

1   **DARK FERMENTATIVE HYDROGEN PRODUCTION BY A HOT SPRING**  
2   **ENRICHMENT CULTURE FROM XYLOSE**

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## Abstract

Dark fermentative hydrogen production by a hot spring culture was studied from different sugars in batch assays and from xylose in continuous stirred tank reactor (CSTR) with on-line pH control. Batch assays yielded hydrogen in following order: xylose > arabinose > ribose > glucose. The highest hydrogen yield in batch assays was 0.71 mol H<sub>2</sub>/mol xylose. In CSTR the highest H<sub>2</sub> yield and production rate at 45 °C were 1.97 mol H<sub>2</sub>/mol xylose and 7.3 mmol H<sub>2</sub>/h/L, respectively, and at 37 °C, 1.18 mol H<sub>2</sub>/mol xylose and 1.7 mmol H<sub>2</sub>/h/L, respectively. At 45 °C, microbial community consisted of only two bacterial strains affiliated to *Clostridium acetobutylicum* and *Citrobacter freundii*, whereas at 37 °C six Clostridial species were detected. In summary hydrogen yield by hot spring culture was higher with pentoses than hexoses. The highest H<sub>2</sub> production rate and yield and thus, the most efficient hydrogen producing bacteria were obtained at suboptimal temperature of 45 °C for both mesophiles and thermophiles.

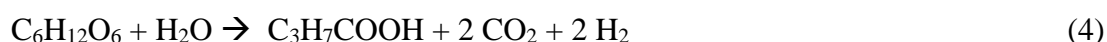
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## 1. Introduction

Lignocellulosic biomass is the most abundant raw material in nature [1]. Lignocellulosic biomass residues such as agricultural crops, pulp and paper industry wastewaters, food processing wastewaters and algae [2] are produced widely. Hydrolysis of lignocellulosic biomass leads to the production of hexoses (glucose, mannose, galactose) and pentoses (xylose, arabinose) [3]. These compounds can be utilized for biological hydrogen production through dark fermentation.

Dark fermentative hydrogen production has been widely studied from glucose (e.g., [4,5,6,7]), whereas less from xylose. Xylose is a degradation product of hemicellulose present in all lignocellulosic materials [8]. Hydrogen production results in a theoretical H<sub>2</sub> yield of 3.33 mol H<sub>2</sub>/mol xylose or 4.0 mol H<sub>2</sub>/mol glucose or 1.67 mol H<sub>2</sub>/mol

xylose or 2.0 mol H<sub>2</sub>/mol glucose when the soluble metabolites of fermentation are acetate (Eq. 1 or 2) or butyrate (Eq. 3 or 4), respectively [9].



The aim of this study was to characterize the substrate spectrum of a hot spring (45 °C) culture previously enriched for hydrogen production from glucose [7]. Furthermore, hydrogen production was studied from xylose in a continuous stirred tank reactor (CSTR). The effects of temperature (37 and 45 °C) on hydrogen production potential and the microbial community in a CSTR were determined.

## 2. Materials and methods

### 2.1. Hydrogen production from different substrates

The ability of hot spring culture to produce hydrogen from different sugars was examined using batch assays. Hexoses, i.e. glucose, galactose, mannose and fructose, pentoses, i.e. xylose, arabinose and ribose, and a disaccharide, sucrose, were used as substrates. Anaerobic 120 mL serum bottles with 50 mL working volume were used. A medium used contained substrate investigated (50 mM) and one liter of medium contained 10.7 g NaH<sub>2</sub>PO<sub>4</sub>, 3.2 g Na<sub>2</sub>HPO<sub>4</sub>, 0.6 g NH<sub>4</sub>Cl, 0.125 g KH<sub>2</sub>PO<sub>4</sub>, 0.11 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.1 g MgCl<sub>2</sub>·6H<sub>2</sub>O, 4 g NaHCO<sub>3</sub>, 0.18 g FeCl<sub>2</sub>·4H<sub>2</sub>O, 50 µg H<sub>3</sub>BO<sub>3</sub>, 50 µg ZnCl<sub>2</sub>, 38 µg CuCl<sub>2</sub>·2H<sub>2</sub>O, 41 µg MnCl<sub>2</sub>·2H<sub>2</sub>O, 50 µg (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O, 50 µg AlCl<sub>3</sub>, 50 µg CoCl<sub>2</sub>·6H<sub>2</sub>O, 50 µg NiCl<sub>2</sub>·6H<sub>2</sub>O, 0.5 mg EDTA, 2 g yeast extract, 26.3 µg Na<sub>2</sub>SeO<sub>3</sub>·5H<sub>2</sub>O, 32.9 µg NaWO<sub>4</sub>·2H<sub>2</sub>O, 0.013 g Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, 0.5 mg Resazurin, 0.24 g Na<sub>2</sub>S·9H<sub>2</sub>O and vitamin solution (DSMZ medium No141, German Collection of Microorganisms and Cell Cultures). Initial pH of the medium was 6.8. Each substrate was examined in duplicate. First batch assay bottles were inoculated (2 % v/v) with a hot spring culture originally enriched from 45 °C Hisarkoy hot spring samples on glucose for hydrogen production [7] and subsequent bottles with enrichment from

79 previous batch assay with same substrate giving the highest hydrogen yields. Three  
80 sequential batch incubations were done with each substrate examined. The bottles were  
81 incubated at 37 °C for 48 h. Gas production was measured by using a syringe method.

