Bioprospecting Thermophilic Microorganisms from Icelandic Hot Springs for Hydrogen and Ethanol Production†

Perttu E. P. Koskinen,*,‡ Chyi-How Lay,§ Steinar R. Beck,||,⊥ Katariina E. S. Tolvanen,‡ Anna H. Kaksonen,‡ Jóhann Örlygsson,|| Chiu-Yue Lin,§ and Jaakko A. Puhakka‡

Institute of Environmental Engineering and Biotechnology, Tampere University of Technology, Post Office Box 541, FIN-33101, Tampere, Finland, Department of Environmental Engineering and Science, Feng Chia University, Taichung, 40724, Taiwan, and Faculty of Natural Resource Sciences, University of Akureyri, Borgir Nordurslod, Akureyri, Iceland

Received May 27, 2007. Revised Manuscript Received September 4, 2007

Fermentations can be used to produce sustainable energy carriers, such as hydrogen and ethanol (EtOH), from biomass or organic waste materials. The aim of this research was to prospect efficient H_2- and EtOH-producing thermophilic microorganisms derived from hot spring environments in Iceland. Hydrogen- and EtOH-producing enrichment cultures were obtained from various hot spring samples over a temperature range of 50–78 °C. The temperature dependencies for the most promising enrichments were determined with a temperature-gradient incubator. One of the enrichments (33HL) produced 2.10 mol of H_2/mol of glucose at 59 °C. Another enrichment (9HG), dominated by bacteria closely affiliated with *Thermoanaerobacter thermohydrosulfuricus*, produced 0.68 mol of H_2/mol of glucose, and 1.21 mol of EtOH/mol of glucose at 78 °C. Hydrogen and EtOH production by 9HG was characterized further in a continuous-flow bioreactor at 74 °C. The highest H_2 and EtOH yields of 9HG were obtained at pH 6.8 ± 0.3. Lactate production decreased the H_2 and EtOH yields in the continuous-flow bioreactor, and the yields were lower than those obtained in the batch fermentations. In conclusion, the thorough batch screening of Icelandic hot spring samples indicated some promising enrichments for H_2 or H_2 plus EtOH production from carbohydrate materials.

Introduction

Today, most of the energy demands are met by nonrenewable energy sources, resulting in resource depletion, environmental deterioration, and public health problems. Therefore, a demand to develop novel renewable energy-harvesting technologies and introduce sustainable energy carriers exists. Energy carriers, such as hydrogen and ethanol, can be produced from renewable energy sources. Hydrogen has many superior properties compared to all of the other energy carriers. It has the highest energy value per mass unit of all substances; its sources are globally distributed; and it can be converted to electricity efficiently and without air emissions. Bioethanol is a valuable renewable energy carrier in the transport sector. It can be used as such or mixed with gasoline to fuel vehicles.

Microbial fermentations have potential for combining organic waste treatment with simultaneous H_2 or ethanol production.[1,2] Carbohydrate-based materials are the most suitable substrates for fermentative H_2 or ethanol production.[3] Fermentative H_2 production has been successfully demonstrated with numerous kinds of carbohydrate materials and organic wastes, including sugar-based materials, such as sugar beet[4] and sugar factory wastewater,[5] starch-based materials, such as potato[6] and wheat[7] starch, and cellulose-based materials, such as *Miscanthus*.[8] In addition, promising pilot-scale demonstrations of dark fermentative H_2 production from industrial carbohydrate-containing wastewaters have been reported.[9]

Hydrogen production through dark fermentation has received increasing attention in the past few years. High H_2 production rates, up to 15 L h^{-1} L^{-1} of the reactor volume (i.e., 360 L day^{-1} L^{-1}),[10] have been achieved with mesophilic H_2 fermentations, and the long-term stability of the H_2 fermentations has been demonstrated.[11,12] Thermophilic H_2 fermentation may have

† Presented at the International Conference on Bioenergy Outlook 2007, Singapore, April 26–27, 2007.

* To whom correspondence should be addressed: Institute of Environmental Engineering and Biotechnology, Tampere University of Technology, Post Office Box 541, FIN-33101, Tampere, Finland. Telephone: +358-3-3115-11. Fax: +358-3-3315-2869. E-mail: perttu.koskinen@tut.fi.

‡ Tampere University of Technology.

§ Feng Chia University.

|| University of Akureyri.

⊥ Present address: Technological Institute of Iceland, IceTec, Keldnaholt, Reykjavik, Iceland.

(1) Levin, D. B.; Pitt, L.; Love, M. *Int. J. Hydrogen Energy* **2004**, *29*, 173–185.

(2) Kapdan, I. K.; Kargi, F. *Enzyme Microb. Technol.* **2006**, *38*, 569–582.

(3) Hawkes, F. R.; Dinsdale, R.; Hawkes, D. L.; Hussy, I. *Int. J. Hydrogen Energy* **2002**, *27*, 1339–1347.

(4) Hussy, I.; Hawkes, F. R.; Dinsdale, R.; Hawkes, D. L. *Int. J. Hydrogen Energy* **2005**, *30*, 471–483.

(5) Ueno, Y.; Otsuka, S.; Morimoto, M. J. *Ferment. Bioeng.* **1996**, *82*, 194–197.

(6) Logan, B. E.; Oh, S.-E.; Kim, A. S.; van Ginkel, S. *Environ. Sci. Technol.* **2002**, *36*, 2530–2535.

(7) Hussy, I.; Hawkes, F. R.; Dinsdale, R.; Hawkes, D. L. *Biotechnol. Bioeng.* **2003**, *84*, 619–626.

(8) de Vrije, T.; de Haas, G. G.; Tan, G. B.; Keijsers, E. R. P.; Claassen, P. A. M. *Int. J. Hydrogen Energy* **2002**, *27*, 1381–1390.

(9) Ren, N.; Li, J.; Li, B.; Wang, Y.; Liu, S. *Int. J. Hydrogen Energy* **2006**, *31*, 2147–2157.

(10) Wu, S.-Y.; Hung, C.-H.; Lin, C.-N.; Chen, H.-W.; Lee, A.-S.; Chang, J.-S. *Biotechnol. Bioeng.* **2006**, *93*, 934–946.

