate around 1800–2000 $mg\,l^{-1}$ may inhibit the growth of X_9. The two strains work together to yield hydrogen from microcrystalline cellulose.

4. Conclusions

This study produced directly biohydrogen from microcrystalline cellulose using dark fermentation. Three strains isolated from a

hydrogen-producing reactor were found to have high biohydrogen yield from microcrystalline cellulose. One strain that exhibited the highest hydrogen yield among the three tested isolates was identified as *C. acetobutylicum* X_9. At 37 °C and pH 5.0, the mono-culture tests with X_9 revealed that principal cellulose hydrolysis and hydrogen production occurred with a 5-h time lag. The cellulose was then promptly hydrolyzed and hydrogen was produced primarily in the following 3–6 h. The corresponding hydrogen yield, maximum hydrogen production rate, and cellulose hydrolysis ratio reached 755 ml l^{-1} medium, 6.4 mmol H_2 h^{-1} g^{-1} dry cell, and 68.3%, respectively. Equivalently, the hydrogen yield was 3.6 mmol H_2 g^{-1} cellulose. The end liquid products were primarily acetate and butyrate.

The co-culture of *C. acetobutylicum* X_9 with strain *E. harbinense* B_{49}, which can produce hydrogen efficiently from monosaccharides but directly from microcrystalline cellulose, yielded hydrogen immediately following initiation of fermentation. The hydrogen yield, maximum hydrogen production rate, and cellulose hydrolysis ratio of 1810 ml l^{-1} medium, 55.4 mmol H_2 h^{-1} g^{-1} dry cell, and 77.6%, respectively, were achieved. The corresponding end liquid products were acetate, ethanol, and butyrate. The equivalent hydrogen yield reached 8.1 mmol H_2 g^{-1} cellulose. This yield is the highest amongst those reported in literature to produce hydrogen from cellulose materials. The strain B_{49} rapidly removed reduced sugar produced by cellulose hydrolysis by X_9, hence improved cellulose hydrolysis and subsequent hydrogen production rates.

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