Ethanol production from biomass by a moderate thermophile, *Clostridium* AK_1

JOHANN ORLYGSSON

Faculty of Natural Resource Science, University of Akureyri, Borgir v. Nordurslod, 600 Akureyri, Iceland.

E-mail: jorlygs@unak.is (corresponding author)

ABSTRACT

The ethanol production ability of *Clostridium* strain AK_1, a moderate thermophilic bacterium was studied. Growth of strain AK_1 was observed at temperatures between 40 and 55°C with optimal growth at 45°C. Optimum pH for growth was at pH 6.5 but growth was observed from pH 4.5 to 7.5. Fermentation of glucose resulted in the production of ethanol (major) and acetate (minor) as well as hydrogen and carbon dioxide. The effect of increased initial substrate (glucose) concentration was investigated and good correlation was observed between increased substrate loadings and end product formation up to 20 mM. At ≥ 30 mM glucose concentrations, end product formation levelled off and the substrate was not completely degraded. In addition, the pH at the end of fermentation dropped from 6.5 (control without glucose) to 5.3 (at ≥ 30 mM glucose). The ability to utilize various carbon substrates was tested, with positive growth observed on xylose, glucose, fructose, mannose, galactose, sucrose, lactose, starch, xylan and pectin. The end products in all cases were the same as for glucose. By varying the liquid to gas phase ratio during glucose fermentation in a batch a clear correlation was found between increased ethanol production at low gas-to-liquid conditions and less acetate and hydrogen. End product formation from fermentation of various hydrolysates was performed by using (5.0 g L^{-1} [dw]) of cellulose (whatman paper), newspaper, grass (*Phleum pratense*), barley straw (*Hordeum vulgare*), and hemp (stem and leaves of *Cannabis sativa*). The biomass was pretreated with either a weak acid (HCl) or a weak alkali (NaOH) as well as enzymes (Celluclast® and Novozyme 188). The strain produced most ethanol (7.4 mM ethanol g^{-1}) from non- chemically pretreated cellulose hydrolysate but less from paper and lignocellulosic biomasses (between 0.2 to 2.1 mM g^{-1}). The ethanol production from lignocellulosic biomass was, however, enhanced significantly by acid and alkali pretreatment with the highest production from hemp (3.5 mM ethanol g^{-1}).

Keywords: ethanol, hot spring, *Clostridium*, carbohydrates, lignocellulose

YFIRLIT

Framleiðsla á etanóli úr lífmassa með Clostridium tegund, AK_1

Etanólframleiðsluhæfileiki bakteríustofnsins *Clostridium* AK_1 sem er hitakær baktería einangruð úr íslenskum hver var rannsökuð. Stofninn vex við hitastig á milli 40 til 55°C en hámarksvöxtur var við 45°C. Kjörsýrustig