(11) Lin, C.-Y.; Chang, R.-C. *Int. J. Hydrogen Energy* **2004**, *29*, 715–720.

(12) Yu, H.-Q.; Mu, Y. *Biotechnol. Bioeng.* **2006**, *94*, 988–995.

10.1021/ef700275w CCC: $37.00
Published on Web 10/18/2007

many advantages as compared to mesophilic fermentation but has remained less studied. High temperatures favor the stoichiometry of H_2 production, resulting in higher H_2 yields as compared to mesophilic systems.[13] Furthermore, thermophilic fermentation is considered to have a narrower spectrum of end products as compared to mesophilic fermentation.[14] Elevated temperature conditions may also reduce the enrichment of contaminating microorganisms during H_2 fermentation processes.[13] Further, some thermophilic bacteria can produce ethanol from lignocellulosic biomass, a process that is not feasible by brewer's yeast.[15] The use of inexpensive lignocellulosic materials, such as forestry wastes, agricultural residues, grasses, and other low-cost biomass, can significantly reduce the cost of ethanol production compared to the conventional methods based on yeast fermentation.[16]

H_2 production through dark fermentation occurs during intermediary steps of anaerobic degradation of organic material. The end products of H_2 fermentation include gases (H_2 and CO_2) and soluble metabolites (organic acids and alcohols). The metabolic pathways of microorganisms determine the efficiency of H_2 production through dark fermentation.[17] The maximum H_2 yield, 4 mol of H_2/mol of glucose, is achieved when acetate is the sole soluble metabolite of fermentation (eq 1).

$$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 4H_2 + 2HCO_3^- + 4H^+ \quad (1)$$

The production of more reduced end products is undesirable because they contain hydrogen equivalents that are not released as H_2 gas.[3] Ethanol production from glucose is not directly associated with the production of H_2 (eq 2).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3CH_2OH + 2HCO_3^- + 2H^+ \quad (2)$$

However, coupled ethanol and acetate production pathways exist (eq 3), which have a theoretical maximum of 2 mol of H_2/mol of glucose.[18,19]

$$C_6H_{12}O_6 + 3H_2O \rightarrow CH_3CH_2OH + CH_3COO^- + 2H_2 + 2HCO_3^- + 3H^+ \quad (3)$$

Lactate production is not associated with hydrogen production and is therefore unfavorable for H_2 fermentation (eq 4).

$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOO^- + 2H^+ \quad (4)$$

Fermentative metabolism can be affected by the concentration of the end products, i.e., end-product inhibition by H_2 or organic acids and alcohols. The partial pressure of hydrogen (pH_2) especially can have a significant effect on the fermentation. Some thermophilic microorganisms may change their metabolism from acetate to lactate production when the pH_2 increases, thereby decreasing the H_2 yields and production rates.[14]

Hot springs are a potential source for thermophilic, H_2- and ethanol-fermenting microorganisms. In this study, geothermal springs in Iceland were bioprospected for H_2- and ethanol-fermenting microorganisms. Samples were collected from hot springs in various regions in Iceland and screened for the presence of anaerobes that could produce H_2 and ethanol from glucose and cellulose. Two promising enrichment cultures were further characterized in a temperature-gradient incubator, and one thermophilic enrichment was evaluated in a continuous-flow bioreactor. Information on thermophilic H_2 and ethanol fermentations is still scanty, and the results of this work help to further the progress in this promising area.

Table 1. Hydrogen and Soluble Metabolite Production by Initial Enrichment Cultures from Icelandic Hot Spring Samples Incubated at the Corresponding *in Situ* Temperatures

T (°C)	substrate[a]	sample	H_2 (mmol)	EtOH[b] (mmol)	HAc[c] (mmol)	HBu[d] (mmol)
50	G	18H	1.71	0.48	0.62	0.55
50	G	22HL	1.54	0.62	0.29	0.45
50	G	33H	1.90	0.75	0.74	0.55
60	G	7H	1.10	0.95	0.89	0.07
60	G	22H	0.19	1.32	1.15	0.03
60	G	25H	0.75	1.26	0.65	0.01
60	G	28H	0.97	1.73	0.91	0.01
60	G	29H	0.85	0.97	0.66	0.00
60	G	33HL	2.41	0.72	1.14	0.42
65	G	5H	0.75	1.26	0.67	0.02
65	G	44H	1.46	0.67	0.85	0.38
70	G	19HL	0.68	0.74	0.61	0.03
78	G	9HG	0.59	1.18	0.27	0.01
78	G	11H	0.52	0.11	0.83	0.01
78	G	13HG	1.13	0.10	1.08	0.00
70	C	PC2H	0.60	0.05	0.52	0.01

[a] G, glucose (1.5 mmol); C, cellulose. [b] EtOH, ethanol. [c] HAc, acetate. [d] HBu, butyrate.

Experimental Section

Batch Enrichment of Thermophilic H_2- and Ethanol-Producing Microorganisms from Hot Springs. Liquid and sediment samples for the enrichment of thermophilic H_2 and ethanol producers were collected in 120 mL serum bottles or 50 mL Falcon tubes from hot springs in Hveragerdi, Hveravellir, and Krafla regions in Iceland. The *in situ* temperatures of the samples were measured with a temperature probe. The pH values of the samples were measured in the laboratory. Samples were stored at +4 °C until used for enrichments. The enrichments were set up in 50 mL anaerobic serum bottles with 15 mL of medium and anaerobic (N_2) head space. A basal anaerobic medium[20] with the following changes was used: glucose (100 mM) or cellulose (~0.1 g/15 mL, Whatman grade number 1 filter paper, Brentford, Middlesex, U.K.), yeast extract (2 g/L), and the vitamin solution 141 from DSMZ. A phosphate-buffer solution (77.5 mM NaH_2PO_4 and 22.5 mM Na_2HPO_4) and a bicarbonate buffer (46.7 mM $NaHCO_3$) were used. The enrichment bottles were inoculated with 1.5 mL of the sample material from a hot spring. The bottles were incubated at various temperatures from 50 to 78 °C, depending upon the *in situ* temperature of the sample. Gas production of the batch cultures was determined as previously described.[21]

