

Ethanol production from monosugars and lignocellulosic biomass by thermophilic bacteria isolated from Icelandic hot springs

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ABSTRACT

Seven strains of thermophilic bacteria were isolated from several Icelandic geothermal areas on various carbohydrates (glucose, xylose, xylan, pectin, cellulose). Phylogenetic studies (16S rRNA) revealed that four of the isolates belong to the genus *Thermoanaerobacterium*, two to *Thermoanaerobacter* and one to *Paenibacillus*. The *Thermoanaerobacterium* strains had pH optima at low pH's (pH 5.0 – 6.0), the *Thermoanaerobacter* at slightly acidic to neutral pH's (pH 6.0 – 7.0) and the *Paenibacillus* strain at pH 8.0. Similarly there was a clear distinction of temperature optima between the various genera; *Thermoanaerobacterium* strains had temperature optima close to 60°C, *Thermoanaerobacter* at 70°C and the *Paenibacillus* at 50°C. Ethanol tolerance was from low (MIC = 1.6% v/v) for *Thermoanaerobacter* to moderately high (MIC = 3.2% v/v) for the *Thermoanaerobacterium* and *Paenibacillus* strains. Ethanol production capacity on 20 mM of glucose and xylose showed that six of the strains produced between 1.0 to 1.5 mol-EtOH mol⁻¹ glucose and 0.4 to 1.3 mol-EtOH mol⁻¹ xylose, respectively. One strain showed much lower yields. Strain AK17 gave the best yields on glucose and xylose with 1.5 mol-EtOH mol⁻¹ glucose and 1.1 mol-EtOH mol⁻¹ xylose, respectively. Other end products analyzed in the culture broth were acetate and hydrogen but in lower amounts. Growth on 0.75 % (w/v) hydrolysates made from cellulose (Whatman paper), non inked paper, inked paper, glossy paper, saw dust and grass (*Phleum pratense*) resulted in good ethanol production yields for most of the strains. Strain AK17 produced 43.4 mM of ethanol from cellulose, 21.2 mM from grass, between 14.4 to 23.3 mM from the three types of paper hydrolysates and 3.2 mM from sawdust. Other strains produced less ethanol from biomass hydrolysates but its production was in correlation to lower ethanol production yields from monosugar fermentation. Other end products from hydrolysates were, as in the case of monosugar fermentation, acetate and hydrogen, but in lower amounts.

Keywords: Thermophilic anaerobic bacteria, hydrolysate, lignocellulosic biomass, bioethanol, Icelandic hot springs

YFIRLIT

Framleiðsla á etanóli úr einsykrum og flóknum lífmassa með hitakærum bakteríum einangruðum úr íslenskum hverum

Sjö stofnar hitakærra baktería voru einangraðar frá nokkrum hverasvæðum á Íslandi á mismunandi kol-efnagjöfum (glúkósi, xýlósi, xýlan, pektín, sellulósi). Raðgreining á 16S rRNA bakteríanna gefur til kynna að fjórir stofnanna tilheyri ættkvíslinni *Thermoanaerobacterium*, tveir tilheyra *Thermoanaerobacter* og einn *Paenibacillus*. *Thermoanaerobacterium* stofnarnir uxu best við pH 5.0 – 6.0, *Thermoanaerobacter* við pH 6.0 – 7.0 og *Paenibacillus* við pH 8.0. Einnig var breytileiki hvað varðar það hitastig sem stofnarnir uxu best við; *Thermoanaerobacterium* stofnarnir uxu best við 60°C, *Thermoanaerobacter* við 70°C og the *Paenibacillus* við 50°C. Etanólþol var lágt (MIC = 1.6% v/v) fyrir *Thermoanaerobacter* en mun hærra (MIC = 3.2% v/v) fyrir *Thermoanaerobacterium* og *Paenibacillus* stofnana. Etanól framleiðslugeta á 20 mM af glúkósa og xýlósa sýndu að sex af sjö stofnum framleiddu á milli 1.0 til 1.5 mól-EtOH mól⁻¹ glúkósa og 0.4 til 1.3 mól-EtOH mól⁻¹ xýlósa. Einn stofn framleiddi mun minna etanól. Besta nýtnin var hjá stofni AK17 eða 1.5 mól-EtOH mól⁻¹ glúkósa og 1.1 mól-EtOH mól⁻¹ xýlósa. Aðrar lokaafurðir voru ediksýra og vetni en í mun minna magni. Vöxtur var einnig athugaður í 0.75% (w/v) “hýdrólýsötum” sem voru gerð úr sellulósa (Whatman pappír), ólituðum pappír, lituðum pappír, glanspappír, viðarsagi og vallarfoxgrasi (*Phleum pratense*). Flestir stofnarnir gáfu ágæta etanólframleiðslu. Stofn AK17 framleiddi 43.4 mM etanól úr sellulósa, 21.2 mM úr vallarfoxgrasi, á milli 14.4 og 23.3 mM úr hinum þremur gerðum af pappír og 3.2 mM úr viðarsaginu. Aðrir stofnar framleiddu minna af etanóli en framleiðsla þeirra var í réttu hlutfalli við etanólframleiðslu þeirra á glúkósa og xýlósa. Aðrar afurðir úr hýdrólýsötum voru þær sömu og á einsykrunum eða ediksýra og vetni.

