Bioprospecting thermophilic ethanol and hydrogen producing bacteria from hot springs in Iceland

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ABSTRACT

The diversity of thermophilic hydrogen- and ethanol-producing anaerobes in various geothermal areas has been largely unresolved. The aim of this study was to investigate the phylogenetic relationship between ethanol and hydrogen-producing anaerobes at temperatures varying from 50 to 75°C. Extensive enrichments on various carbon substrates (glucose, xylose, cellulose [Whatman paper and cellulose powder], pectin and xylan) were done from 48 samples collected from the geothermal area in Grensdalur (Hveragerði, SW Iceland). Enrichments were regarded as positive if they showed good ethanol and/or hydrogen production. After repeated enrichments and end point dilutions, partial 16S rRNA from 59 samples were analysed, as well as end product formations from glucose and xylose. At moderate temperatures (50 to 60°C) most of the bacteria were phylogenetically most closely related to the genera of *Caloramator*, *Clostridium* and *Thermoanaerobacterium*, whereas at higher temperatures (70 to 75°C) *Caldicellulosiruptor* and *Thermoanarobacter* dominated the microbial flora. At lower temperatures the main flow of carbohydrate fermentation was towards ethanol and butyrate, whereas at higher temperatures, acetate and hydrogen (and CO_2) were the main end products formed. Thus, a clear correlation between phylogenetic relationship and end product formation was observed in this ecological survey of thermophilic anaerobes in the geothermal area investigated. The study indicates the presence of bacteria in geothermal springs that could potentially be used for hydrogen and ethanol production from organic matter.

Keywords: Bioprospecting, thermophilic, anaerobic bacteria, hydrogen, ethanol

YFIRLIT

Skyldleikagreining á etanól- og vetnisframleiðandi bakteríum einangruðum úr heitum hverum á Íslandi

Fjölbreytileiki hitakærra, loftfirrtra etanól- og vetnisframleiðandi baktería í margvíslegum jarðhitasvæðum hefur ekki verið kannaður til hlítar. Markmið þessarar rannsóknar var að rannsaka fjölbreytileika slíkra baktería við mismunandi hitastig (50-75°C), bæði hvað varðar lífeðlisfræði og skyldleika þeirra. Ítarlegar auðgunartilraunir á margvíslegum hvarfefnum (glúkósa, xýlósa, sellulósa [Whatman pappír og sellulósa duft], pektíni

og xýlani) voru framkvæmdar úr 48 sýnum sem tekin voru í Grensdal við Hveragerði. Auðgun var álitin jákvæð ef hún sýndi af sér góða vetnis- og/eða etanólframleiðslu. Eftir endurteknar auðganir og einangranir með þynningum voru 59 sýni greind með 16S rRNA raðgreiningu. Öll sýnin voru einnig ræktuð á glúkósa og xýlósa og etanól og vetnisframleiðsla þeirra mæld. Þau sýni sem áttu uppruna sinn við lægri hitastig sem auðgað var við (50 til 60°C) innihéldu bakteríur sem greindust aðallega til ættkvíslanna *Caloramator*, *Clostridium* og *Thermoanaerobacterium* en bakteríur sem einagruðust við hærri hitastigin (70 til 75°C) greindust eingöngu til ættkvíslanna *Caldicellulosiruptor* og *Thermoanaerobacter*. Niðurbrot einsykra hjá auðgunarræktunum við lægri hitastigin leiddu aðallega til framleiðslu á etanóli og smjörsýru en við hærri hitastigin voru meginlokaafurðir ediksýra og vetni. Greinileg tengsl voru því á milli skyldleika baktería, hitastigs og framleiðslu lokaafurða í þessari vistræðilegu rannsókn á örverum úr hverum í Grensdal. Rannsóknarniðurstöður gefa til kynna að margar bakteríanna sem einangraðar voru er hugsanlega hægt að nota til framleiðslu á endurnýjanlegri orku úr lífmassa í formi etanóls og vetnis.

INTRODUCTION

The progressive depletion of non-renewable energy sources worldwide, together with the fact that their use has resulted in environmental deterioration and public health problems, has led to development of new renewable energy harvesting technologies (Zaldivar et al. 2001, Sánchez & Cardona 2008). The use of solar energy to grow energy crops and lignocellulosic residues is a good example of the generation of renewable energy (Hamelinck et al. 2005) but both ethanol and hydrogen can be produced by fermentation from such biomass. The complexity of the production processes depends on the feedstock. Thus, implemented technologies span the gamut from simple conversion of sugar- and starch-based biomass to multi-stage conversion of lignocellulosic material (Sánchez & Cardona 2008). Lignocellulosic material is the most abundant biopolymer on Earth and its annual production is estimated at approximately 50 billion tons (Claassen et al. 1999). Many types of lignocellulosic materials have been tested for biofuel production, varying from crop residues to hardwood, waste paper and municipal solid waste (Zaldivar et al. 2001). Various pre-treatment methods have been developed for the utilization of lignocellulosic material prior to fermentation (Sánchez & Cardona 2008). One of the main problems in bioethanol production from lignocellulosic material is that most yeast species cannot ferment the pentose containing sugars (e.g. xylose and arabinose) which often comprise a substantial portion of the biomass. Hydrogen production through mesophilic fermentation has received increasing attention in the past few years with high production rates and stabilities (Wu et al. 2006, Lin & Chang 2004, Yu & Mu 2006). Thermophilic H_2 fermentation has many advantages over mesophilic fermentation, but has remained less studied. High temperatures favor the stoichiometry of H_2 production resulting in higher H_2 yields compared to mesophilic systems (van Groenestijn et al. 2002, van Niel et al. 2003). Furthermore, thermophilic fermentation results in less variety of end products as compared to those of mesophilic fermentation (van Niel et al. 2003). High hydrogen yields (more than 3 mol-H_2 mol-hexose^{-1}) in pure cultures have been observed by extremophiles like *Caldicellulosiruptor sp.* (de Vrije et al. 2007, Zeidan & van Niel 2010) and *Thermotoga elfii* (van Niel et al. 2002) as well as in thermophilic enrichment cultures originating from Icelandic hot springs (Koskinen et al. 2008b).