Temperature-Gradient Experiments. The optimum temperatures and temperature dependencies of two enrichment cultures were characterized using a temperature-gradient incubator (Test Tube Oscillator, Terratec, Blackmansbay, Tasmania, Australia). The temperature gradients were set up from 37 to 70 °C for enrichment 33HL (fifth subculture from the original enrichment shown in Table 1) and from 54 to 90 °C for 9HG (sixth subculture), respectively. The enrichments were incubated in 25 mL anaerobic tubes with 7 mL of the medium, and the growth was measured with an optical

(13) van Groenestijn, J. W.; Hazewinkel, J. H. O.; Nienoord, M.; Bussmann, P. J. T. *Int. J. Hydrogen Energy* **2002**, *27*, 1141–1147.
(14) van Niel, E. W.; Claassen, P. A. M.; Stams, A. J. M. *Biotechnol. Bioeng.* **2003**, *81*, 255–262.
(15) Dien, B. S.; Cotta, M. A.; Jeffries, T. W. *Appl. Microbiol. Biotechnol.* **2003**, *63*, 258–266.
(16) Lin, T.; Tanaka, S. *Appl. Microbiol. Biotechnol.* **2006**, *69*, 627–642.
(17) Hallenbeck, P. C.; Benemann, J. R. *Int. J. Hydrogen Energy* **2002**, *27*, 1185–1193.
(18) Hwang, M. H.; Jang, N. J.; Hyun, S. H.; Kim, I. S. *J. Biotechnol.* **2004**, *111*, 297–309.
(19) Ren, N. Q.; Gong, M. L. *Eng. Life Sci.* **2006**, *6*, 403–409.
(20) Örlygsson, J.; Houwen, F. P.; Svensson, B. H. *Swed. J. Agric. Sci.* **1993**, *23*, 45–54.
(21) Owen, W. F.; Stuckey, D. C., Jr.; Young, L. Y.; McCarty, P. L. *Water Res.* **1979**, *13*, 485–492.

density. Gas production was determined as previously described.[21] Organic acids and alcohols were analyzed as end-point concentrations after the cultures reached the maximum optical density.

Continuous-Flow Bioreactor Experiments for H_2 and Ethanol Production. The production of H_2 and ethanol by 9HG was characterized in a continuous-flow, completely mixed bioreactor (volume of 0.45 L and height/diameter ratio of 7). The medium used was as described above. The bioreactor was operated at 74 °C, and the pH was controlled by adjusting the feed pH with 5 M NaOH or 37% HCl. The bioreactor was operated for 52 days at different pH values. The hydraulic retention time (HRT) was maintained constant at 19 h, corresponding to a glucose loading rate of 0.95 g h^{-1} L^{-1}. Gas production was measured using a wet gas meter (Ritter Apparatebau, Bochum, Germany).

Chemical Analyses. The composition of product gas in the batch enrichment assays was measured with a Perkin Elmer gas chromatograph (GC), equipped with a 1010 Carboxen GC Plot capillary column (Supelco, Bellefonte, PA) and a thermal conductivity detector (TCD). The oven, injector, and detector temperatures were 65, 200, and 200 °C, respectively. Nitrogen was used as the carrier gas. The composition of gases (H_2 and CO_2) from bioreactor and temperature-gradient incubator tests was measured with another GC–TCD, HP 5890II, using a 6 ft Porapak N packed column (80/100 mesh) and N_2 as the carrier gas. The oven, injector, and detector temperatures were 80, 110, and 110 °C, respectively. The concentrations of ethanol and other organic alcohols and organic acids were measured with a Perkin Elmer or HP 5890II gas chromatograph as previously described.[22] The substrate consumption and lactate and formate concentrations were analyzed with a Waters 510 liquid chromatograph with a Shodex Sugar SH1011 column (Showa denko K.K., Tokyo, Japan) and a Δn −1000 refraction index detector (WGE Dr. Bures GmbH & Co. KG, Dallgow, Germany). The mobile phase was 0.01 N H_2SO_4. Biomass was estimated as volatile suspended solids (VSS) according to APHA standards methods,[23] as optical density at 600 nm with an Ultraspec 500 Pro Visible spectrophotometer (Amersham Biosciences, Piscataway, NJ), or both.

Molecular Characterization of the 9HG Community. The diversity and dynamics of the community in enrichment 9HG was analyzed by PCR–DGGE (polymerase chain reaction–denaturing gradient gel electrophoresis) of partial 16S rRNA genes followed by their sequencing. Duplicate samples of the reactor liquid were taken from the bioreactor during operation and stored at −20 °C. DNA was extracted from 1 mL of samples with the VIOGENE Blood and Tissue Genomic DNA kit (Proteogenix SA, Fegersheim, France). Partial bacterial 16S rRNA genes of the community DNA were amplified using primer pair GC-BacV3f[24] and 907r,[25] as previously described.[22] DGGE was performed with the INGENYphorU2x2 system (Ingeny International BV, Goes, The Netherlands) using 8% polyacrylamide gels (acrylamide/bisacrylamide gel stock solution of 37.5:1) with a denaturing gradient from 40 to 60% (100% denaturing solution contains 7 M of urea and 40% formamide). Gels were run at 60 °C in 1× TAE with 100 V for 21.5 h and stained with SYBR Gold (Molecular Probes, Inc., Eugene, OR). The dominant bands were excised from the gel, eluted in 25 μL of sterile H_2O (overnight at +4 °C), and re-amplified for sequencing as previously described.[22] Sequence data was analyzed with Bioedit software[26] (version 7.0.5.2) and compared to sequences in GenBank (http://www.ncbi.nlm.nih.gov/blast/). The existence of chimeras was analyzed using CHIMERA_CHECK software [version 2.7; Center for Microbial Ecology, Michigan State University (http://rdp.cme.msu.edu/cgis/chimera.cgi?su=SSU)]. Further, the presence of archaea in the bioreactor was studied from the DNA extracts using a nested PCR procedure, as previously described.[22]

The accession numbers of the gene sequences submitted to GenBank were EF595570, EF595571, and EF595572.