INTRODUCTION

Biofuels have gained increased interest in recent years due to environmental and economic reasons (Qureshi et al. 2006). Today, most of the energy demands are met by non-renewable energy sources, resulting in resource depletion, environmental deterioration and public health problems (Sanches & Cardona 2008). Therefore, there is a demand to develop novel renewable energy harvesting technologies and to introduce sustainable energy carriers. Bioethanol as an alternative to fossil fuels has been extensively studied and its annual, worldwide production is about 51 million liters (Renewable Fuels Association 2008). About 90% of all ethanol is derived from sugar or starch based crops by fermentation (first generation ethanol); the rest is produced chemically. The world's largest ethanol producers are Brazil and the USA, which together account for about 87% of the global ethanol production. Fuel ethanol is produced in Brazil mainly from sugar cane and in the USA from corn (Renewable Fuels Association, 2008).

Fermentation technologies for sugar and starch based crops are well developed, but have been strongly debated since the biomass used is of high value for the food and feed applications. Therefore second generation

ethanol production has been an interesting alternative because it is made from non-edible sources such as lignocellulosic material, which comprises mainly cellulose, hemicelluloses and lignin. Except for lignin, these long-chain polysaccharides can be hydrolysed to produce a mixture of hexoses (C₆) and pentoses (C₅) (Badger 2007, Winters 2007). Although, hydrolysis of lignocelluloses to monosugars, is an extra step, lignocelluloses are highly diverse and abundantly available. Additionally, the feedstock cost is lower for lignocelluloses compared to agricultural crops (Mann 2004).

The most common way of bioethanol production today is by fermentation using the yeast *Saccharomyces cerevisiae* with high ethanol yields from starch based substrates (almost 2 moles mol⁻¹ of glucose). In the past decades thermophilic bacteria have gained more attention because of fast growth rates and their ability to degrade a broad variety of both hexoses and pentoses (Sommer et al. 2004, Georgieva & Ahring 2007, Örylgsson & Baldursson 2007, Koskinen et al. 2008). Although, ethanol tolerance of thermophiles is generally less than those of *S. cerevisiae* and the well known mesophilic bacterium *Zymomonas mobilis*, they have several advantages like lower risk of contamination, increased bioconversion

rates and product recovery (Lynd 1989). A variety of ethanol producing thermophilic microorganisms have been isolated and characterized in the past two decades from different environments, including farm soils, sewage plants, riverbanks, thermal springs, sediments, as well as waste composts. Often, with the intention to evaluate and develop them for large-scale ethanol production. These bacteria include *Thermoanaerobacter ethanol-*

icus (Kannan & Mutharasan 1985, Wiegel & Ljungdahl 1986), *Thermoanaerobacter thermo-hydrosulfuricus* (Wiegel et al. 1979), *Thermoanaerobacter mathranii* (Larsen et al. 1997), *Thermoanaerobacter brockii* (Zeikus et al. 1979, Lamed & Zeikus 1980, Lee et al. 1993), *Clostridium thermosaccharolyticum* (renamed *Thermoanaerobacterium thermosaccharolyticum* (Vancanneyt et al. 1987) and *Clostridium thermocellum* (Herrero & Gomez 1980, Lamed & Zeikus 1980).

Hot springs are a potential source for thermophilic, H₂ and ethanol producing microorganisms. The aim of this study was to use newly isolated ethanol producing microorganisms from hot springs in Iceland for production of ethanol from selected waste/biomass material.

MATERIALS AND METHODS

Sampling sites

The seven strains investigated in this study were isolated from various hot springs in Graendalur in the Hengill area in SW-Iceland and from the Krafla area in NE-Iceland. The temperature and pH of the hot springs where the strains were collected is shown in Table 1 as well as the initial temperature and pH used for enrichment from the samples. Isolation and characterization of strain AK17 has already been described earlier (Örlygsson & Baldursson 2007). Temperatures were measured directly from the hot springs but the pH was

Table 1. Strain identification number, the temperature and pH of the hot springs from which the strains originate, and the temperature and pH used for isolation.

Strain	Temperature (site)	pH (site)	Temperature (isolation)	pH (isolation)
AK17	70°C	6.5	65°C	7.0
20-07-X	72°C	6.8	70°C	7.0
25-07-C	50°C	7.4	50°C	7.0
33-07-X ₀	71°C	8.0	70°C	7.0
64-07-X	59°C	7.0	60°C	7.0
66-07-G	62°C	7.4	60°C	7.0
66-07-P	62°C	7.4	60°C	7.0

measured from experimental bottles upon arrival at the laboratory.

Media

The medium (per liter) consisted of: NH₄Cl 0.3 g, NaCl 0.3 g, CaCl₂ 0.11 g, MgCl₂ x 6H₂O 0.1 g, yeast extract 2.0 g, resazurine 1 mg, trace element solution 1 ml, vitamin solution 1 ml and NaHCO₃ 0.8 g. Phosphate buffers were also used where 1 M stock solutions of NaH₂PO₄ and Na₂HPO₄ were made and added to the media to give a buffer capacity of 30 mM at the different pH's used. Carbon and energy sources were 20 mM or in the case of polymers, 3 g L⁻¹. The vitamin solution was according to DSM141. The trace element was as described earlier (Örlygsson & Baldursson 2007). The medium was prepared by adding the buffer to distilled water, which was then boiled for 5-10 min and cooled while flushing with nitrogen. The mixture was then transferred to cultivation bottles using the Hungate technique (Hungate 1969) and then autoclaved. All other components of the medium were added separately through filter sterilized solutions.