The best known ethanol- and hydrogen-producing thermoanaerobes are the thermophilic clostridia and members of the genera *Thermoanaerobacter*, *Thermoanaerobacterium*, *Caldicellulosiruptor* and *Thermotoga*. Many species in these genera can produce high amounts of ethanol and hydrogen from pentoses and hexoses (Wiegel & Ljungdahl 1981, Kublanov et al. 2007, de Vrije et al. 2007, van Niel et al. 2002, Zeidan & van Niel 2010). The main drawback of using thermophiles is their production of by-products (acetate, lactate, butyrate and formate) and low ethanol toler-

ance (Wiegel 1980). However, the fact that the cultures may carry out their ethanol production at high temperatures offers the possibility of removing the alcohol by distillation or evaporation (Claassen et al. 1999). Geothermal springs have been regarded as a potential source for ethanol- and hydrogenproducing bacteria (Wiegel & Ljungdahl 1981, Sommer et al. 2004, Koskinen et al. 2008a). The present investigation examined the bioprospecting of various ethanol- and hydrogen-producing bacteria isolated from more than 40 hot springs varying in temperature and pH. All samples were subjected to partial 16S rRNA analysis and tested for their ethanol and hydrogen production capacity in batch assays.

MATERIALS AND METHODS

Sampling sites

Fourty-four samples were collected in June 2007 from the Hengill area in Grensdalur in SW Iceland. The temperature of the hot springs ranged from 40.0°C to 84.0°C and the pH was between 4.3 and 9.6. Additionally, four samples collected from the same area in 2006 were included in this study.

Medium

The composition of the medium used and its preparation have been described earlier (Orlygsson & Baldursson 2007).

Enrichments and isolations

Sampling and isolations were as described by Sveinsdottir et al. (2009). All samples were inoculated on glucose (50 mM), xylose (50 mM) or 3 g l^{-1} of xylan, pectin or cellulose (both Whatman paper and cellulose powder). The samples were incubated at temperatures slightly below the experimental site temperatures (50°C, 60°C, 70°C and 75°C) and at pH 6.0 or 7.0. Samples were incubated for one week and positive enrichments (determined by overpressure or hydrogen production measured by gas chromatograph) were re-inoculated into the medium with the same carbon source. This was repeated three times (only continued with positive samples), and finally end point dilutions were made (two times). Positive samples were then analyzed for partial 16S rRNA analysis.

16S rRNA sequence analysis

For 16S rRNA analysis, 16S rRNA genes were amplified from DNA with primers F9 and R1544, specific for bacterial genes (Skirnisdottir et al. 2000) with PCR. In most cases 6-700 bp was used for analysis. Methodology concerning sequencing and databases used for blasting as well as making the phylogenetic trees were according to Vesteinsdottir et al. (2010) and references cited in that paper.

End product formation from glucose and xylose

End product formation from glucose and xylose (both 50 mM) was carried out by inoculating 1 ml of a fresh culture into 49 ml of medium containing the carbon substrate. The fermentation time was one week and samples for volatile fatty acids, ethanol and hydrogen were taken and analyzed at the end of the experimental time.

Analytical methods

Ethanol, acetate and hydrogen were measured by gas chromatograph as previously described (Orlygsson & Baldursson 2007, Sveinsdottir et al. 2009).

RESULTS

A total of 44 liquid/mud samples were collected from hot springs in Grensdalur. All samples were inoculated on the six different carbon sources (264 enrichments). After several repeated enrichments the number of positive (H_2 production/overpressure) samples had been reduced to 55 (Table 1). Additionally, four samples obtained in earlier studies were included for further experiments and analyzed for phylogenetic characterization by 16S rRNA analysis.

16S rRNA gene sequence analysis

Samples that were analyzed for 16S rRNA analysis revealed that in most cases pure cul-

Table 1. Strain identification number, the temperature and pH of the hot springs from which the strains originated and the pH used for isolation. Labelling of the samples: G = enriched on glucose, Xo = xylose. Cpa = cellulose (Whatman paper), Cpo = cellulose (powder), Cpa-G = cellulose at start of enrichment but later enriched on glucose, P = pectin, X= xylan.