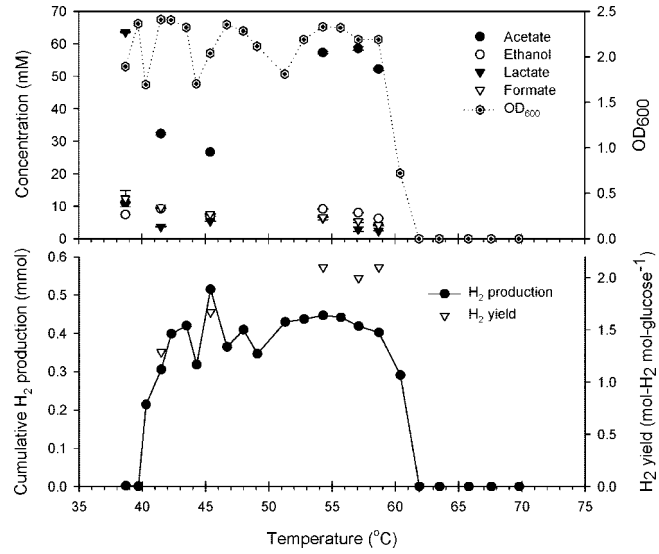

Figure 1. Temperature-gradient profiles of enrichment 33HL. Data are shown for maximum growth (no further increase in OD_{600}) and fermentative metabolites.

Results

Batch Enrichment of Thermophilic Bacteria from Hot Spring Samples. About 80 samples were taken from hot springs in various regions in Iceland and screened for their H_2 and ethanol production potential at the corresponding temperatures (50–78 °C) prevailing at the sample site. The metabolite formation of the most promising H_2- and/or ethanol-producing cultures in the initial incubations was as shown in Table 1. The enrichments had different metabolic patterns. Some enrichments produced H_2 from glucose through acetate (13HG and 11H) or through acetate and butyrate (33HL, 44H, 18H, 22HL, and 33H) (Table 1). Some enrichments produced ethanol as the main soluble metabolite (9HG, 19HL, 5H, 7H, 22H, 25H, 28H, and 29H) along with H_2 from glucose. Enrichment 9HG produced relatively high ethanol yields at the ethanol distillation temperature (78 °C). Further, one enrichment (PC2H) produced H_2 and acetate from cellulose at 70 °C (Table 1).

Effect of the Temperature. The selected H_2- and/or ethanol-producing enrichment cultures were further studied with a temperature-gradient incubator to determine the effect of the temperature on growth and metabolite formation. The enrichments included 33HL (high H_2 yield) and 9HG (high ethanol yield).

Enrichment 33HL grew in the range of 37–61 °C (Figure 1). The optimum temperature for H_2 production by 33HL was between 51 and 59 °C, with a H_2 yield of 2.1 mol of H_2/mol of glucose. The highest quantity of H_2 was produced at 45 °C, but the H_2 yield was only 1.67 mol of H_2/mol of glucose. Acetate was the main soluble metabolite at those temperatures at which

(22) Koskinen, P. E. P.; Kaksonen, A. H.; Puhakka, J. A. *Biotechnol. Bioeng.* **2007**, *97*, 742–758.

(23) American Public Health Association (APHA). *Standard Methods for the Examination of Water and Wastewater*, 19th ed.; American Public Health Association/American Water Works Association/Water Environment Federation: Washington, D.C., 1995.

(24) Muyzer, G.; De Waal, E. C.; Uitterlinden, A. G. *Appl. Environ. Microbiol.* **1993**, *59*, 695–700.

(25) Muyzer, G.; Hottenträger, S.; Teske, A.; Waver, C. In *Molecular Microbial Ecology Manual*; Akkermans, A. D. L., van Elsas, J. D., De Bruijn, F., Eds; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1996; p 3.4.4/1–23.

(26) Hall, T. A. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **1999**, *41*, 95–98.

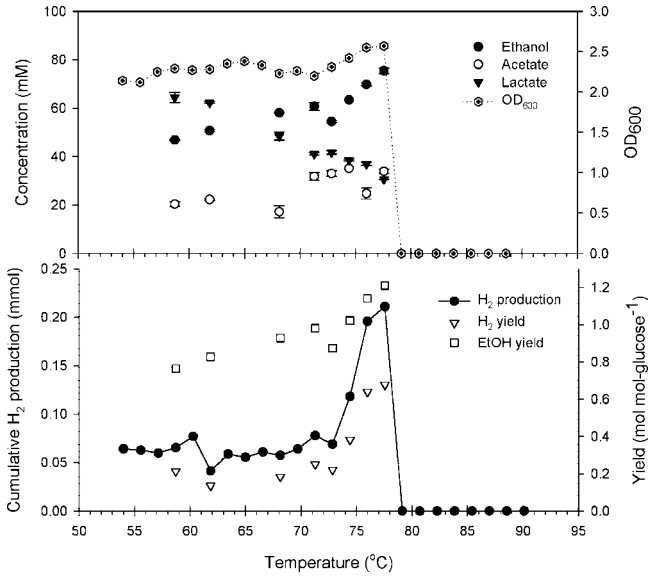

Figure 2. Temperature-gradient profiles of enrichment 9HG. Data are shown for maximum growth (no further increase in OD_{600}) and fermentative metabolites.

H_2 was produced. The highest acetate concentrations were seen in the temperature region between 51 and 59 °C, at which the H_2 yields were also the highest. Other soluble end products included ethanol, lactate, and formate. Enrichment 33HL did not produce H_2 at temperatures of 37–39 °C, likely because of high lactate production. Contrary to the initial batch enrichments with 33HL (Table 1), butyrate was not detected in the temperature-gradient incubation.

Enrichment 9HG grew in the range of 54–78 °C (Figure 2). No growth was detected >78 °C. Hydrogen and ethanol production increased with the temperature, and the improved H_2 yields were associated with increases in acetate concentrations and decreases in lactate production. The optimum temperature of H_2 and ethanol production by 9HG was 78 °C, with corresponding H_2 and ethanol yields of 0.68 mol of H_2/mol of glucose and 1.21 mol of EtOH/mol of glucose, respectively.