## 83 ***2.2. Hydrogen production in a CSTR***

84  
85 Hydrogen was produced continuously at 37 or 45 °C in a CSTR with a working volume  
86 of 0.9 L. The pH was maintained at 5.1 by continuous on-line titration (Metrohm, 719S)  
87 and the reactor was mixed mechanically (40 rpm). The hydraulic retention time (HRT)  
88 was varied between 12.5 and 10 h and the CSTR was inoculated (17 % v/v) with the  
89 hot spring culture. A synthetic feed described above was used with modifications by  
90 omitting resazurin and Na<sub>2</sub>S·9H<sub>2</sub>O and 50 mM xylose was used as substrate. Synthetic  
91 feed was prepared to tap water daily and stored at 4 °C. Gas production was measured  
92 with wet gas meter (Ritter Apparatebau, Bochum, Germany), gas samples were  
93 analyzed daily and liquid samples were taken three times a week.

## 95 ***2.3. Chemical analyses***

96  
97 Gas composition in batch bottles and CSTR was analyzed with a Shimadzu gas  
98 chromatograph GC-2014 equipped with Porapak N column (80/100 mesh) and a  
99 thermal conductivity detector (TCD). The temperatures of injector, oven, and detector  
100 were 110, 80, and 110 °C, respectively. Nitrogen was used as carrier gas at a flow rate  
101 of 20 mL/min.

102  
103 Substrate removal, volatile fatty acids (VFAs) and alcohols were analyzed with  
104 Shimadzu High Performance Liquid Chromatograph (HPLC) equipped with a  
105 refraction index detector (Shimadzu). HPLC was equipped with a Shodex Sugar  
106 SH1011 column (Showa Denko K.K., Japan) for substrate batch assay samples and 0.01  
107 N H<sub>2</sub>SO<sub>4</sub> was used as mobile phase at a flow rate of 0.7 mL/min. For CSTR samples,  
108 HPLC was equipped with Rezex RHM-Monosaccharide column (Phenomenex).  
109 Column was kept at 40 °C and 0.01 N H<sub>2</sub>SO<sub>4</sub> was used as mobile phase at a flow rate  
110 of 0.6 mL/min. All samples were filtrated (0.45 µm) before analysis.

## **2.4. Microbial community analysis with PCR-DGGE**

Bacterial communities were characterized using DNA extraction and polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE) of partial 16S rRNA genes followed by their sequencing. Microbial community samples were taken as duplicate from the reactor three times a week and stored at -20 °C. DNA was extracted from the pellets (sample centrifuged at 10'000xg for 5 min) with a PowerSoil™ DNA isolation kit (MoBio laboratories, Inc.). Partial bacterial 16S rRNA genes were amplified by using a primer pair GC-BacV3f and 907r as previously described by Koskinen et al. [5] by using T3000 Thermocycler (Biometra). DGGE was performed with INGENY phorU2 x 2 –system (Ingeny International BV, GP Goes, The Netherlands) as described by Nissilä et al. [10]. The bands were re-amplified for sequencing as described by Koskinen et al. [5] and sequence data was analyzed with Bioedit-software (version 7.0.5) and compared with sequences in GenBank (<http://www.ncbi.nlm.nih.gov/blast/>).

## **3. Results**

### **3.1 Hydrogen production from various substrates**

In batch assays the highest hydrogen yields (0.71 mol H<sub>2</sub>/mol substrate) were obtained with xylose (Figure 1). During the third enrichment step with arabinose, ribose and glucose H<sub>2</sub> yields of 0.50, 0.26 and 0.06 mol H<sub>2</sub>/mol substrate were obtained, respectively. Highest H<sub>2</sub> yields from arabinose and glucose were 0.61 and 0.54 mol H<sub>2</sub>/mol substrate, respectively, and were obtained during second enrichment step.

Highest hydrogen yields were obtained from xylose, arabinose and were accompanied by production of acetate, butyrate and formate (Table 1). With mannose, galactose, fructose and sucrose only little or no H<sub>2</sub> was produced and the main soluble end product was lactate. Production of lactate lowered pH to below 5.0 and decreased substrate conversion. Also, some ethanol and propionate was produced from all sugars.