Enrichment culture	T °C (site)	pH (site/isolation)	Enrichment culture	T °C (site)	pH (site/isolation)	Enrichment culture	T °C (site)	pH (site/isolation)
50°C			52-07-P	50	5.4/6.0	66-07-G	62	7.4/7.0
1-07-Cpa-G	45	8.0/7.0	65-07-Xo	49	6.7/7.0	66-07-P	62	7.4/7.0
2-07-G	50	7.9/7.0	65-07-X	49	6.7/7.0	67-07-P	66	7.7/7.0
2-07-Cpa-G	50	7.9/7.0						
2-07-Cpa	50	7.9/7.0	**60°C**			**70°C**		
9-07-P	40	4.8/6.0	8-06-G*	58	6.2/6.0	2-06-G*	73	4.3/7.0
9-07-X	40	4.8/6.0	21-07-Xo	60	6.7/7.0	4-07-X	69	8.0/7.0
10-07-P	46	6.6/7.0	21-07-Cpo	60	6.7/7.0	8-07-Cpo	71	8.2/7.0
10-07-X	46	6.6/7.0	21-07-Cpa-G	60	6.7/7.0	8-07-Cpa	71	8.2/7.0
15-06-G*	49	5.8/7.0	24-07-X	60	7.7/7.0	20-07-G	69	7.5/7.0
15-07-Cpa-G	50	7.5/7.0	27-07-X	60	7.7/7.0	20-07-X	69	7.5/7.0
23-07-Cpa-G	57	7.7/7.0	29-07-G	60	9.6/7.0	20-07-Cpo	69	7.5/7.0
25-07-Cpa-G	50	7.4/7.0	29-07-Cpo	60	9.6/7.0	33-07-Xo	71	8.0/7.0
35-07-X	50	7.7/7.0	29-07-Cpa-G	60	9.6/7.0	55-07-Xo	73	5.1/6.0
35-07-Cpa-G	50	7.7/7.0	34-07-X	60	7.4/7.0			
44-07-G	56	5.5/6.0	54-07-Xo	66	5.3/6.0	**75°C**		
44-07-Xo	56	5.5/6.0	54-07-P	66	5.3/6.0	14-07-G	84	8.0/7.0
44-07-P	56	5.5/6.0	63-07-G	60	7.7/7.0	14-07-Xo	84	8.0/7.0
44-07-X	56	5.5/6.0	63-07-Cpa	60	7.7/7.0	14-07-P	84	8.0/7.0
47-07-Xo	53	6.2/6.0	64-07-G	59	7.0/7.0	14-07-X	84	8.0/7.0
47-07-P	53	6.2/6.0	64-07-P	59	7.0/7.0	32-07-G	78	4.9/6.0
52-07-Xo	50	5.4/6.0	64-07-X	59	7.0/7.0	39-06-G*	78	5.5/6.0

tures had been obtained, although several samples, especially those enriched on cellulose, gave bad sequence data, indicating the presence of a mixture of different strains. In some of these cases samples were re-enriched on glucose and then submitted for 16S rRNA analysis. Thus, of the total of 59 samples that were analyzed for partial 16S rRNA analysis, 47 samples revealed good sequences (85%). The data obtained were used to determine the phylogenetic position of the bacteria and are presented for each of the four enrichment temperatures used (Figure 1 A-D).

50°C enrichments

Twenty samples were sequenced for partial 16S rRNA analysis from enrichment cultures at 50°C (Figure 1A; Table 2). Three samples gave bad sequences (not pure cultures) and are not shown in Figure 1A. Most sequenced data (15 out of 17 samples) resulted in organisms that belong to the subphylum containing gram-positive bacteria with a DNA G+C content less than 55 mol% and that clustered with members of the clostridial group. Two major subclusters were observed, *Clostridium* Cluster I (6 enrichments; bacteria in genera *Clostridium* and *Caloramator*) and *Clostridium* Cluster V (9 enrichments; bacteria in genera *Thermoanaerobacter* and *Thermoanaerobaceterium*). Finally two samples were most closely related to species in the genus *Paenibacillus*. Successful sequencing of strains enriched on monosugars, xylan and pectine resulted in bacteria that were closely related to *Thermoanaerobacterium* species except for sample 44-07-G (*Thermoanaerobacter*), 15-06-G (*Caloramator*) and 9-07-X (*Clostridium* Cluster I). Most of the *Thermoanaerobacterium* strains showed a close relationship to each other and to species like *Thermoanaerobacterium aciditolerans*, *Thermoanaerobacterium saccharolyticum*, *Thermoanaerobacterium aotearoense*, *Thermoanaerobacterium* strain AK_{17} and *Thermoanaerobium lactoethylicum*. In general, the phylogenetic relationship between the eight enrichments