Isolations and enrichments

Samples were collected using an extended pole equipped with grip arms on the end. Serum bottles (120 ml) were fixed at the end, opened and completely filled with geothermal liquid/

mud samples and closed with butyl rubber and aluminum caps. A five ml aliquot from each sample was inoculated into 120 ml serum bottles containing 45 ml medium with 2 g L⁻¹ YE and either 20 mM monosugars (glucose or xylose) or 3 g L⁻¹ of xylan, pectin or cellulose. The samples were incubated at temperatures slightly below the experimental site temperatures. In most cases, because of the dense inoculum, it was not possible to follow growth with increased absorbance by using a spectrophotometer. Therefore, after seven days, an aliquot of 5 ml of each enrichment culture was transferred into a new fresh carbon-containing media. This was repeated three times. Positive samples from the third enrichment series were diluted (tenfold dilutions) and inoculated in the same medium with 20 g L⁻¹ of Gelrite® in Hungate roll tubes. Visible colonies were picked up with sterile Pasteur pipettes and inoculated into fresh media. Six isolates were obtained and analyzed for full 16S rRNA sequence analysis.

Determination of minimum inhibition concentration

MIC (*Minimu Inhibitory Concentration*) determination was performed for the seven strains in order to determine their maximum ethanol tolerance. The experiment was carried out in 23 ml serum bottles containing 10 ml medium and different concentrations of ethanol (0%, 0.2%, 0.4%, 0.8%, 1.6%, 3.2%, 6.4% and 8%). The initial glucose concentration was 20 mM of glucose and 2 g L⁻¹ of yeast extract but control samples did not contain any ethanol or glucose. Optical density was measured (OD₆₀₀) in the beginning and at the end of the incubation period (120 h) to determine the MIC's for each strain.

Determination of pH_{opt} and T_{opt}

Estimation of temperature and pH optimum were done at four different temperatures (50°C, 60°C, 70°C and 75°C) and six different pH levels (pH 4, 5, 6, 7, 8 and 9). Determination of growth was performed on spectrophotometer at 600 nm. Log-phase growth rates (μ)

were derived from the absorbance (OD) data using the standard equation $\ln(X/X_0) = (\mu)(t)$, where X is the measured culture OD, X₀ is the initial culture OD, and t is the elapsed time.

Preparation and pretreatment of hydrolysates from complex biomass substrates

Hydrolysates (HL) were made from a number of different biomass: Whatman filter paper (cellulose), white glossy paper (WGP), newspaper with (NPi) and without ink (NP), sawdust and grass (*Phleum pratense*). The Whatman paper consists of 99% cellulose and was used as a control. The grass was dried overnight at 50°C and cut into small pieces (< 3 mm). All paper was shredded and thereafter cut with scissors. Ten grams of each biomass was weighed into separate Waring blenders and water was added until the total mass of 400 g (2.5% dry weight) was reached. Water and raw biomass were mixed together thoroughly for one minute or until homogenized. After that, each mixture was put in 500 ml flasks which were autoclaved for 90 minutes. After cooling, the pH was measured and adjusted if needed with either HCl or NaOH to pH 5.0. Thereafter, 1 ml of Celluclast® and 1 ml of Novozymes 188 were added into each flask and they were placed in 45°C water bath for 68 hours. Finally, the pH level of the HL's was adjusted to pH that suited the optimum of each strain

Physiological experiments – fermentation of monosugars and hydrolysates

End products from fermentation of monosugars and from lignocellulosic HL's were determined by inoculating 1 ml of a fresh culture into 49 ml of medium containing glucose, xylose or HL's from various lignocellulosic materials. The concentration of glucose and xylose was 20 mM and the concentrations of HL's was 30% or 0.75% dw. Experimental bottles containing HL always contained the same amount of all other components as described earlier; the medium was never diluted of other components like salts, trace elements

Table 2. Carbon substrates used for isolation of seven thermophilic bacteria strains. Also shown is the environmental factors used during enrichment and isolations and the closest phylogenetic genus.

Strain	Genus	Carbon source	T °C	pH
AK17	<i>Thermoanaerobacterium</i>	Glucose	60	6
20-07-X	<i>Thermoanaerobacter</i>	Xylan	70	7
25-07-C	<i>Paenibacillus</i>	Cellulose	50	7
33-07-Xo	<i>Thermoanaerobacter</i>	Xylose	70	7
64-07-X	<i>Thermoanaerobacterium</i>	Xylan	60	7
66-07-G	<i>Thermoanaerobacterium</i>	Glucose	60	7
66-07-P	<i>Thermoanaerobacterium</i>	Pectine	60	7

or vitamins. Fermentation time was one week and samples for volatile fatty acids, ethanol and hydrogen were taken and analyzed at the beginning and end of the experiment.