### **3.2 Continuous hydrogen production in a CSTR**

Hydrogen production from xylose was studied first at 37 °C and then the CSTR temperature was increased to 45 °C. Considerably higher maximum and mean H<sub>2</sub> yields were obtained at 45 °C (1.97 and 1.46 mol H<sub>2</sub>/mol xylose), than at 37 °C (1.18 and 0.34 mol H<sub>2</sub>/mol xylose), respectively. Similarly, the hydrogen production rate and content increased with increasing temperature, i.e., from 1.71 to 7.28 mmol H<sub>2</sub>/h/L and from 23.7 to 48.4 %, respectively (Figure 2). The results were also affected by short-term operational interferences and addition of inoculum on day 8 (Table 2). Three process upsets occurred during continuous operation at 45 °C; oxygen leaked to the reactor on day 38, temperature decreased to 20 °C on day 45, and pH decreased to below 5.0 on day 55. Regardless of process upsets, hydrogen production recovered and stabilized shortly after each upset. The main soluble metabolites were acetate and butyrate with low amounts of ethanol at 37 °C (Figure 2.D). The acetate/butyrate ratio increased from 0.74 to 0.88 when the temperature increased from 37 to 45 °C, respectively.

### 3.2. Microbial community analysis

Microbial communities at different time points during CSTR operation were characterized with PCR-DGGE-sequencing (Figure 3, Table 3). At 37 °C the microbial communities consisted mainly of Clostridial species (6 species). *Citrobacter freundii*, was present at 45 °C. *Clostridium acetobutylicum* was a dominant species at both temperatures and was the only bacterium at 45 °C in addition to *C. freundii*. *Clostridium butyricum* was present only in the beginning of operation, while *Clostridium tyrobutyricum* was present over the whole continuous operation at 37 °C.

## 4. Discussion

### 4.1 Hydrogen production from pentoses and hexoses

In batch assays the highest hydrogen yield of 0.71 mol/mol xylose corresponded with 21 % of the theoretical yield with acetate as the only soluble metabolite. Also from arabinose and ribose 15 and 8 % of theoretical H<sub>2</sub> yield was obtained, whilst on glucose

the yield was only 2 %. This indicates that microbial community favored pentoses over hexoses in H<sub>2</sub> fermentation. On glucose, galactose, mannose, fructose and sucrose lactate was the as main soluble end product. This indicates that hexoses and sucrose favored lactic acid bacteria over H<sub>2</sub> producing bacteria leading to lactic acid production instead of H<sub>2</sub> fermentation. Furthermore, decrease in pH likely inhibited hydrogen producing bacteria.

Unlike batch assays (37 °C) the hydrogen production rate and yield from xylose in the CSTR at 37 °C remained at 1.7 mmol H<sub>2</sub>/h/L and 0.34 mol H<sub>2</sub>/mol xylose, respectively. Increasing the temperature from 37 to 45 °C, however, increased the average hydrogen production rates and yields to 9.9 mmol H<sub>2</sub>/h/L and 1.97 mol H<sub>2</sub>/mol xylose (59 % of theoretical yield), respectively. Hydrogen production from glucose with the same hot spring culture resulted in hydrogen yields of 0.9 and 1.71 mol H<sub>2</sub>/mol glucose at 37 and 45 °C, respectively, corresponding to 23 and 43 % of the theoretical yield [11]. In the CSTR the H<sub>2</sub> yields from xylose were in the range of the results reported earlier (Table 4).

Hexoses and pentoses have different fermentation pathways [12]. In the batch assays of this study, pentose fermentation led to H<sub>2</sub>, butyrate, formate and acetate production whereas more lactate was produce from hexoses. This further suggests that pentose sugars are preferred substrates for this culture. In the continuous cultures, acetate and butyrate were produced at same amounts from xylose, while butyrate was the main fermentation product from glucose followed by acetate production [11].

Prakasham et al. [13] reported higher H<sub>2</sub> production with xylose compared to glucose. However, these results are contrary to those obtained by Kim and Kim [14] who studied H<sub>2</sub> production from different carbohydrates using a thermophilic mixed culture enriched from anaerobic digester sludge. They concluded that H<sub>2</sub> production capability decreased in the following order sucrose > galactose > glucose > cellobiose > starch > xylose. Also Jianzheng et al. [15] obtained higher H<sub>2</sub> production rates and yields from hexoses (glucose, fructose and galactose) than from pentose (arabinose) using a mesophilic mixed culture enriched from digested sludge.