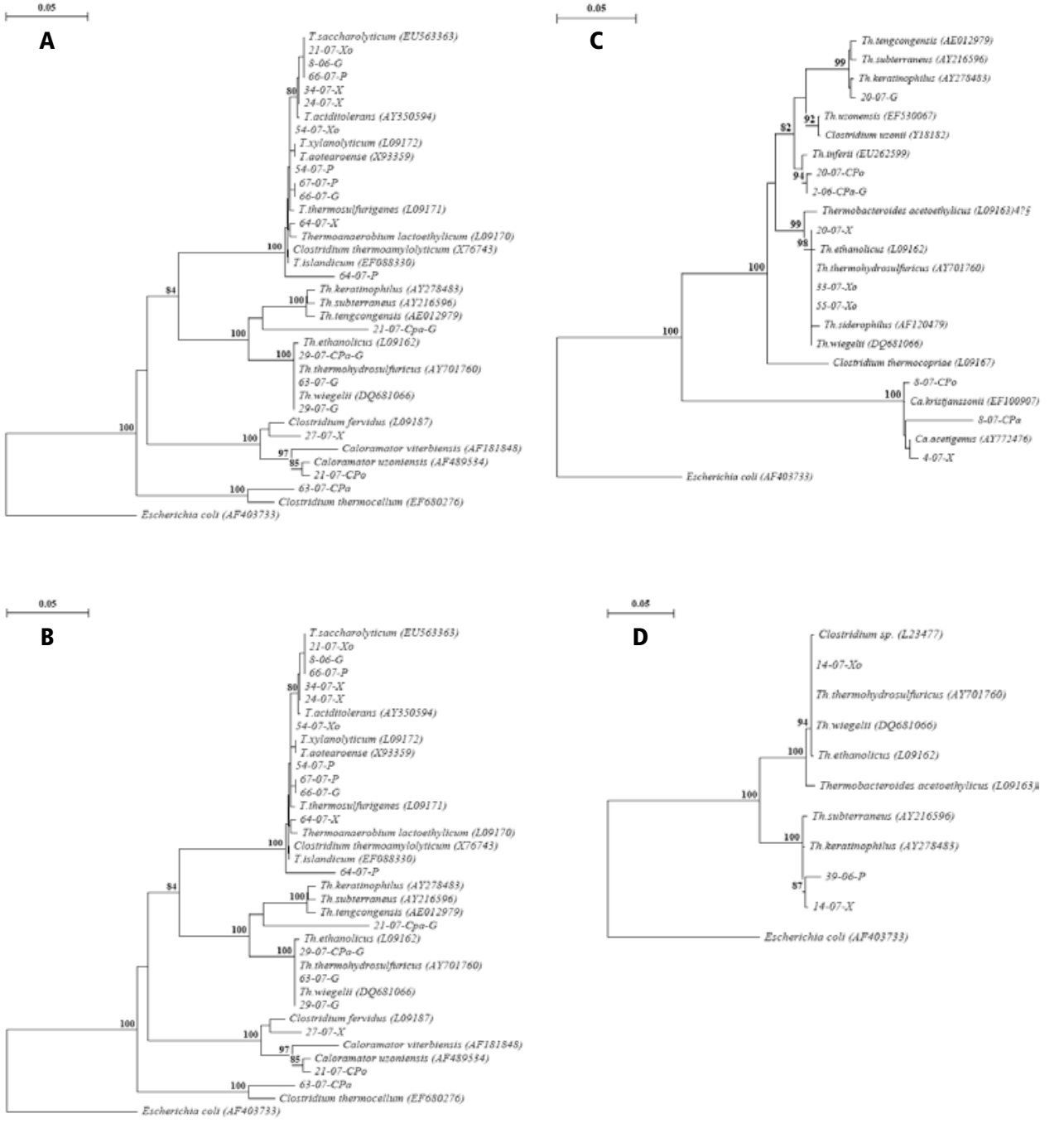

Figure 1. Phylogeny of the strains based on the 16S rRNA gene partial sequences (≈500 bp) from enrichments at 50°C (**A**), 60°C (**B**), 70°C (**C**) and 75°C (**D**). The phylogenetic tree was generated using a distance matrix and neighbor joining algorithms with 500 bootstraps. Only supported bootstrap values (>80%) are shown. *Escherichia coli* (AF403733) was selected as an out-group. The scale bar indicates 0.05 substitutions per nucleotide position.

Table 2. The main phylogenetic and physiological data for all enrichment cultures. End product formation from glucose and xylose (both 50 mM) are shown as well as the closest phylogenetic position of the enrichment in percentages.