Continuous-Flow Bioreactor Experiment with Enrichment 9HG. The effect of pH on H_2 and ethanol production by enrichment 9HG was determined in continuous-flow bioreactor experiments at 19 h of HRT and 74 °C. The pH ranges in these experiments were 6.0 ± 0.5 (days 0–28), 5.3 ± 0.2 (days 29–39), and 6.8 ± 0.3 (days 40–52). The H_2 concentrations varied between 15 and 35%, and the gas phase mainly consisted of CO_2 (Figure 3). The concentrations and production rates of H_2 were highest when the reactor pH was 6.8 ± 0.3, while pH of 5.3 ± 0.2 resulted in the lowest H_2 production rates (Table 2). The maximum H_2 production rate, 1.35 mmol h^{-1} L^{-1}, was on day 45, with a yield of 0.42 mol of H_2/mol of glucose. No methane was detected.

During the startup of continuous operation at 6.0 ± 0.5, the lactate concentration increased rapidly (Figure 3). On day 8, the lactate concentration started to decrease, while the concentration of ethanol increased. Ethanol concentrations were highest when the reactor was operated at pH 6.0 ± 0.5, while the ethanol yield was highest at pH 6.8 ± 0.3 (Table 2). The concentration and yield of ethanol remained low when the reactor pH was 5.3 ± 0.2. The CO_2 production rates in the reactor correlated with the corresponding ethanol concentrations as expected according to eq 2. The acetate concentrations were highest when the reactor was operated at pH 6.8 ± 0.3. The acetate concentrations in the reactor correlated with the H_2 production rates, suggesting that acetate and H_2 shared the same pathway

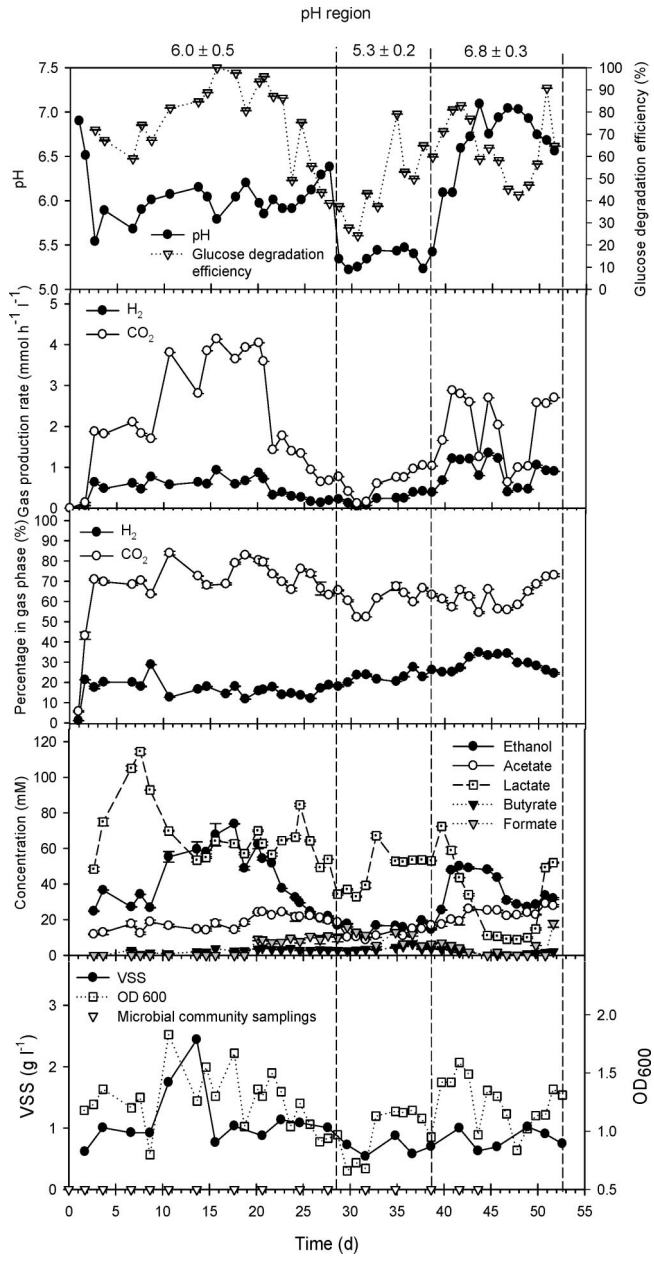

Figure 3. Performance data for the continuous-flow bioreactor maintained with enrichment 9HG at 74 °C.

(eq 1). The lactate concentrations were highest when the reactor pH was 6.0 ± 0.5 and remained high also after the pH was lowered to 5.3 ± 0.2. The lactate concentrations decreased when the pH was adjusted to 6.8 ± 0.3. The concentrations of formate, butyrate, and butanol were less than 15, 5 (Figure 3), and 0.2 mM, respectively (data not shown). The efficiency of the glucose consumption in the reactor varied considerably, and glucose was only partially consumed throughout the 52 day experiment. Complete glucose consumption was achieved only on day 16 when the reactor pH was 5.8. The biomass concentrations in the reactor were generally less than 1 g of VSS L^{-1}. The biomass concentrations were too low or the HRT was too short to achieve complete glucose consumption.

Carbon mass balances were calculated from the bioreactor for the three pH regions. The carbon recoveries were highest, up to 87%, for pH 6.0 ± 0.5 and 5.3 ± 0.2 (Table 3). The carbon in the bioreactor was mainly directed to lactate, ethanol, acetate, and CO_2 production. It should be noted that ethanol was not monitored from the gas phase. Some of the etha-