Strain identification

For 16S rRNA analysis, 16S rRNA genes were amplified from DNA with primers F9 and R1544, specific for bacterial genes (Skirmisdottir et al. 2000) with PCR. In most cases 6-700 bp was used for analysis (strains were both fully and partially sequenced). The PCR products were sequenced with universal 16S rRNA primers: F9, F515, F1392, R357, F1195 and R1544 by using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Subsequently the DNA was analyzed with 3730 DNA analyzer from Applied Biosystems. The nucleotide sequence was displayed and analyzed with Sequencer (Gene Code Corporation) (Skirmisdottir et al. 2000). Sequences from 16S rRNA gene analysis were uploaded to the NCBI database using the nucleotide-nucleotide BLAST (BLASTn). Ribosomal Database Project was also used to obtain sequences of related strains. The most similar sequences obtained from the databases were aligned with the results from the sequencing in the program BioEdit and ClustalX where final alignments were done to generate phylogenetic trees. The program TreeCon was used to view the trees. *Escherichia coli* (AE000406) was selected as out-group.

Analytical methods

Ethanol, acetate and hydrogen were measured by gas chromatograph as previously described (Örlygsson & Baldursson 2007).

RESULTS

Isolations of bacterial strains

– phylogenetic studies

Originally, more than sixty strains were isolated on various carbon substrates from several hot springs in Graendalur in SW-Iceland and from the Krafla area in NE-Iceland. Twelve of these strains were selected for further studies based on good growth rates and high ethanol yields from monosugar fermentations. In this study, seven strains (Table 1) were characterized both phylogenetically and physiologically, by studying fermentation spectrum on monosugars (glucose and xylose) as well as on HL's made from various lignocellulosic biomasses. Additionally, strain AK17 was included in present study, an isolate already isolated at our laboratory (Örlygsson & Baldursson 2007). Three of the seven strains were isolated on monosugars, two on xylan, one on pectin and one on cellulose (Table 2). Six of the strains belonged either to *Thermoanaerobacterium* or *Thermoanaerobacter*. The four *Thermoanaerobacterium* strains (AK17, 64-07-X, 66-07-G and 66-07-P) are phylogenetically very close when compared to each other (Figure 1). Their closest relatives are *Clostridium thermoamylolyticum* (99.0 – 99.2%) and *Thermoanaerobacterium aciditolerans* (99.0 – 99.4%). The two strains (20-07-X and 33-07-Xo) that belonged to *Thermoanaerobacter*

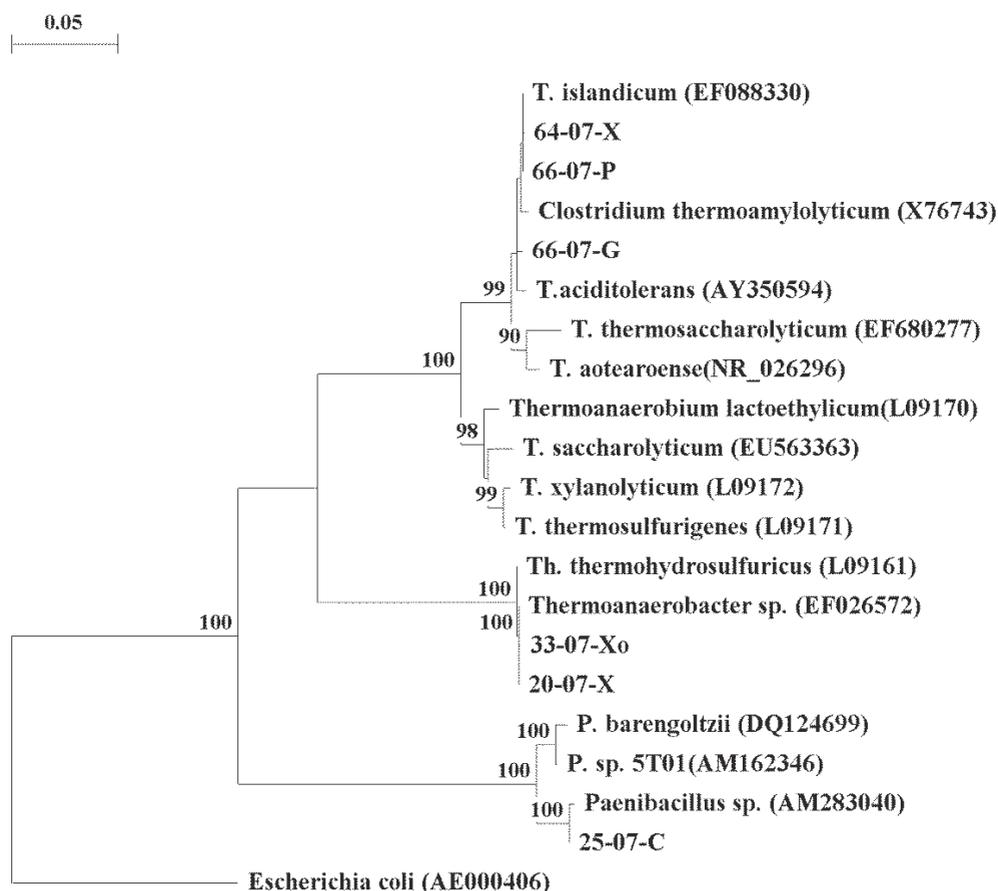


Figure 1. Phylogeny of the strains in this study based on the 16S rRNA gene partial sequences (600-700 bp). The phylogenetic tree was generated using a distance matrix and neighbor joining algorithms with 300 bootstraps. Only supported bootstrap values (>95%) are shown. *Escherichia coli* (AE000406) was selected as an out-group. The scale bar indicates 0.05 substitutions per nucleotide position.