Products	Ethanol (mM)		Acetate (mM)		Butyrate (mM)		H_2 (mmol L^{-1})		Phylogeny	
Substrates	Glucose	Xylose	Glucose	Xylose	Glucose	Xylose	Glucose	Xylose		
50°C samples										
2-07-G	21	32	21	21	0	0	30	27	Not analysed	
15-06-G	66	31	16	17	0	0	22	20	*Caloramator viberbiensis*	(94%)
44-07-G	28	51	16	15	0	0	21	23	*Thermoanaerobacter* subcluster B	(>98%)
44-07-Xo	35	39	21	23	0	0	24	29	Not analysed	
47-07-Xo	25	16	16	10	0	0	22	18	Not analysed	
52-07-Xo	28	27	16	15	0	0	20	27	*Thermoanaerobacterium* AK17	(98%)
65-07-Xo	7	57	8	12	12	0	20	14	*Thermoanaerobacterium saccharolyticum*	(100%)
1-07-Cpa-C	8	14	6	9	2	1	0	0	*Clostridium* AK14	(99%)
2-07-Cpa-C	9	21	6	14	1	1	6	3	*Clostridium* sp. strain P2	(99%)
15-07-Cpa-	8	4	19	4	1	8	9	1	*Clostridium* sp. strain P2	(99%)
23-07-Cpa-	12	24	10	13	1	0	4	5	*Paenibacillus* sp. HM06-04	(99%)
25-07-Cpa-	67	17	15	14	1	1	17	15	*Paenibacillus* sp. HM06-04	(98%)
35-07-Cpa-	47	6	17	6	1	1	19	8	*Caloramator viberbiensis*	(94%)
2-07-Cpo	40	31	15	18	1	0	8	12	Not analysed	
9-07-P	19	22	14	17	0	0	20	23	*Thermoanaerobacterium saccharolyticum*	(98%)
10-07-P	2	21	11	18	14	1	24	15	Not analysed	
44-07-P	25	21	21	14	6	0	31	23	*Thermoanaerobacterium* AK17	(99%)
47-07-P	26	32	16	20	0	0	20	32	*Thermoanaerobacterium* AK17	(98%)
52-07-P	24	30	17	20	0	0	22	29	*Thermoanaerobacterium* AK17	(98%)
9-07-X	3	6	6	10	7	6	15	19	*Clostridium* AK14	(98%)
10-07-X	10	7	12	12	11	15	24	34	Bad sequence	
35-07-X	2	4	9	13	14	16	26	32	*Thermoanaerobacterium saccharolyticum*	(99%)
44-07-X	21	26	14	15	0	0	18	22	*Thermoanaerobacterium* AK17	(99%)
65-07-X	1	44	8	17	13	1	24	29	Bad sequence	
60°C samples										
8-06-G	24	31	15	17	0	0	25	23	*Thermoanaerobacterium saccharolyticum*	(98%)
29-07-G	35	52	3	3	0	0	10	3	*Thermoanaerobacter* subcluster B	(>98%)
63-07-G	13	11	9	10	0	0	1	9	*Thermoanaerobacter* subcluster B	(>98%)
64-07-G	24	31	3	4	0	0	17	12	Not analysed	
66-07-G	52	65	18	17	0	0	14	18	*Thermoanaerobacterium thermosulfurigenes*	(100%)
21-07-Xo	31	30	18	16	0	0	34	33	*Thermoanaerobacterium saccharolyticum*	(99%)
54-07-Xo	1	1	1	1	0	0	0	0	*Thermoanaerobacterium aciditolerans*	(98%)
21-07-Cpa-	19	11	13	45	0	0	10	2	*Caloramator viterbiensis*	(97%)
29-07-Cpa-	12	12	38	20	0	1	1	4	*Thermoanaerobacter* subcluster B	(>98%)
63-07-Cpa	16	4	17	12	0	0	2	0	*Clostridium thermocellum*	(92%)
21-07-Cpo	16	0	18	30	0	0	9	11	*Thermoanarobacter* sp	(< 90%)
29-07-Cpo	12	11	39	20	0	2	1	4	Not analysed	
54-07-P	39	25	21	14	0	0	16	25	*Thermoanaerobacterium aciditolerans*	(98%)
64-07-P	36	27	16	13	0	0	16	13	*Thermoanaerobacterium aciditolerans*	(98%)
66-07-P	41	50	24	20	0	0	28	26	*Thermoanaerobacterium saccharolyticum*	(99%)
67-07-P	42	50	18	5	0	0	27	6	*Thermoanaerobacterium aciditolerans*	(95%)
24-07-X	39	39	15	14	0	0	24	18	*Thermoanaerobacterium saccharolyticum*	(98%)
27-07-X	27	24	17	10	0	0	23	12	*Caloramator fervidus*	(97%)
34-07-X	41	33	19	16	14	16	27	19	*Thermoanaerobacterium saccharolyticum*	(99%)
64-07-X	37	47	31	24	0	1	15	16	*Thermoanaerobacterium aciditolerans*	(98%)
70°C samples										
2-06-G	16	24	2	20	0	0	11	20	*Thermoanaerobacter* subcluster A	(>97%)
20-07-G	2	1	14	15	0	0	23	18	*Thermoanaerobacter* subcluster A	(>97%)
33-07-Xo	50	43	3	4	0	0	4	7	*Thermoanaerobacter* subcluster B	(>97%)
55-07-Xo	8	8	7	7	0	0	9	12	*Thermoanaerobacter* subcluster B	(>98%)
8-07-Cpa	14	1	18	11	0	0	31	3	*Caldicellulosiruptor acetigenium*	(97%)
8-07-Cpo	2	1	16	12	0	0	10	2	*Caldicellulosiruptor kristjanssonii*	(96%)
20-07- Cpo	13	17	7	10	0	0	6	6	*Thermoanaerobacter* subcluster A	(>97%)
4-07-X	2	3	13	13	0	0	21	19	*Caldicellulosiruptor acetigenus*	(100%)
20-07-X	39	37	2	6	0	0	9	15	*Thermoanaerobacter* subcluster B	(>99%)
75°C samples										
14-07-G	1	1	2	12	0	0	0	11	Not analysed	
32-07-G	1	1	1	1	0	0	0	0	Not analysed	
39-06-G	1	1	12	12	0	0	20	11	*Thermoanaerobacter* subcluster A	(>97 %)
14-07-Xo	5	9	11	14	0	0	16	17	*Thermoanaerobacter* subcluster B	(>99%)
14-07-P	3	4	16	15	0	0	27	31	Not analysed	
14-07-X	2	3	15	21	0	0	21	25	*Thermoanaerobacter* subcluster B	(>99%)

that belong to *Thermoanaerobacterium* ranged from 97.7% to 100% with the five *Thermoanaerobacterium* species showed in Figure 1A. In many cases, sequence analysis of samples that originated from cellulose paper (Cpa) enrichments resulted in sequences with bad quality. These samples were therefore reinoculated and enriched on glucose and therafter analyzed phylogenetically as well (a total of six samples; labeled with Cpa-G in Figure 1A). Four of these samples showed a close relationship with *Clostridium* Cluster I and two belonged to the genus *Paenibacillus*. Enrichments 35-07-Cpa-G and 15-06-G showed 99.7% similarity to each other and were closely related to *Caloramator viterbiensis* (98.3% homology). Analysis of enrichments 1-07-Cpa-G and 9-07-X resulted in species with a close relationship to *Clostridium* AK_{14} and *Clostridium mesophilum* strain SW408.