Table 2. Summary of Performance Data (±Standard Deviations) for the Continuous-Flow Bioreactor Maintained at 74 °C with Enrichment 9HG at Different pH Values

	measured value		
parameter	pH 6.0 ± 0.5	pH 5.3 ± 0.2	pH 6.8 ± 0.3
time period	days 16–21	days 36–39	days 42–46
actual pH	6.0 (0.2)	5.4 (0.1)	6.8 (0.2)
H_2 composition in product gas (%)	15.2 (2.4)	24.8 (2.4)	32.3 (3.0)
H_2 production rate (mmol h^{-1} L^{-1})	0.75 (0.14)	0.36 (0.08)	1.15 (0.21)
H_2 yield[a] (mol of H_2/mol of glucose)	0.16 (0.03)	0.12 (0.03)	0.32 (0.08)
ethanol concentration (mM)	61.4 (10.0)	16.0 (2.5)	47.7 (2.8)
ethanol yield[a] (mol of EtOH/mol of glucose)	0.65 (0.07)	0.28 (0.03)	0.69 (0.07)
lactate concentration (mM)	63.3 (4.6)	53.2 (0.5)	18.1 (10.9)
lactate yield[a] (mol of lactate/mol of glucose)	0.68 (0.05)	0.94 (0.11)	0.27 (0.12)
glucose degradation efficiency (%)	93.6 (7.6)	56.9 (6.7)	68.1 (11.2)
VSS (g L^{-1})	0.89 (0.14)	0.64 (0.08)	0.66 (0.05)

[a] Yields were calculated per glucose degraded.

Table 3. Carbon Mass Balances (±Standard Deviation) for the Bioreactor at 74 °C Maintained with Enrichment 9HG at Different pH Values

	pH region		
	6.0 ± 0.5	5.3 ± 0.2	6.8 ± 0.3
time period	days 16–21	days 36–39	days 42–46
carbon in substrates (mmol of C h^{-1} L^{-1})			
glucose–carbon loading rate	31.6	31.6	31.6
residual glucose–carbon in bioreactor	2.03 (2.41)	13.63 (2.13)	10.08 (3.54)
glucose–carbon consumption rate	29.57 (2.41)	17.97 (2.13)	21.52 (3.54)
carbon in products (mmol of C h^{-1} L^{-1})			
ethanol[a]	6.47 (1.06)	1.68 (0.26)	5.02 (0.3)
acetate	2.09 (0.46)	1.52 (0.26)	2.52 (0.34)
lactate	10.01 (0.72)	8.40 (0.09)	3.67 (2.34)
butanol	0.18 (0.05)	0.18 (0.05)	0.20 (0.11)
butyrate	0.68 (0.15)	0.99 (0.28)	0.19 (0.17)
formate	0.19 (0.26)	0.39 (0.25)	0.10 (0.09)
CO_2[b]	3.87 (0.24)	0.95 (0.14)	2.28 (0.64)
biomass[c]	2.14 (0.27)	1.49 (0.20)	1.80 (0.46)
carbon recovery (%)	86.6 (4.4)	86.8 (9.6)	73.4 (4.1)

[a] Ethanol in the liquid phase was included, and ethanol in the gas phase was not analyzed. [b] CO_2 in the liquid phase was ignored. [c] Biomass was calculated on the basis of VSS values, assuming the biomass composition $C_5H_7O_2N$.[27]

nol may have been evaporated due to the high temperature used (74 °C), but this was not accounted for in the carbon mass balances.

Microbial Community Analyses of Enrichment 9HG. Bacterial community samples were taken at intervals from the bioreactor throughout the time course. DNA extraction followed by PCR–DGGE of partial 16S rRNA genes of the bacterial community was used to characterize the dominant bacteria in these samples (Figure 4 and Table 4). The initial inoculum, i.e., the batch enrichment culture 9HG, was also characterized.

The initial bioreactor inoculum was dominated by organisms very closely affiliated with *Thermoanaerobacter thermohydrosulfuricus* (100%) (OTU BR-9HG-A).[28] This organism dominated the bioreactor culture throughout the bioreactor operation. On days 21 and 32, bacteria closely affiliated with *Bacillus circulans* (99.3%) (BR-9HG-B) and *Clostridium baratii* (99.4%) (BR-9HG-C),[29] respectively, were also detected in bioreactor samples. *B. circulans* and *C. baratii* are mesophilic bacteria and were likely present in the feed tank (kept at +4 °C) or feed tubing of the bioreactor. Because of the extreme culture conditions, the bioreactor feed was not sterilized.

No amplification products of archaeal 16S rRNA genes were obtained from the DNA extracts of the inoculum culture or from the bioreactor samples with a nested PCR procedure, while the positive control (DNA from anaerobic digester sludge) had a high amplification yield (data not shown). This result indicated the absence of archaea in the 9HG enrichment and the bioreactor.

Discussion

In general, the potential of thermophiles in ethanol and especially H_2 production has been somewhat overlooked in the literature. The results of this study demonstrate that numerous H_2- and/or ethanol-producing thermophilic enrichment cultures could be obtained from liquid and sediment samples collected from hot springs in Iceland.

Enrichment Culture 33HL. High temperature favors the kinetics and thermodynamics of H_2 production.[13] Thermophilic microorganisms have, therefore, higher H_2 yields than mesophilic organisms. The highest H_2 yields have been reported for thermophilic isolates, such as *Thermotoga maritima* with 4 mol of H_2/mol of glucose at 80 °C[30] and *Caldicellulosiruptor saccharolyticus* with 3.3 mol of H_2/mol of glucose at 70 °C.[31] Mixed microbial cultures have generally lower H_2 yields as compared to pure cultures because of the presence of hydrogen-nonproducing or hydrogen-consuming organisms. Mixed cultures are, however, preferred in bioprocesses with waste materials because sterilization is not feasible. The H_2 yield of enrichment culture 33HL, 2.10 mol of H_2/mol of glucose, is comparable with other studies using thermophilic mixed cultures. The H_2 yield of 33HL is close to that previously reported,[32] 2.2 mol of H_2/mol of hexose at 60 °C, obtained with sludge inoculum from a municipal sewage treatment plant. The yield is 2-fold higher than the previously reported[33] 1.11 mol of H_2/mol of glucose at 55 °C, obtained with inoculum from a continuous, H_2-producing bench-scale reactor. Among the highest H_2 production yields by thermophilic mixed cultures are 2.59 mol of H_2/mol of hexose at 60 °C with aerated activated sludge inoculum[5] and 2.47 mol of H_2/mol of glucose[34] at 70 °C with inoculum from a bench-scale, methanogenic, dairy manure-treating reactor. The 33HL produced H_2 together with acetate, which has a theoretical maximum of 4 mol of H_2/mol of glucose (eq 1).