#### ***4.2 Microbial communities responsible for continuous H<sub>2</sub> production***

At 37°C six species from the genus *Clostridia* was detected in the CSTR. *Clostridium acetobutylicum* and *Clostridium tyrobutyricum*, known H<sub>2</sub> producers, were present over the whole experiment period at both 37 and 45 °C. Hydrogen production with *C. acetobutylicum* from glucose [16] and cassava wastewater [17] has been reported with hydrogen yields of 1.79 and 2.41 mol H<sub>2</sub>/mol glucose, respectively. Optimum growth temperature of *C. acetobutylicum* is 37 °C [18] and it grows on xylose, although glucose and arabinose are more preferred substrates [19]. *C. tyrobutyricum* produces acetate, butyrate, H<sub>2</sub> and CO<sub>2</sub> as fermentation products from glucose with hydrogen yield of 1.35 mol H<sub>2</sub>/mol glucose [20]. In a continuous bioreactor *C. tyrobutyricum* produced hydrogen with a rate and yield of 7.2 L H<sub>2</sub>/L/d and 1.65 mol H<sub>2</sub>/mol hexose, respectively [21]. The metabolism of *C. tyrobutyricum* on xylose and glucose depends strongly on pH, temperature and substrate concentration [22,23]. Increasing the temperature from 37 to 45 °C further enriched the community and retained *C. acetobutylicum* and *Citrobacter freundii*, that has an optimum growth temperature of 37 °C [24]. These results indicate that the use of 45 °C, a suboptimal temperature for both mesophiles and thermophiles, selectively enriched efficient hydrogen producing bacteria.

Anaerobic microbial cultures enriched from one hot spring (Hisarkoy) have been studied under a variety of environmental conditions i.e. at different pH [7], temperature ([11], this study), and using different substrates (this study) (Figure 4). Different main metabolic products of fermentation including H<sub>2</sub>, ethanol or lactic acid were produced at different conditions. The community analyses revealed prompt community responses to changes in environmental conditions. These studies also revealed that the hot spring environment harbored a very diverse microbial community. Especially in continuous CSTR's with completely mixed biomass the washout of cells is compensated by fast enrichment of new desired microorganisms under given conditions. This emphasizes that changes in fermentation patterns in CSTR's are due to changes in community structures rather than metabolic changes within the bacteria.

## 5. Conclusions

The Hisarkoy hot spring enrichment culture produced hydrogen from many sugars and favored pentoses over hexoses. Batch assays yielded 0.71 mol H<sub>2</sub>/mol xylose (21 % from the maximum yield). Hydrogen was produced continuously from xylose in a CSTR both at 37 and 45 °C. The highest average hydrogen production rate and yield, 170 mmol H<sub>2</sub>/d/L and 1.46 mol H<sub>2</sub>/mol xylose, respectively, were obtained at suboptimal temperature of 45 °C for mesophiles and thermophiles. *Clostridium acetobutylicum* (T<sub>opt</sub> 37 °C) and *Citrobacter freundii* (T<sub>opt</sub> 37 °C) were the only bacterial species remaining at 45 °C and thus, responsible for xylose fermentation to hydrogen.