60°C enrichments

Eighteen of the 20 samples that were sequenced for partial 16S rRNA analysis from 60°C revealed good sequences (Figure 1B; Table 2). Most samples revealed bacteria with a close relationship to *Thermoanaerobacterium* (11 samples), then *Thermoanaerobacter* (4 samples) and *Clostridium* and *Caloramator* species (3 samples). Most of the *Thermoanaerobacterium* enrichments showed very close sequence homology (between 97.0 to 100%) to each other and to species like *Thermoanaerobacterium saccharolyticum*, *Thermoanaerobacterium aciditolerans* and *Clostridium thermoamylolyticum*. Sample 64-07-X showed a very low homology with other *Thermoanaerobacterium* species (89.1% homology with *Clostridium thermoamylolyticum*) but the sequence data for this sample was of low quality. Four enrichments were classified in the *Thermoanaerobacter* genus. There was a clear distinction between two subclusters in the genus, one containing e.g. *Thermoanaerobacter keratinophilus, Thermoanaerobacter tengcongensis* and *Thermoanaerobacter subterraneus* (hereafter referred to as *Thermoanaerobacter* subcluster A) and another containing e.g. *Thermoanaerobacter thermohydrosulfuricus*, *Thermoanaerobacter wiegelii* and *Thermoanaerobacter ethanolicus* (hereafter referred as *Thermoanaerobacter* subcluster B). Enrichments 63-07-G, 29-07-Cpa-G and 29-07-G were identical and belong to *Thermoanaerobacter* subcluster B but enrichment 21-07-Cpo was of bad quality though it showed the closest homology with *Thermoanaerobacter* subcluster A. Three enrichments were classified in the family *Clostridiaceae*. Enrichments 27-07-X and 21-07-Cpa-G belonged to the genus *Caloramator* but 63-07-Cpa belonged to *Clostridium* Cluster III with its closest homology with *Clostridium thermocellum* (95.2%).

70°C enrichments

Nine enrichment cultures at 70°C were analyzed for 16S rRNA and all gave good data for phylogenetic analysis. The four cultures originating from glucose and xylose all belonged to the genus *Thermoanaerobacter* whereas the four strains analyzed on polymers belonged to either *Thermoanaerobacter* or *Caldicellulosiruptor* (Figure 1C; Table 2). The 16S rRNA sequence analysis of enrichments 33-07-Xo and 55-07-Xo were identical and these strains were very close (98.0 - 100%) to wellknown species in *Thermoanaerobacter* subcluster B. Enrichment 20-07-X was also closely related to bacteria in subcluster B and had a 98.0% homology with 33-07-Xo and 55-07-Xo. Enrichments 20-07-G, 2-06-G and 20-07-Cpo, however, showed more than a 96% sequence homology with *Thermoanaerobacter* subcluster A. Finally, three enrichments included bacteria that belong to the genus *Caldicellulosiruptor*.

75°C enrichments

From six 16S rRNA sequence analyses of the highest temperature enrichment samples, only three could be used for phylogenetic studies. These three samples all revealed bacteria that belonged to the *Thermoanaerobacter* genus (Figure 1D; Table 2). Enrichments 14-07-X

Table 3. The ethanol and hydrogen yield from selected enrichment cultures given in mol-EtOH mol-glucose^{-1} and xylose and mol-H_2 mol-glucose^{-1} and xylose.

Temperature °C	Enrichment	Ethanol yield mol-EtOH mol-Glucose^{-1}	Ethanol yield mol-EtOH mol-Xylose^{-1}	Hydrogen yield mol-H_2 mol-Glucose^{-1}	Hydrogen yield mol-H_2 mol-Xylose^{-1}
50	2-07-G	0.42	0.64	0.84	0.76
50	15-06-G	1.32	0.62	0.62	0.56
50	44-07-Xo	0.70	0.78	0.67	0.82
50	25-07-CPa	1.34	0.34	0.48	0.42
50	35-07-Cpa	0.94	0.12	0.69	0.69
50	44-07-P	0.50	0.42	0.87	0.65
50	47-07-P	0.52	0.64	0.56	0.90
50	52-07-P	0.48	0.60	0.62	0.81
50	10-07-X	0.20	0.14	0.67	0.95
50	35-07-X	0.04	0.08	0.73	0.90
50	65-07-P	0.02	0.88	0.67	0.81
60	29-07-G	0.70	1.04	0.28	0.09
60	66-07-G	1.04	1.30	0.39	0.50
60	21-07-Xo	0.62	0.60	0.95	0.92
60	54-07-P	0.78	0.50	0.50	0.70
60	66-07-P	0.82	0.98	0.78	0.73
60	67-07-P	0.84	1.00	0.76	0.17
60	24-07-X	0.78	0.78	0.67	0.50
60	34-07-X	0.82	0.66	0.76	0.53
60	64-07-X	0.74	0.94	0.42	0.45
70	33-07-Xo	1.00	0.86	0.11	0.20
70	20-07-X	0.78	0.74	0.25	0.42
70	8-07-Cpa	0.28	0.02	0.87	0.08
75	14-07-P	0.06	0.08	0.45	0.87
75	14-07-X	0.04	0.06	0.42	0.70

and 39-06-P belonged to the *Thermoanaerobacter* subcluster A whereas enrichment 14-07-Xo belonged to *Thermoanaerobacter* subcluster B.