Table 3. Determination of T_{opt} for seven thermophilic bacterial strains. Generation time and maximum optical density of strains is shown as well as isolation temperature.

Strain	Genus	Isolation T°C	T_{opt} °C	Generation time (h)	OD _{max(600nm)}
AK17	<i>Thermoanaerobacterium</i>	65	58	1,24	1,27
20-07-X	<i>Thermoanaerobacter</i>	70	70	2,00	0,94
25-07-C	<i>Paenibacillus</i>	50	50	0,71	1,82
33-07-Xo	<i>Thermoanaerobacter</i>	70	70	1,22	1,46
64-07-X	<i>Thermoanaerobacterium</i>	60	60	1,01	1,24
66-07-G	<i>Thermoanaerobacterium</i>	60	60	1,95	1,63
66-07-P	<i>Thermoanaerobacterium</i>	60	60	0,96	1,47

Table 4. Determination of pH_{opt} for seven thermophilic bacterial strains. Generation time and maximum optical density of strains is shown as well as the isolation pH.

Strain	Genus	Isolation pH	pH_{opt}	Generation time (h)	$\text{OD}_{\text{max}(600\text{nm})}$
AK17	<i>Thermoanaerobacterium</i>	6	6	0,40	1,24
20-07-X	<i>Thermoanaerobacter</i>	7	6	0,93	1,14
25-07-C	<i>Paenibacillus</i>	7	8	0,39	1,59
33-07-Xo	<i>Thermoanaerobacter</i>	7	7	0,83	1,35
64-07-X	<i>Thermoanaerobacterium</i>	7	5	0,90	1,07
66-07-G	<i>Thermoanaerobacterium</i>	7	5	1,33	1,78
66-07-P	<i>Thermoanaerobacterium</i>	7	5	1,99	1,37

Table 5. Minimum inhibitory concentrations of ethanol for seven thermophilic bacterial strains. The final optical density (OD) was used as indicator of growth; ++++ = OD > 1.0; +++ = OD between 0.7 and 1.0; ++ = OD between 0.3 and 0.7; + OD below 0.3 but above control.

Strain	Genus	% vol / vol							
		0.0	0.2	0.4	0.8	1.6	3.2	6.2	8.0
AK17	<i>Thermoanaerobacterium</i>	++++	++++	++++	++++	+++	++	-	-
20-07-X	<i>Thermoanaerobacter</i>	++++	++++	++++	++++	++	-	-	-
25-07-C	<i>Paenibacillus</i>	++++	++++	++++	++++	+++	+	-	-
33-07-Xo	<i>Thermoanaerobacter</i>	++++	++++	++++	++++	++	-	-	-
64-07-X	<i>Thermoanaerobacterium</i>	++++	++++	++++	++++	+++	++	-	-
66-07-G	<i>Thermoanaerobacterium</i>	++++	++++	++++	++++	+++	+	-	-
66-07-P	<i>Thermoanaerobacterium</i>	++++	++++	++++	++++	+++	++	-	-

showed 99.4% homology to each other and their closest relatives are *Thermoanaerobacter thermohydrosulfuricus* and *Thermoanaerobacter* sp. Strain 25-07-C was the only strain isolated on cellulose and is phylogenetically far away from the other strains belonging to the genus *Paenibacillus* (Figure 1).

Physiological properties of strains

The strains were isolated at temperatures between 50 to 70°C. During isolations they were incubated at temperatures slightly below their natural environmental temperatures. Thus, it was not surprising that most of the strains had similar temperature optimum as their isolation temperature (Table 3). The generation time for the strains varied from 0.71 h (strain 25-07-C) to 2.00 h (strain 20-07-X). There is a correlation between the phylogenetic relationship of the strains and the temperature optimum. *Paenibacillus* (25-07-C) has the lowest T_{opt}

(50°); the four *Thermanaerobacterium* strains grew best at 60°C and the two *Thermoanaerobacter* strains at 70°C. Similarly initial pH optimum was determined for all strains (Table 4). Strikingly, all strains grew best at pH different from their environmental pH. Again, the fastest growing strain was 25-07-C but strain 66-07-P had the slowest growth rate. As for the temperature optimum there is a clear relationship between the phylogenetic status and the pH optimum. All *Thermoanaerobacterium* strains have low initial pH optimum (pH 5.0 – 6.0) whereas *Thermoanaerobacter* species grow best at pH between 6.0 and 7.0 and the *Paenibacillus* strain had pH optimum at pH 8.0.

Ethanol tolerance

Table 5 shows the ethanol tolerance of the seven strains. The four *Thermoanaerobacterium* species and the *Paenibacillus* strain showed ethanol tolerance up to 3.2% (v/v) but the

Table 6. End product formation (in mM) from glucose (20 mM) and xylose (20 mM) by seven thermophilic bacterial strains.