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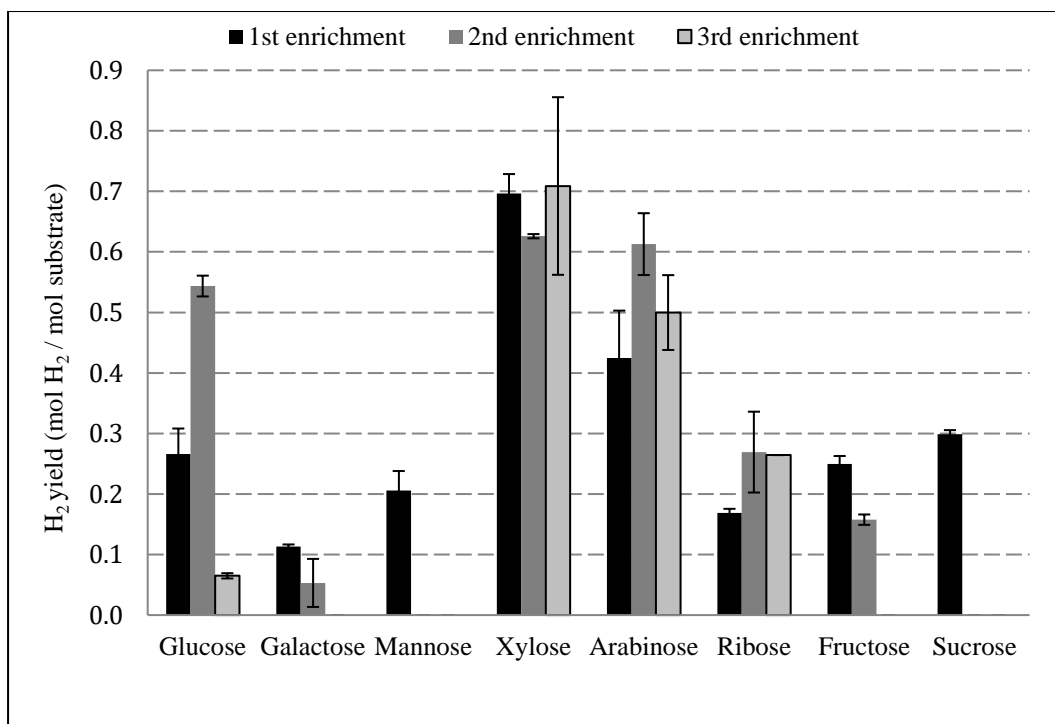
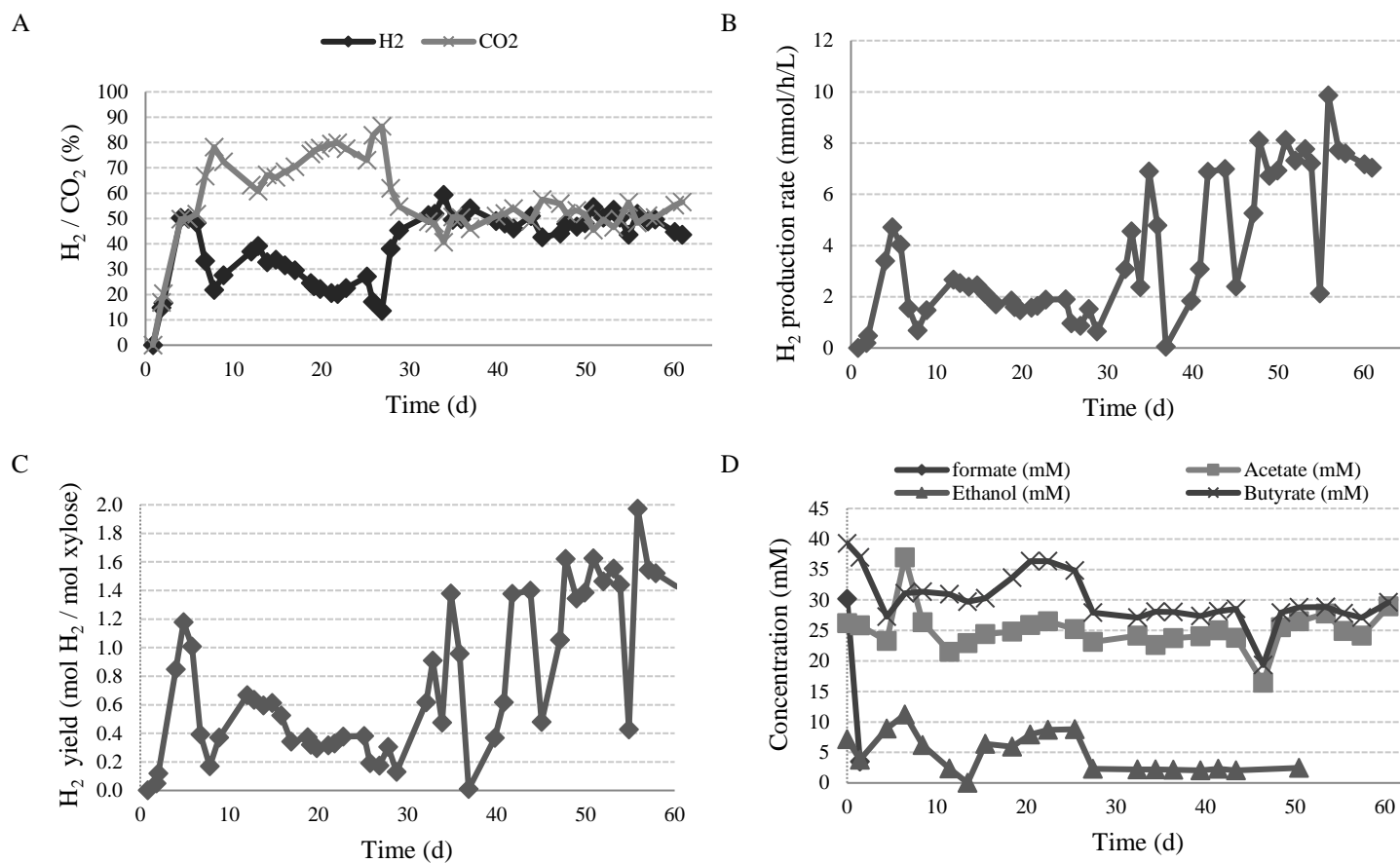


Figure 1. H<sub>2</sub> yields obtained from three subsequent enrichments of hot spring culture using different substrates.