End product formation

50°C enrichments

The two enrichment cultures 15-06-G and 25-07-Cpa-G that produced the highest amounts of ethanol from glucose belonged to the genera *Caloramator* and *Paenibacillus*, respectively (Tables 2 and 3), both with more than 1.3 mol-EtOH mol-glucose^{-1} (assuming 100% glucose degradation). The best ethanol producers on xylose were enrichments 44-07-G and 65-07-Xo (> 1.0 mol-ethanol mol-xylose)$^{-1}$ which belonged to *Thermoanaerobacter* and *Thermoanaerobacterium*, respectively. Other „good ethanol producers“ on glucose (> 0.7 mol-EtOH mol-glucose^{-1}) were 44-07-Xo (not phylogenetically analyzed), 35-07-Cpa-G (*Caloramator viterbiensis*) and 2-07-Cpo (not phylogenetically analyzed). High ethanol yields (> 0.6 mol-EtOH mol-xylose^{-1}) on xylose were produced by 2-07-G (not phylogenetically analyzed), 15-06-G (*Caloramator viterbiensis*), 44-07-Xo (not phylogenetically analyzed), 2-07-Cpo (not phylogenetically analyzed), 47-07-P (*Thermoanaerobacterium*), 52-07-P (*Thermoanaerobacterium*) and 65-07-X (not phylogenetically analyzed). Several strains produced substantial amounts of butyrate, especially those enriched on pectine and xylan. The best hydrogen producing enrichments on glucose were 2-07-G (not phylogenetically analyzed) and 44-07-P (*Thermoanaerobacterium*), and on xylose 44-07-P and 35-07-X (both *Thermoanaerobacterum*) (Tables 2 and 3).

60°C enrichments

All „high ethanol-producing enrichments“ at 60°C belonged to *Thermoanaerobacterium*. Enrichment 66-07-G produced more than 1.0 mol-EtOH mol-glucose^{-1}, but many xylan and pectine enrichments produced between 0.72 to 0.84 mol-EtOH mol-glucose^{-1} (54-07-P, 64-07-P, 66-07-P, 67-07-P, 24-07-X, 34-07-X, 64-07-X; Tables 2 and 3). Four cultures produced more than 1.0 mol-EtOH mol xylose^{-1}; 29-07-G (*Thermoanaerobacter*), 66-07-G, 66-07-P and 67-07-P (all *Thermoanaerobacterium*), and four others produced slightly lower amounts (0.6 to 1.0 mol EtOH mol-xylose^{-1}) 8-06-G, 21-07-Xo, 24-07-X and 34-07-X (all *Thermoanaerobacterium*) (Table 3). Enrich-

ment 34-07-X produced butyrate from both glucose and xylose as well as ethanol and acetate. Other enrichments at 60°C produced only negligible amounts of butyrate. The best hydrogen producing enrichments on glucose were 8-06-G, 21-07-Xo, 66-07-P, 67-07-P and 34-07-X, all belonging to *Thermoanaerobacterium*. The best hydrogen producers on xylose were the same as on glucose except for 67-07-P (low H_2 production) as well as 54-07-P (*Thermoanaerobacterium*).

70 and 75°C enrichments

Only two enrichments from 70 and 75°C (both belong to *Thermoanaerobacter* subcluster B) produced substantial amounts of ethanol from the two mono-sugars tested: 33-07-Xo and 20-07-X (Tables 2 and 3). In general, end product formation was directed more towards acetate and hydrogen instead of ethanol and no butyrate was observed at these temperatures. The highest hydrogen amounts from glucose fermentation at 70°C were from enrichments 20-07-G (*Thermoanaerobacter* subcluster A), 8-07-Cpa (*Caldicellulosiruptor*) and 4-07-X (*Caldicellulosiruptor*) and on xylose, 2-06-G (*Thermoanaerobacter* subcluster A). The best hydrogen produing strains from 75°C (all *Thermoanaerobacter*) on glucose were 39-06-G, 14-07-P and 14-07-X but the last two were also good hydrogen producers on xylose.

Hydrogen and ethanol yields of selected enrichments

Table 3 summarizes the best ethanol and hydrogen producing bacteria with the focus upon on the yield of ethanol and hydrogen. The maximum theoretical yield of ethanol and hydrogen from glucose are 2.0 and 4.0 moles, respectively. The maximum yield of ethanol and hydrogen from xylose are 1.67 and 3.33 moles, respectively. The enrichments that produced most ethanol usually had an ethanol/acetate ratio between 2.0 and 3.0, whereas the good hydrogen-producing bacteria had ratio lower than 1.0.

DISCUSSION

The interest in using thermophilic bacteria for biofuel production is mainly because of their capacity to degrade a broader spectrum of carbohydrates present in lignocellulosic biomass as compared to yeasts and *Zymomonas mobilis* (Taylor et al. 2009). Many thermophilic bacteria are known to produce ethanol and hydrogen from carbohydrates, especially species within the genera of *Clostridium, Thermoanaerobacter*, *Thermoanaerobacterium*, *Caloramator*, *Caldicellulosiruptor* and *Thermotoga*, Cook et al. 1996, Kublanov et al. 2007, Seyfried et al. 2002, van Niel et al. 2002, Wiegel et al. 2006). In the present study 59 enrichment samples were analyzed for partial 16S rRNA from various hot springs in Grensdalur, SW Iceland. Forty-seven samples revealed good sequences, indicating pure cultures, and were used for phylogenetic comparison. More than two thirds of the samples revealed bacteria that belong to the genera *Thermoanaerobacter* and *Thermoanaerobacterium*. Clearly, temperature is a major factor concerning the isolation of strains from these two genera. All of the 19 *Thermoanaerobacterium* isolates were obtained from enrichments at 50 - 60°C, but the majority of the *Thermoanaerobacter* strains were isolated at 70 - 75°C (9 out of 12 strains). Most of the recognized *Thermoanaerobacter* species have temperature optima above 65°C. The only exceptions are *Thermoanaerobacter brockii* (T_{opt} = 55 to 60°C; Cayol et al. 1995) and *Thermoanaerobacter uzonensis* (T_{opt} = 61°C; Wagner et al. 2008). Most *Thermoanaerobacterium* species, however, have a temperature optimum below 65°C, with the only exceptions being *Thermoanaerobacterium polysaccharolyticum* (T_{opt} = 65-70°C) and *Thermoanaerobacterium zeae* (T_{opt} = 65 to 68°C (Cann et al. 2001).