Strain	Genus	Glucose (20mM)			Xylose (20mM)		
		Ethanol (mM)	Acetate (mM)	Hydrogen mmol L ⁻¹	Ethanol (mM)	Acetate (mM)	Hydrogen mmol L ⁻¹
AK17	<i>Thermoanaerobacterium</i>	29,9	7,5	11,8	21,3	8,3	0,0
20-07-X	<i>Thermoanaerobacter</i>	3,2	3,1	2,0	5,1	6,5	4,7
25-07-C	<i>Paenibacillus</i>	26,1	12,5	0,0	18,8	11,4	0,0
33-07-Xo	<i>Thermoanaerobacter</i>	29,5	6,3	3,8	15,3	7,5	3,8
64-07-X	<i>Thermoanaerobacterium</i>	22,9	9,9	13,4	19,2	10,4	17,3
66-07-G	<i>Thermoanaerobacterium</i>	20,8	7,1	9,9	8,9	3,7	3,2
66-07-P	<i>Thermoanaerobacterium</i>	25,1	10	12,7	25,4	8,1	8,0

two *Thermoanaerobacter* species had lower ethanol tolerance (1.6%).

End product formation from glucose and xylose

Before determining the ability of the strains to produce ethanol from lignocellulosic biomass, growth characteristics on monosugars (glucose and xylose) was performed. The four *Thermoanaerobacterium* strains all showed similar end product formation on glucose fermentation, i. e. high ethanol yields and low acetate formation (Table 6). The ratio between ethanol and acetate varies between 2.3 (64-07-X) to 4.0 (AK17) and the ethanol yield (mol ethanol per mol glucose) varies from 1.05 (66-07-G) to 1.50 (AK17) which corresponds to 52 – 75% of theoretical yield (100% yield is referred as 2 mol-EtOH/mol-glucose). All *Thermoanaerobacterium* strains produced similar amounts of acetate (7.1 to 10.0 mM) and hydrogen (9.9 to 13.4 mmol l⁻¹) from glucose. The carbon recovery calculated from analyzed end products ranged from 69.9 (66-07-G) to 93.6% (AK17). On xylose, similar fermentation spectrum was observed for three of the four *Thermoanaerobacterium* strains, with high ethanol concentrations and the ethanol and acetate ratio of 1.9 to 3.2. The ethanol yield was 57.4 to 75.2% for three strains but only 26.8% for strain 66-07-G (100% yield is referred as 1.67 mol-EtOH/mol-xylose). Acetate production was in good correlation with ethanol (32 - 54% compared to ethanol) but hydrogen production varied greatly for the four strains.

The two *Thermoanaerobacter* strains show-

ed great variation in fermentation end products. Strain 33-07-Xo showed similar fermentation pattern as the *Thermoanaerobacterium* strains i.e. high ethanol concentrations in the fermentation broth both on glucose and xylose. Strain 20-07-X, however, was a poor ethanol and acetate producer. Glucose was not analyzed in the culture broth but assuming all glucose was degraded very low carbon recoveries were observed for strain 20-07-X (16.5%) compared to 33-07-Xo (89.6%).

The *Paenibacillus* strain was a good ethanol producer, both on glucose and xylose, but no hydrogen was detected during fermentation on either of the monosugars. Carbon recovery on glucose was very good or 96.4%.

End product formation from hydrolysates

All seven strains were inoculated into medium containing hydrolysates containing 7.5 g L⁻¹ (0.75 w/v) from six different types of biomass (cellulose, WGP, NPi, NP, sawdust and grass). In general, ethanol production from the HL's were high to low in the following order; cellulose > WGP > grass > NP > NPi > saw dust (Figure 2). Highest ethanol production was observed on cellulose for all strains except by the poor ethanol producer 20-07-X. Strain AK17 produced 43.6 mM of ethanol from cellulose HL whereas strain 20-07-X produced only 3.4 mM. The amount of end products produced from cellulose HL correlated well with end product formation observed on glucose alone (Table 6). The amount of ethanol produced from grass was between 33 to 60% of that from cellulose HL except for the poor