390 Figure 2. H<sub>2</sub> and CO<sub>2</sub> percentages (A), hydrogen production rates (B) and yields (C)  
 391 and soluble metabolites produced (D) in a CSTR fed on xylose at 37 °C (days 0-27)  
 392 or at 45 °C (days 28-63).

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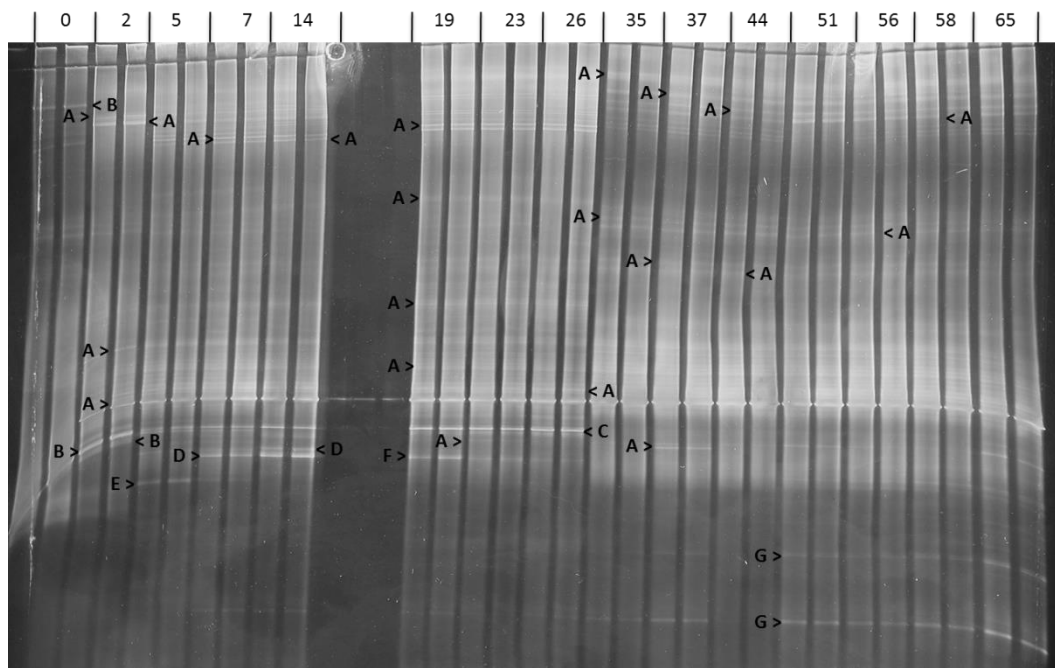


Figure 3. Bacterial community profile in a CSTR at different time points (in days) determined with PCR-DGGE of partial 16S rRNA genes. See Table 3 for the labeled bands.

	Substrate	T (°C)	pH	H <sub>2</sub> yield (mol H <sub>2</sub> /mol substrate)	EtOH yield (mol H <sub>2</sub> /mol substrate)	Lactate yield (mol H <sub>2</sub> /mol substrate)	Microbial community	Reference
Hisarkoy hot spring sample	Glucose	60	5.0	0.9	<b>1.5</b>	0	<i>Thermoanaerobacteria</i> (2 species)	[11]
		50	5.0	0	0	<b>1.3</b>	<i>Bacillus coagulans</i>	[11]
		45	5.0	<b>1.7</b>	0	0	<i>Clostridium</i> sp.	[11]
		37	>5.5	0.1-0.3	<b>0.7-1.2</b>	0	<i>Clostridium</i> <i>chartatabidum</i>	[7]
			5.3	<b>1.4</b>	0.2	0	<i>Clostridium butyricum</i> , <i>Clostridium ramosum</i>	[7]
			4.9	0.7	0.1	<b>0.6</b>	<i>Bacillus coagulans</i>	[7]
	Xylose	45	5.1	<b>1.5</b>	0	0	<i>Clostridium</i> <i>acetobutylicum</i> , <i>Citrobacter freundii</i>	This study
		37	5.1	<b>0.3</b>	0.1	0	<i>Clostridium</i> <i>acetobutylicum</i> , <i>Clostridium tyrobutyricum</i>	This study

399

400 Figure 4. Effects of process conditions (substrate, temperature and pH) on hydrogen, ethanol and lactate production yields with enrichment  
401 cultures from the same hot spring sample and the main bacteria responsible for the main metabolic products.

Table 1. Soluble metabolites and substrate removal with hot spring culture using different substrates ( $\pm$  standard deviation).