Analysis showed that eleven of the 16S rRNA samples from 50 to 60°C strains belonged to bacteria of the genera *Clostridium*, *Caloramator* and *Paenibacillus*. At higher temperatures no species were observed that belonged to these genera. The *Clostridium* species isolated were most closely related to

Clostridium AK_{14} and C. *thermocellum*. Strain AK_{14} was indeed isolated from the same geothermal area (Orlygsson & Baldursson 2007) as the samples in the present study. The three isolates that were closely related to *Caloramator* showed the closest relationship with *Caloramator viterbiensis* (between 96.6 to 97.2% homology), but a full 16S rRNA analysis needs to be done to determine whether the enrichment contained bacteria that are new species.

Two samples were classified as belonging to the genus *Paenibacillus*. The optimum growth rate for the majority of the members of this genus has been reported to be 28-30°C (Shida et al. 1997), although several species have been described as growing at slightly higher temperatures and are cellulolytic (Wang et al. 2008). Members of this genus are aerobic, or facultatively anaerobic, and several species have been reported to produce ethanol from sugar fermentation (Marwoto et al. 2004).

From samples enriched at 70°C three samples were analyzed for bacteria that belong to *Caldicellulosiruptor*. Members of this genus have been reported to be cellulolytic and good hydrogen (and acetate) producers (Bredholt et al. 1999, van Niel et al. 2002). Today, seven species are phylogenetically classified as belonging to this genus and are mainly of hot spring origin.

Recently, ethanol and hydrogen production from carbohydrates by thermoanaerobes has been increasingly investigated. The reason for this is mostly because of the vast available biomass of lignocellulosic material on Earth today, as well as the economic, sociological and environmental issues that have been debated concerning first-generation ethanol production from starch- and sugar-based biomass (Tenenbaum, 2008, Metzger & Huttermann 2009). Many of the strains isolated in the present investigation produced considerable amounts of ethanol from glucose and xylose. Most of the „high ethanol" producing species belonged to the genera *Thermoanaerobacterium* and grew at temperatures between 50 to 60°C. Additionally, several of the enrichment cultures that belonged to *Clostridium*, *Caloramator* and *Paenibacillus* produced up to or more than 1 mol-EtOH mol-glucose^{-1}. The three *Caldicellulosiruptor* species produced mostly acetate and hydrogen, a well-known property of members of this genus (Nielsen et al. 1993, Mladenovska et al. 1995, Bredholt et al. 1999, Zeidan & van Niel 2010).

The yields of ethanol and hydrogen in the present study have to be taken with caution regarding the culture conditions used. The maximum yield of hydrogen from glucose by fermentation is 4 moles-H_2 mol-glucose^{-1} degraded if the end product formation is directed solely to acetate as the volatile end product (Hawkes et al. 2002). Lower yields are obtained when end product formation is directed to ethanol, lactate and butyrate. Similarly, ethanol yields among thermophiles have been reported to be up to 1.9 mol-EtOH mol-hexose^{-1}, but generally much lower yields (between 0.5 - 1.5) are obtained. Of great importance regarding yields of both hydrogen and ethanol under batch conditions is the liquid-to-gas (L-G) ratio used. A high L-G ratio favors the production of reduced end products, whereas the low L-G ratios favor hydogen production (Almarsdottir et al. 2010). This is caused by the inhibitory effects of the accumulating hydrogen (high partial pressure of H_2) during growth. In the present investigation the L-G was 0.71 (50 ml liquid in a 120 ml bottle), which explains the relatively low hydrogen yields obtained as compared to ethanol yields. Additionally, initial substrate concentrations are another factor of importance. Neither glucose or xylose were analyzed in the present study but it has been shown that substrate concentrations between 30 and 50 mM may be inhibitory for thermophilic bacteria (Sveinsdottir et al. 2009, Almarsdottir et al. 2010). Thus, it is not surprising that both carbon recoveries and hydrogen and ethanol yields for the data presented (Table 3) were in many cases very low when assuming 100% degradation of both xylose and glucose. For instance, for the enrichments at 50°C, in the only case where ethanol yields were higher

than 1 mol-EtOH mol-glucose^{-1} (15-06-G and 25-07-Cpa-G) the carbon recovery yields reached more than 80%. In most cases the recoveries were much lower (calculated from the data in Table 3). Similar observations were seen at higher temperatures. Thus, for more exact yields of both hydrogen and ethanol, various environmental factors like initial substrate concentrations, the partial pressure of hydrogen, the pH and temperature have to be investigated in more detail. This has already been done with several of the isolates in the present study (Sveinsdottir et al. 2009) where enrichments 33-07-Xo (*Thermoanaerobacter*), 64-07-X and 66-07-P (both *Thermoanaerobacterium*) showed more than 50% higher yields in ethanol production by simply lowering initial glucose concentrations from 50 to 20 mM.

ACKNOWLEDGEMENTS

This work was supported by the The Technology Development and Innovation Fund, administered by the Icelandic Centre for Research (Rannís, project no. 081303408), Nordic Energy Research Fund (NER, project 06-Hydr-C13) and the Research Fund of the University of Akureyri.

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Manuscript received 26 March 2010
Accepted 3 September 2010