var við pH 6.5 en mælanlegur vöxtur var við sýrustig á milli pH 4.5 og 7.5. Niðurbrot á glúkósa leiddi aðallega til framleiðslu á etanóli en einnig ediksýru, vetni og koltvísýrings. Áhrif mismunandi upphafsstyrks af glúkósa á framleiðslu lokaafurða var rannsakaður hjá bakteríunni. Við lágan upphafsstyrk glúkósa voru bein tengsl á milli styrks hvarfefnis og myndefna, þ.e. aukning á glúkósa leiddi til línulegrar aukningar á lokaafurðum. Hins vegar þegar styrkur glúkósa náði 30 mM kom í ljós greinleg hindrun og glúkósinn var ekki brotinn fullkomlega niður. Sýrustigið í lok gerjunar lækkaði einnig með auknum styrk glúkósa en það var 6.5 án glúkósa og fór niður í 5.3 við 30 mM upphafsstyrk glúkósa en lækkaði ekki frekar við hærri glúkósastyrk. Hæfileiki stofnsins til að brjóta niður mismunandi kolefnisgjafa leiddi í ljós að hann brýtur niður xýlósa, glúkósa, frúktósa, mannósa, galaktósa, súkrósa, laktósa, sterkju, xýlan og pektín. Lokaafurðir við niðurbrot þessara hvarfefna var í öllum tilfellum etanól, ediksýra, vetni og koltvísýringur. Með því að nota mismunandi hlutfall á milli gas og vökvafasa við niðurbrot á glúkósa kom í ljós að styrkur etanóls var mestur þegar gasfasinn var hlutfallslega lítill miðað við vökvaræktina en styrkur ediksýru og vetnis minnstur. Vöxtur var einnig athugaður í 0.5% (wv^{-1}) "hýdrólýsötum" sem búin voru til úr sellulósa (Whatman pappír), dagblaðapappír, vallarfoxgrasi (*Phleum pratense*), hálmi úr byggi (*Hordeum vulgare*), og hampi (stilkar og laufblöð (*Cannabis sativa*)). Lífmassinn var formeðhöndlaður með veikri sýru (HCl) eða veikum basa (NaOH) sem og ensímum. Án efnaformeðhöndlunar framleiddi stofninn mest etanól úr sellulósa (7.4 mM etanól g^{-1}) en mun minna úr öðrum lífmassa (0.2 til 2.1 mM g^{-1}). Hins vegar var hægt að auka þessa framleiðslu til muna með því að formeðhöndla lífmassann og hæsta gildið fékkst úr hampi eða 3.5 mM etanól g^{-1}. Aðrar lokaafurðir voru þær sömu og áður, þ.e. etanól, ediksýra, og $H_2 + CO_2$.

INTRODUCTION

Recent challenges of global energy crisis and climate changes caused by increase of CO_2 in the atmosphere are leading to the search for alternative and renewable, non-fossil energy sources (Zaldivar et al. 2001; Ballesteros et al. 2006). Ethanol production from biomass is an interesting alternative to fossil fuels. In 2010, 86.8 billion litres of ethanol were produced worldwide, mostly in the USA and Brazil (Renewable Fuels Association, 2011). However, most of the increase of bioethanol production is from starch and soluble sugar-based biomass like sugar cane juice and corn starch (Bai et al. 2008). This first generation ethanol production has led to an intensive food versus fuel debate worldwide. Lignocellulose is the most abundant biopolymer on earth and the main component of plant biomass. Fermentation of lignocellulosic biomass (e.g. wood, straw, grass and waste material from, for example, agricultural and municipal activities and the pulp and paper industry) therefore constitutes a potential alternative for second generation ethanol production (Binod et al. 2010; Sarkar et al. 2012). However, the lack of suitable microorganisms for efficient conversion of ligcocellulosic biomass into ethanol has been one of the major bottlenecks preventing commercialization of second generation ethanol production (Taylor et al. 2009).

Several mesophilic ethanol producing microorganisms have been extensively studied for this purpose, with special focus on genetic engineering to increase ethanol tolerance or yields. Thermophilic bacteria have been proposed as good candidates for ethanol production from lignocellulosic biomass (Taylor et al. 2009). Most studies have been on Gram positive, strictly anaerobic bacteria that belong to the class Clostridia and the phylum Firmicutes. Many of these strains are of geothermal origin and have a temperature optimum above 60°C and belong to the genera of *Clostridium, Thermoanaerobacter* and *Thermoanaerobacterium* (Lamed & Zeikus, 1980; Ben Bassat et al. 1981; Wiegel & Ljungdahl, 1981; Fardeau et al. 1996, Sveinsdottir et al. 2009). The main reason for this increased interest is because of the robustness of these microbes to tolerate high temperatures and other environmental factors, e.g. tolerance to high sulphide concentrations. Secondly, many thermophiles have a much broader substrate range as compared to the commonly used *Sacchcaromyces cerevisare and Zymomonas mobilis*. Finally, several species have shown very good ethanol yields, often between 1.5 to 1.9 mol ethanol