Substrate	Lactate (mM)	Formate (mM)	Acetate (mM)	Propionate (mM)	Ethanol (mM)	Butyrate (mM)	Substrate conversion (%)
<b>Glucose</b>							
1 <sup>st</sup> enrichment	52.5 (1.5)	18.4 (0.3)	11.7 (2.4)	0.4 (0.2)	6.1 (80.3)	16.2 (0.3)	100 (0)
2 <sup>nd</sup> enrichment	24.8 (2.3)	26.3 (0.8)	14.4 (0.4)	0.3 (0.04)	5.8 (0.6)	25.1 (0.1)	100 (0)
3 <sup>rd</sup> enrichment	105.7 (10.1)	7.7 (0.1)	5.3 (0.2)	0.04 (0.06)	5.2 (0.4)	6.7 (0.2)	100 (0)
<b>Galactose</b>							
1 <sup>st</sup> enrichment	70.5 (2.1)	15.9 (0.1)	6.5 (0.1)	0.2 (0.1)	7.9 (0.3)	12.2 (0.1)	100 (0)
2 <sup>nd</sup> enrichment	84.3 (4.0)	11.6 (1.1)	5.1 (0.4)	0.3 (0.05)	7.9 (0.9)	8.1 (2.1)	100 (0)
3 <sup>rd</sup> enrichment	95.4 (0.7)	9.2 (0.04)	4.7 (0.1)	0.2 (0)	8.5 (0.04)	3.4 (0.05)	91.7 (3.5)
<b>Mannose</b>							
1 <sup>st</sup> enrichment	84.6 (2.3)	14.4 (0.4)	7.0 (0.4)	0.1 (0.1)	5.3 (0.9)	14.4 (0.8)	100 (0)
2 <sup>nd</sup> enrichment	128.4 (8.7)	0.9 (1.1)	1.6 (0.1)	0.1 (0.1)	4.8 (0.2)	1.7 (0.1)	96.4 (0.5)
3 <sup>rd</sup> enrichment	130.9 (11.5)	0.1 (0)	1.2 (0.04)	0.2 (0.3)	4.5 (0.2)	0.8 (0)	91.7 (11.5)
<b>Xylose</b>							
1 <sup>st</sup> enrichment	0.4 (0.1)	24.7 (7.8)	15.5 (4.3)	0.6 (0.1)	5.6 (0.3)	27.3 (2.4)	100 (0)
2 <sup>nd</sup> enrichment	1.0 (1.0)	29.6 (1.3)	19.4 (1.5)	0.3 (0.1)	4.5 (0.1)	26.0 (0.8)	100 (0)
3 <sup>rd</sup> enrichment	1.0 (1.0)	27.7 (0.8)	20.3 (2.7)	0.2 (0.03)	4.2 (0.05)	28.0 (3.3.)	97.4 (3.4)
<b>Arabinose</b>							
1 <sup>st</sup> enrichment	11.0 (0.7)	23.8 (2.3)	15.6 (1.1)	0.4 (0.02)	9.7 (0.2)	22.2 (1.4)	100 (0)
2 <sup>nd</sup> enrichment	3.2 (0.8)	25.7 (0.2)	16.3 (0.1)	0.2 (0.05)	4.3 (0.3)	24.2 (0.1)	100 (0)
3 <sup>rd</sup> enrichment	6.2 (2.4)	19.2 (3.5)	17.5 (1.2)	0.1 (0.01)	9.6 (1.5)	23.1 (0.1)	99.9 (0.1)
<b>Ribose</b>							
1 <sup>st</sup> enrichment	21.2 (2.2)	4.2 (0.5)	13.2 (0.2)	0.5 (0.03)	7.0 (0.3)	14.1 (0.4)	100 (0)
2 <sup>nd</sup> enrichment	0.1 (0.01)	0.3 (0.3)	23.8 (0.3)	0.5 (0.03)	5.4 (0.3)	16.9 (0.8)	100 (0)
3 <sup>rd</sup> enrichment	3.3 (0)	0.6 (0)	26.7 (0)	0.3 (0)	5.7 (0)	16.4 (0)	99.8 (0)
<b>Fructose</b>							
1 <sup>st</sup> enrichment	68.6 (5.5)	13.9 (1.0)	8.5 (0.3)	0.4 (0.3)	4.5 (1.1)	12.5 (0.4)	100 (0)
2 <sup>nd</sup> enrichment	87.1 (15.6)	12.9 (0.7)	7.4 (0.3)	0.1 (0.03)	4.5 (0.1)	10.3 (0.1)	100 (0)
3 <sup>rd</sup> enrichment	132.6 (2.8)	1.5 (0.1)	1.5 (0.03)	0.7 (0.7)	4.4 (0.4)	0.9 (0.01)	99.0 (0.2)
<b>Sucrose</b>							
1 <sup>st</sup> enrichment	77.0 (7.2)	21.4 (0.4)	11.4 (0.8)	0.5 (0.1)	7.1 (0.4)	16.6 (0.9)	53.5 (2.9)
2 <sup>nd</sup> enrichment	133.5 (1.0)	0.5 (0.6)	1.5 (0.1)	1.4 (0.1)	4.4 (0.2)	1.8 (0.2)	53.5 (2.9)
3 <sup>rd</sup> enrichment	137.0 (1.8)	0.4 (0.2)	0.8 (0.03)	1.7 (0.4)	4.4 (0.2)	0.8 (0)	58.9 (2.5)

Table 2. Changes in reactor parameters and process upsets during the reactor experiment.