Enrichment Culture 9HG. In batch cultivations, enrichment 9HG produced H_2 and ethanol at an ethanol distillation

(27) Fang, H. H. P.; Liu, H. *Bioresour. Technol.* **2002**, *82*, 87–93.
(28) Rainey, F. A.; Ward, N. L.; Morgan, H. W.; Toalster, R.; Stackebrandt, E. *J. Bacteriol.* **1993**, *175*, 4772–4779.
(29) Hutson, R. A.; Thompson, D. E.; Collins, M. D. *FEMS Microbiol. Lett.* **1993**, *108*, 103–110.
(30) Schröder, M.; Selig, M.; Schönheit, P. *Arch. Microbiol.* **1994**, *161*, 460–470.
(31) van Niel, E. W.; Budde, M. A. W.; de Haas, G. G.; van der Maar, F. J.; Claassen, P. A. M.; Stams, A. J. M. *Int. J. Hydrogen Energy* **2002**, *27*, 1391–1398.
(32) Shin, H. S.; Youn, J. H. *Biodegradation* **2005**, *16*, 33–44.
(33) Oh, Y.-K.; Kim, S. H.; Kim, M.-S.; Park, S. *Biotechnol. Bioeng.* **2004**, *88*, 690–698.
(34) Kotsopoulos, T. A.; Raymond, J. Z.; Angelidaki, I. *Biotechnol. Bioeng.* **2006**, *94*, 296–302.

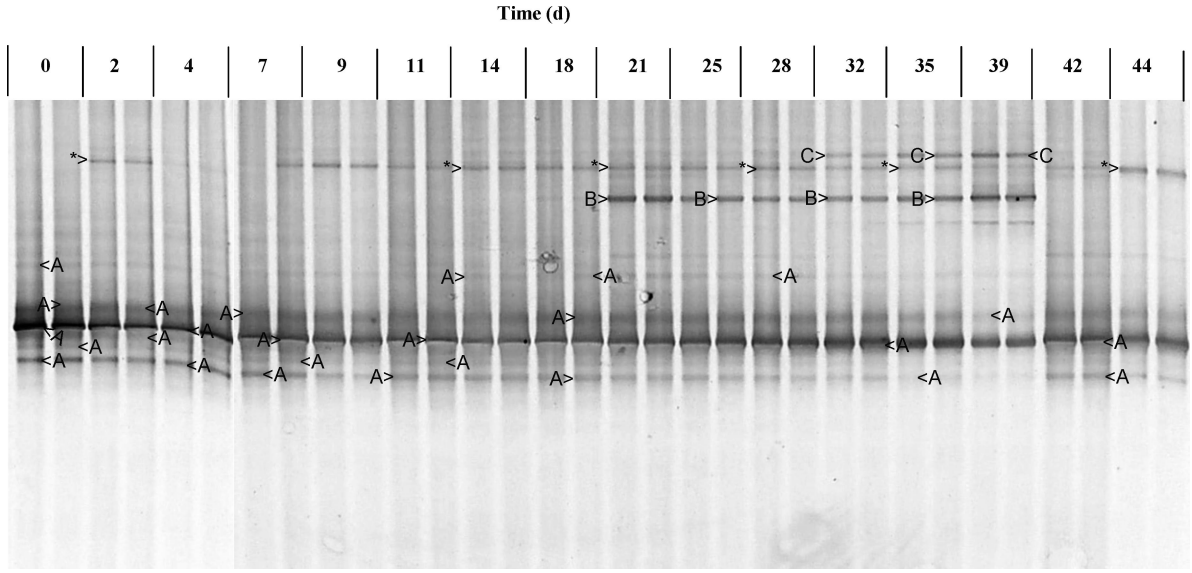

Figure 4. Bacterial community profile determined with PCR–DGGE of partial 16S rRNA genes of the continuous-flow bioreactor maintained with enrichment 9HG. See Table 4 for the labeled bands. Time 0 represents the batch enrichment inoculum. (*) Chimerical sequence (an artifact).

Table 4. Affiliations of 16S rRNA Gene Sequences of Bands Excised from the DGGE Gel of the Continuous-Flow Bioreactor at 74 °C Maintained with Enrichment 9HG

OTU (acc)[a]	label[b]	SL[c]	family[d]	affiliation (acc)[e]	sim (%)[f]
BR-9HG-A (EF595570)	A	523	*Thermoanaerobacteriaceae*	*Thermoanaerobacter thermohydrosulfuricus* DSM 567 (L09161)	100
BR-9HG-B (EF595571)	B	549	*Bacillaceae*	*Bacillus circulans* WSBC 20030 (Y13062)	99.3
BR-9HG-C (EF595572)	C	523	*Clostridiaceae*	*Clostridium baratii* ATCC43756 (X68175)	99.4

[a] Operational taxonomic unit with an accession number. [b] Band label in Figure 4. [c] Sequence length (bp). [d] Family according to Ribosomal Database Project II or GenBank databases. [e] Closest species in the GenBank database with an accession number. [f] Similarity (%).

temperature of 78 °C with H_2 and ethanol yields of 0.68 mol of H_2/mol of glucose and 1.21 mol of EtOH/mol of glucose, respectively. The H_2 and ethanol yields increased with the temperature in the batch assays, and these trends were associated with decreased lactate production.

In the continuous-flow bioreactor experiment at 74 °C, H_2 and ethanol production by 9HG was influenced by the pH of the culture. Lactate formation limited the H_2 and ethanol production in the bioreactor. Lactate formation decreased at pH 6.8 ± 0.3 and increased when the pH was lowered (Table 2). The maximum yields in the bioreactor, 0.42 mol of H_2/mol of glucose and 0.76 mol of EtOH/mol of glucose, were substantially lower than those determined in batch experiments for 9HG. This difference was attributed to a higher lactate production in the bioreactor as compared to batch cultures.

The decrease in H_2 yields because of lactate formation is a well-known concern in mesophilic and thermophilic H_2 fermentation.[33,35,36] In thermophilic cultures, it has been shown that lactate concentrations generally increase with the increasing substrate concentration.[33,35] The 100 mM glucose concentration used in this study was relatively high, which may partly explain the high lactate concentrations observed in the bioreactor with enrichment 9HG.