mol-hexose^{-1} degraded, which is close to the theoretical maximum yield of 2.0 mol ethanol mol-hexose^{-1}. Most attention has been on the ethanol production capacity of thermophilic bacteria from simple sugars but more recently also from complex, lignocellulosic biomass (Ahring et al. 1999; Georgieva et al. 2008; Lin et al. 2010, Almarsdottir et al. 2012; Sigurbjornsdottir & Orlygsson, 2012). Little attention has however been placed on ethanol producing thermoanaerobes growing at more moderate temperatures. In the present study, the ethanol production capacity of a new species, *Clostridium* AK_1, a moderate thermophile isolated from a hot spring in Grensdalur (Orlygsson & Baldursson, 2007), in SW Iceland was investigated in detail.

MATERIALS AND METHODS

Culture Media

The medium (per litre) consisted of: NH_4Cl 0.3 g, NaCl 0.3 g, $CaCl_2$ 0.11 g, $MgCl_2$ x $6H_2O$ 0.1 g, yeast extract 2.0 g, resazurin 1 mg, trace element solution 1 ml, vitamin solution 1 ml and $NaHCO_3$ 0.8 g. Phosphate buffers were also used where 1 M stock solutions of NaH_2PO_4 and Na_2HPO_4 were made and added to the media to give a buffer capacity of 30 mM. The vitamin solution was according to DSM141. The trace element was as described earlier (Örlygsson & Baldursson, 2007). The moderately thermophilic strain AK_1 used in this study was isolated as described earlier by Orlygsson & Baldursson (2007).

Determination of growth

Cell concentration was determined by measuring absorbance at 600 nm by a Perkin Elmer Lambda-25 UV-Vis spectrophotometer. Maximum (specific) growth rate (μ_{max}) for each growth experiment was derived from the absorbance data (OD_{600}) using the equation: $\ln(x/x_0) = (\mu)(t)$, where x is the measurement of optical density of the culture, x_0 is the initial optical density of the culture, t is the elapsed time and μ denotes the maximum growth rate. All experiments were done in duplicate and average values are reported.

Determination of pH_{opt} and T_{opt}

The optimum pH for growth was determined by incubating the strain at 45°C and at a pH from 3.0 to 9.0 with increments of 0.5 pH units. The media pH was adjusted with acid (HCl) or base (NaOH) as needed. The optimum temperature for growth was determined by incubating the strain at pH 6.5 and at temperatures from 30°C to 60°C.

Effect of substrate concentration

The effect of increased glucose concentration was tested by growing strain AK_1 at an initial glucose concentration from 5 to 200 mM. Control samples did not contain glucose, only yeast extract. After fermentation, glucose, hydrogen, acetate and ethanol were measured as well as the final pH in the experimental bottles. Experiments were done in 117.5 mL serum bottles with 50 mL of liquid medium.

Substrate utilization

The ability of strain AK_1 to utilize different substrates was tested using the BM medium supplemented with various filter sterilized substrates (20 mM or 2 g L^{-1}). The substrates tested were: xylose, ribose, arabinose, glucose, fructose, galactose, mannose, sucrose, lactose, lactate, formate, succinate, malate, pyruvate, oxalate, crotonate, glycerol, inositol, starch, cellulose, xylan, sorbitol, pectin, casamino acids, peptone, beef extract, tryptone, alanine, aspartate, glycine, glutamate, serine, theronine, histidine and cysteine. Growth was observed by increase in optical density. Where growth was detected, hydrogen, acetate and ethanol were analysed. Experiments were done in 23 mL serum bottles with 10 mL of liquid medium.

Effect of liquid-gas (L-G) volume ratio on hydrogen production

The influence of partial hydrogen pressure (ρH_2) on end product formation was investigated with different ratios of liquid and gas phases when grown on 20 mM glucose. The liquid phase varied from 2 to 90 mL in serum bottles with a total volume of 117.5 mL; thus, the L-G

volume ratio varied from 0.017 to 3.27. After 5 days of incubation, the glucose content and the end products formed (hydrogen, acetate and ethanol) were measured.