Day	Reactor parameters / Process failures
0	Starting the reactor as batch, pH 5.1, 37°C
1	Continuous flow started with 1.2 mL/min (HRT = 12,5 h)
8	Addition of inoculum (100 mL)
17	Flow rate increased to 1.5 mL/min (HRT = 10 h)
26	Mixing was stopped during night
27	Temperature was increased to 45°C
38	Oxygen leak to the reactor
45	Temperature temporarily decreased to 20°C
55	pH decreased to below 5.0 due to titrator failure

Table 3. Affiliation of DGGE fragments determined by their 16S rRNA genes from CSTR reactor samples obtained at 37 or 45 °C.

BM <sup>a</sup>	Family <sup>b</sup>	Affiliation (acc) <sup>c</sup>	Sim (%) <sup>d</sup>	SL (bp) <sup>e</sup>
A	Clostridia	<i>Clostridium acetobutylicum</i> (FM994940)	91.5-99.8	408-508
B	Clostridia	<i>Clostridium butyricum</i> (FR734080)	91.0-99.1	435-437
C	Clostridia	<i>Clostridium tyrobutyricum</i> (GU227148)	97.2	460
D	Clostridia	<i>Clostridium</i> sp. (FJ805840)	92.3-95.2	444-454
E	Clostridia	<i>Clostridium diolis</i> (FJ947160)	92.7	476
F	Clostridia	Uncultured <i>Clostridia</i> (EU887962)	92.5	489
G	Enterobacteriaceae	<i>Citrobacter freundii</i> (HM756481)	99.6-99.8	451-476

<sup>a</sup> Band mark in Figure 3

<sup>b</sup> Family according to Ribosomal Database Project II

<sup>c</sup> Closest species in GenBank with accession number

<sup>d</sup> Similarity (%) of various bands

<sup>e</sup> Sequence length (base pairs)

Table 4. Hydrogen production rates and yields obtained in batch assays or in continuous reactors.

Culture	Reactor	Xylose (g/L)	T (°C)	pH	HRT (h)	H <sub>2</sub> (%)	H <sub>2</sub> yield (mol H <sub>2</sub> /mol xylose)	H <sub>2</sub> production rate (mmol H <sub>2</sub> /d/L)	Reference
<b>Batch assay</b>									
Sewage sludge	Batch	18.8 <sup>a</sup>	35	6.5	-	54	1.3	250	[25]
Sewage sludge	Batch	18.8 <sup>a</sup>	35	6.0	-	55	2.25	nr	[26]
Pure culture <sup>b</sup>	Batch	18.8 <sup>a</sup>	37	7.5		nr	0.73	210	[27]
Pure culture <sup>c</sup>	Batch	10	60	7.0	-	nr	2.19	260	[28]
BioH <sub>2</sub> reactor	Batch	0.5	70	6.8	-	nr	1.62	nr	[29]
Pure culture <sup>d</sup>	Batch	16.2	40	7.0	-	nr	2.0	nr	[30]
BioH <sub>2</sub> reactor	Batch	2	70	7.0	-	nr	1.84	nr	[31]
Pure culture <sup>e</sup>	Batch	5	75	7.0	-	40	2.8	1.3	[32]
Hot spring	Batch	7.5	37	6.5	-	28	0.71	17.2	This study
<b>Continuous reactor</b>									
Sewage sludge	Chemostat	18.8 <sup>a</sup>	35	7.1	12	32	0.70	100	[26]
Sewage sludge	Chemostat	18.8 <sup>a</sup>	50	7.1	12	42	1.40	240	[33]
Compost	CSTR	2	55	5.0	22	nr	1.70	60	[34]
BioH <sub>2</sub> reactor	CSTR	1.0	70	6.7	72	31	1.36	2.6	[29]
Hot spring	CSTR	7.5	45	5.1	10	48	1.46	170	This study

<sup>a</sup> 20 g COD/L, <sup>b</sup> *Clostridium butyricum*, <sup>c</sup> *Thermoanaerobium thermosaccharolyticum*, <sup>d</sup> *Enterobacter* sp., <sup>e</sup> *Thermotoga neapolitans*  
nr: not reported