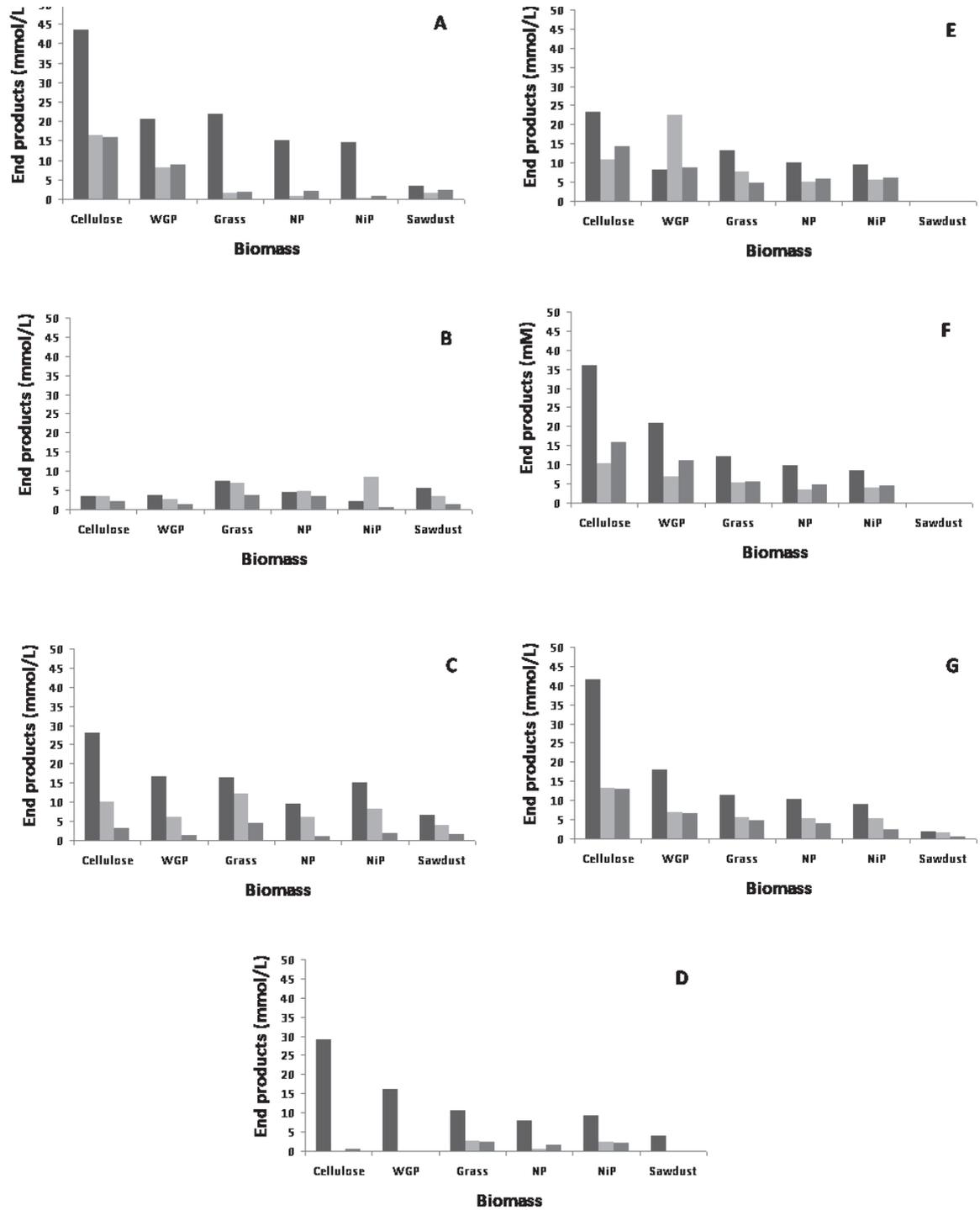


Figure 2. End product formation from biomass (7.5 g L⁻¹) types tested for (A) AK17, (B) 20-07-X, (C) 25-07-C (D) 33-07-Xo (E) 64-07-X (F) 66-07-G (G) 66-07-P. From left to right for biomass types are ethanol, acetate and hydrogen.

ethanol producer 20-07-X where it was slightly higher. Sawdust HL gave the lowest ethanol production by all strains.

DISCUSSION

From previous studies on thermophilic, saccharolytic and anaerobic bacteria a substantial collection of ethanol and hydrogen producing bacteria have been obtained. Many of these bacteria can degrade both five and six carbon monosugars. For a viable second generation bioethanol production we need strains that can convert all the main carbohydrates constituents of lignocellulosic material to ethanol (Wright 1988 Lynd 1996, von Sivers & Zacchi 1995).

In the present study the potential of some of these isolates to produce ethanol from monosugars (glucose and xylose) as well as from HL's made from various lignocellulosic biomasses were tested. The seven strains were subjected to a screening program based on ethanol tolerance and yield of ethanol from monosugars. Earlier experiments showed that increased concentration of sulfuric acid (0, 0.75 and 1.5%) used in pretreatment of lignocellulosic biomass had little effect (results not showed) and it was therefore decided to use only heat (121°C) and no acid on the biomass for 90 min.

Phylogenetic studies on strain AK17, revealed that this bacterium belongs to the genus *Thermoanaerobacterium*. The phylogeny of the strain and its capability of hydrogen and ethanol production has already been thoroughly investigated (Örlygsson & Baldursson 2007, Koskinen et al. 2008). Three other strains that were isolated in the present study were closely related (less than 1.0 % difference was found within the four strains) to strain AK17 as well as with *Clostridium thermoamylolyticum* and *Thermoanaerobacterium aciditolerans*, the latter a well known ethanol and hydrogen producer (Kublanov et al. 2007). Two strains belong to the genus *Thermoanaerobacter* and were closely related to *Thermoanaerobacter thermohydrosulfuricus* also known as a good ethanol producer (Van-

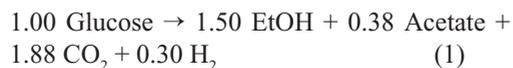
canneyt et al. 1987, Kannan & Mutharasan 1985, Wiegel et al. 1979). Strain 25-07-C belongs to *Paenibacillus* with closest relationship with *Paenibacillus* (AM283040), but several species within this genus are known for their ethanol production (Marwoto et al. 2004).