Pretreatment of biomass and hydrolysate preparation

Hydrolysates (HL) were made from whatman filter paper (cellulose), hemp (*Cannabis Sativa*) – leaves and stem fibres, newspaper, barley straw (*Hordeum vulgare*) and grass (*Phleum pratense*). Whatman paper consists of 99% cellulose and was therefore used as a control. Hydrolysates were prepared according to Almarsdottir et.al. (2012) yielding a final dry weight of 25 g L^{-1}. Chemical pretreatment with acid (0.5% H_2SO_4) or base (0.5% NaOH) (control was without chemical pretreatment) were done before autoclaving for 30 minutes (121°C). After heating, the bottles were cooled down to room temperature and the pH adjusted to 5.0 by adding either HCl or NaOH. Two enzymes were added to each bottle, Celluclast® (75 U g^{-1}) and Novozyme 188 (20 U g^{-1}), and incubated in a water bath at 45°C for 68h. After the enzyme treatment the pH was measured again and adjusted to pH 6.5 which is the pH optimum for the strain. The solutions were then filtered through 0.45 μm filters into sterile bottles to collect the hydrolysates.

Fermentation of hydrolysates

Fermentation of carbohydrates present in hydrolysates by strain AK_1 was done in 23 mL serum bottles. The medium (8 mL) was supplemented with 2 mL of hydrolysate in each sample (total liquid volume 10 mL), giving a final concentration of 5.0 g L^{-1}. The control sample contained no hydrolysate, only yeast extract, which was present in all experimental bottles.

Analytical methods

Hydrogen, ethanol and volatile fatty acids were measured by gas chromatography as previously described (Orlygsson & Baldursson 2007). Glucose was analysed by a slight modification of the method from Laurentin & Edwards (2003). A liquid sample (400 μL) was mixed with 2 mL of anthrone solution (0.2% anthrone in 72% sulphuric acid). The sample was boiled for 11 minutes and then cooled down on ice. Optical density was then measured at 640 nm.

RESULTS

Temperature and pH optimum of strain AK_1

The strain AK_1 grows in a very narrow temperature range (40 to 55°C), with optimal growth at 45°C (generation time 1.9h). Optimum pH for growth was at pH 6.5 (generation time; 1.6h), but growth was observed from pH 4.5 to 7.5. Thus, all experiments were performed at 45°C and at pH 6.5.

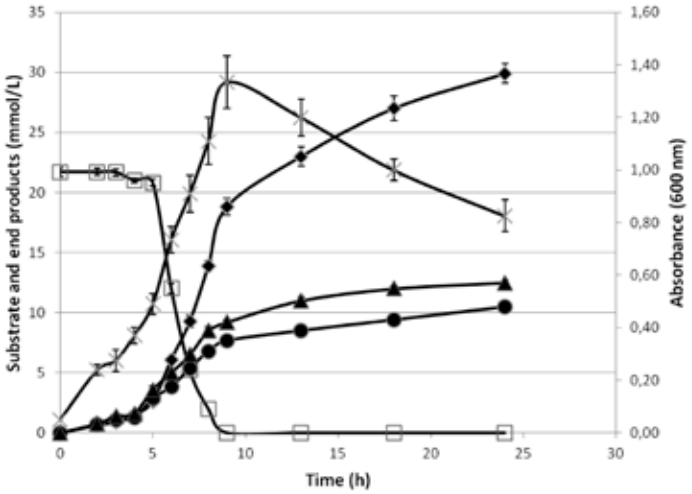

Figure 1. Kinetics of glucose (20 mM) degradation to end products. Also shown is the optical density (growth) measured at 600 nm. Standard deviations are shown as error bars. In some cases the error bars are smaller than the symbols. Glucose (□), Ethanol (♦), Acetate (●), Hydrogen (▲). Growth (X).