The partial pressure of H_2 (pH_2) is known to affect the growth and metabolism of H_2-fermenting organisms. Hydrogen production acts as an electron sink during fermentation and serves to dispose of excess electrons released during substrate oxidation. Hydrogenases especially are inhibited at high pH_2 levels, resulting in the accumulation of reducing equivalents and decreased metabolic activity.[14] Thermophilic organisms can solve this metabolic block by producing more reduced products, such as ethanol, lactate, or alanine, as hydrogen sinks.[14,37] Thus, elevated pH_2 values could be a regulatory signal, leading to increased ethanol yields in thermophiles. Indeed, a 100-fold increase in the ethanol/acetate ratio has been previously reported[38] for *Clostridium thermocellum* cultured at an elevated hydrostatic pressure of 17.3 MPa (i.e., at increased pH_2 in the liquid phase) as compared to the ratio determined for normal atmospheric pressure conditions. Alternatively, increases in the pH_2 may shift the fermentative metabolism toward lactate formation, which is unfavorable in this case.

Glucose remained only partially consumed in the bioreactor, regardless of the relatively long HRT of 19 h and low glucose loading rate of 0.95 g h^{-1} L^{-1} used. The rate of glucose utilization was substantially lower than reported for mesophilic, suspended-cell bioreactors.[11,39] The low glucose utilization rate in our thermophilic bioreactor was likely due to a low biomass content. The low cell density of thermophilic cultures is considered a limitation of thermophilic H_2 fermentation bioprocesses.[40] Improving biomass retainment in the thermophilic bioreactor with enrichment 9HG requires further optimization of the bioreactor design and operation.

Community Composition of 9HG. The enrichment 9HG was dominated by bacteria closely affiliated with *T. thermohydrosulfuricus* (Figure 4 and Table 4), previously known as *Clostridium thermohydrosulfuricum*, originally isolated from

(35) Levin, D. B.; Islam, R.; Cicek, N.; Sparling, R. *Int. J. Hydrogen Energy* **2006**, *31*, 1496–1503.
(36) Noike, T.; Takabatake, H.; Mizuno, O.; Ohba, M. *Int. J. Hydrogen Energy* **2002**, *27*, 1367–1371.
(37) Kengen, S. W. M.; Stams, A. J. M.; de Vos, W. M. *FEMS Microbiol. Rev.* **1996**, *18*, 199–137.
(38) Bothun, G. D.; Knutson, B. L.; Berberich, J. A.; Strobel, H. J.; Nokes, S. E. *Appl. Microbiol. Biotechnol.* **2004**, *65*, 149–157.
(39) Lin, C.-Y.; Chang, R.-C. *J. Chem. Technol. Biotechnol.* **1999**, *74*, 498–500.
(40) Hallenbeck, P. C. *Wat. Sci. Technol.* **2005**, *52*, 21–29.

extraction juices of sugar beet factories.[41] *T. thermohydrosulfuricus* belongs to the cluster V of the genus *Clostridium*.[42] *T. thermohydrosulfuricus* is an obligately anaerobic, spore-forming bacterium and capable of using a wide variety of carbohydrates, such as xylan, xylose, starch, cellobiose, glucose, and sucrose, with the main products of H_2, CO_2, ethanol, acetate, and lactate.[43] EtOH yields of 1.4 mol of EtOH/mol of glucose have been reported for *T. thermohydrosulfuricus* (*C. thermohydrosulfuricum*).[44] On the basis of Lee et al.,[43] the temperature maximum of *T. thermohydrosulfuricus* is 78 °C with the optimal pH from 6.9 to 7.5, which is in agreement with our results (T_{max} of 78 °C and optimal pH 6.8 ± 0.3 of the pH values studied).[43]

Conclusions

Potential thermophilic microorganisms for sustainable energy production (H_2 and/or ethanol) were enriched from Icelandic hot springs over a wide temperature range (50–78 °C). The batch enrichments had different metabolic patterns, including high H_2 or high ethanol yields from glucose. One enrichment produced a high H_2 yield directly from cellulose at 70 °C. The 33HL enrichment had a H_2 yield of 2.10 mol of H_2/mol of glucose at 59 °C. The 9HG, dominated by bacteria closely affiliated with *T. thermohydrosulfuricus*, produced 0.68 mol of H_2/mol of glucose and 1.21 mol of EtOH/mol of glucose at 78 °C in batch incubations, but the yields were lower in a continuous-flow bioreactor at 74 °C. H_2 and ethanol production of 9HG in the bioreactor was dependent upon the pH, with an optimum pH range of 6.8 ± 0.3. Lactate production decreased the H_2 and ethanol yields in the bioreactor. In summary, promising bacterial enrichments were obtained from Icelandic hot spring samples, but the full H_2 and ethanol production potential of the enrichments has yet to be revealed.

Acknowledgment. This research was funded by the Academy of Finland (HYDROGENE project, 107425), Nordic Energy Research (BIOHYDROGEN project, 28-02), Tampere University of Technology Graduate School (to P.E.P.K.), Feng Chia University (Grant 94GB66), and National Science Council of Taiwan (NSC 95-2221-E-035-094).

EF700275W

(41) Matteuzzi, D.; Hollaus, F.; Biavati, B. *Int. J. Syst. Bacteriol.* **1978**, *28*, 528–531.

(42) Collins, M. D.; Lawson, P. A.; Willems, A.; Cordoba, J. J.; Fernandez-Garayzabal, J.; Garcia, P.; Cai, J.; Hippe, H.; Farrow, J. A. E. *Int. J. Syst. Bacteriol.* **1994**, *44*, 812–826.

(43) Lee, Y.-E.; Jain, M. K.; Lee, C.; Lowe, S. E.; Zeikus, J. G. *Int. J. Syst. Bacteriol.* **1993**, *43*, 41–51.

(44) Wiegel, J.; Ljungdahl, L. G.; Rawson, J. R. *J. Bacteriol.* **1979**, *139*, 800–810.