Ethanol production capacity among thermoanaerobes has been well known for many years now and is potentially thought as a possible future renewable energy source (Cook et al. 1991, Sommer et al. 2004). *Thermoanaerobacter ethanolicus* has been reported to have the highest ethanol yields from glucose and xylose. Ethanol yields from glucose were reported to be 1.9 mol-EtOH mol⁻¹ glucose in batch (Wiegel & Ljungdahl 1981) and in continuous cultures (Lacis & Lawford 1988, Lacis & Lawford 1991). Another well know ethanol producing thermoanaerobe is *Thermoanaerobacter thermohydrosulfuricus* that produces 1.5 mol-EtOH mol⁻¹ glucose (Wiegel et al. 1979). The four *Thermoanaerobacterium* strains in present study produced more than 1.0 mol-EtOH and three strains more than 0.4 mol-EtOH from glucose and xylose, respectively. The best ethanol producer on glucose was strain AK17 with 1.5 mol-EtOH mol⁻¹ glucose (75% of theoretical yield) but strain 66-07-P produced most ethanol from xylose (1.27 mol-EtOH mol⁻¹ xylose; 76% of theoretical yield). The two *Thermoanaerobacter* strains showed a very different ethanol production yields; strain 33-07-Xo produced 29.5 mM ethanol from 20 mM of glucose (73.8% of theoretical yield) and 15.3 mM from xylose whereas strain 20-07-X produced nine times less amounts from glucose and three times less amounts from xylose. This can be caused of either insufficient glucose degradation or production of other end products that were not analyzed in the culture broth, e.g. lactate. The *Paenibacillus* strain (25-07-C) was a good ethanol producer with 65% and 56% yields from glucose and xylose, respectively. Ethanol production has been reported with *Paenibacillus polymyxa* yielding up to 0.74 mol-EtOH mol⁻¹-glucose (Nakashimada et al. 2000, Marwoto et al.

2004) and *Paenibacillus* sp. strain JDR-2 under oxygen limitations (Chow et al. 2007). Members of the genus *Paenibacillus* are facultative anaerobic, spore forming organisms and thermophilic strains have been reported (Wang et al. 2008). Some of these bacteria excrete diverse assortments of extracellular polysaccharide hydrolyzing enzymes, including xylanases, cellulases, amylases, gelatinases, ureases and β -galactosidases (Velázquez et al. 2004).

Other end products produced were hydrogen and acetate, both well know end products from sugar fermentation by species within *Thermoanaerobacter*, *Thermoanaerobacterium* and *Paenibacillus* (Nakashimada et al. 2000, Marwoto et al. 2004, Wiegel & Ljungdahl 1981, Vancanneyt et al. 1987).

To compare ethanol yields from cellulosic and hemicellulosic material HL's (7.5 g L⁻¹) were made from paper, grass and sawdust as well as from pure cellulose (Whatman paper). High ethanol yields were obtained from the cellulose but due to various lignin content of some of other biomass types, lower yields were observed (Figure 2). The Whatman paper consists of 99% glucose. This reflects that if all the glucose that is bound in the paper cellulose is released during hydrolysis and enzyme treatment, a glucose concentration of 41.7 mM would have been present in the final media mixed with the HL's. The end product stoichiometry of glucose fermentation by strain AK17 is (data derived from Table 6):



Thus, from the cellulose HL's 41.7 mM of glucose would theoretically give 62.5 mM of ethanol. The actual ethanol concentration found in the fermentation broth of strain AK17 was however 43.6 mM (control subtracted) or 70% than the theoretical yield according to equation 1. The most probably reason for this low yields is because of the high initial glucose concentrations causing substrate inhibition (Sommer et al. 2004). Indeed, different load-

ings of cellulose HL's have shown that ethanol yields for strain AK17 ranged from 97% at 5.0 g L⁻¹ hydrolysate to 26% at 17.5 g L⁻¹ hydrolysate HL caused by undegraded glucose residues in the culture broth (results not shown).

The fermentation of strain AK17 on other HL's showed lower ethanol production. Similar values were obtained for the WGP and grass; 20.6 to 21.9 mM. The other paper types (both NP and NPi) gave slightly lower ethanol production (14.6 to 15.2 mM) but much lower yields were observed from sawdust. The other strains produced less ethanol from the various HL's but was generally proportional to lower yields from monosugars (Tables 6 and Figure 2).

The yields of ethanol produced in present study can be regarded as relatively good when compared to other studies. Sommer and co-workers (Sommer et al. 2004) showed that thermophilic bacteria produced between 9.8 – 25.7 mM of ethanol from undiluted wheat straw hydrolysate (60 g L⁻¹). *Thermoanaerobacter ethanolicus* produced 24 mM of ethanol when cultivated in steam exploited birch wood hemicelluloses hydrolysate (0.8 w/v) (Wiegel et al. 1983) and *Clostridium thermosaccharolyticum* produced 40 mM of ethanol in oak sawdust pretreated with 1% sulfuric acid (Liu et al. 1988). Clearly, at high hydrolysate concentrations, the yield of ethanol decreases dramatically. Microorganisms producing promising yields on pure glucose and xylose do not necessarily do well in pretreated hydrolysate that contains inhibitory compounds like acetate, furfural and lignin degradation products (Watson et al. 1984, Palmqvist & Hahn-Hagerdal 2000).

Some investigation of ethanol production in pretreated lignocellulosic biomass is an important screening criterion when considering a microorganism for real-life applications (Hahn-Hagerdal et al. 1993, Zacchi et al. 1988, Wyman 1999, Hinman et al. 1989). Grass or waste paper is likely to be the future substrates in Icelandic ethanol process, based on the conversion of both the cellulose and the hemicelluloses fraction into ethanol. The strains isolat-

ed here are therefore promising candidates for such applications.

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