Kinetics of glucose degradation

During growth on glucose (20 mM), a relatively short lag phase occurs (3 to 4h) before glucose degradation and end product formation starts (Figure 1). The doubling time during ex-ponential growth phase is 1.86 h and the ethanol production rate is 4.0 mM h^{-1}. Maximun optical density is obtained within 10h but end product formation continues throughout the incubation period. Glucose is completely degraded to various end product with the following stoichometry:

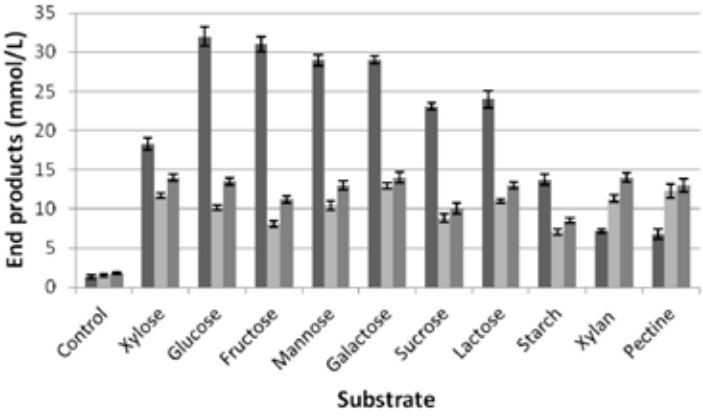

Figure 2. End product formation from various substrates by strain AK_1. Data represent the average of two replicate experiments. Standard deviations are shown as error bars. Hydrogen values are corrected to volume units. Columns from left to right; ethanol, acetate and hydrogen.

1.0 Glucose → 1.50 Ethanol + 0.38 Acetate + 0.55 H_2 + 1.88 CO_2 (Eq. 1).

Thus, ethanol yields are 1.5 mol EtOH mol $glucose^{-1}$, or 8.33 mM g^{-1} glucose. The carbon balance is almost complete or 94% (± 1.5%).

Ethanol production from sugars and other substrates

Strain AK_1 grew on various types of carbohydrates as the sole carbon and energy source (Figure 2). Of the three pentoses tested AK_1 only degraded xylose but all four hexoses tested (glucose, frucose, galactose, mannose) were utilized as well as the disaccharides sucrose and lactose. AK_1 also degraded starch, xylan and pectine. All of the other substrates tested were not degraded. The end products formed on all substrates were the same as for glucose: ethanol, acetate, H_2 + CO_2. The ratio of ethanol and acetate was similar on all hexoses and disaccharides (between 2.2 to 2.9) but lower on xylose (1.6). The amount of hydrogen was always proportional to acetate production.

Effect of initial glucose loadings on ethanol production

To investigate the ability of the strain to grow on high substrate concentrations, the strain was incubated with different initial glucose concentrations. At low substrate loadings (5, 10, 20 mM), a complete degradation of glucose occurred and a similar end product spectrum was observed as earlier, i.e. ethanol, acetate and hydrogen (Figure 3). At 30 mM, only 68% of the glucose was degraded and much less at higher (≥ 50 mM) concentrations. This was also reflected in similar amounts of end products at high initial glucose concentrations compared to the lower substrate concentrations. The pH was measured after fermentation and decreased from low glucose loadings (pH 6.1) to high (≥ 50mM) loadings (pH 5.3).

Effect of partial pressure of hydrogen

The influence of partial pressure of hydrogen on hydrogen production by strain AK_1 has been previously studied (Orlygsson & Baldursson 2007). In that study, the theoretical yield of hydrogen increased from 18 to 34% when the L-G volume ratio was changed from 3.27 to 0.02. In this case it is assumed that the theoretical yield is 4 moles of hydrogen per mole degraded hexose and acetate is the

only volatile end product (Hawkes et al. 2002). This experiment was repeated in the present investigation but now volatile end products and ethanol formation were also analysed to gain further insight into the metabolism of the bacterium. Again, similar yields of hydrogen were observed, varying from 0.4 to 1.6 mol-H_2 mol glucose^{-1} (10 to 40% of theoretical yield). As expected, lower H_2 yields followed the de-crease in acetate and increase in ethanol production (Figure 4).

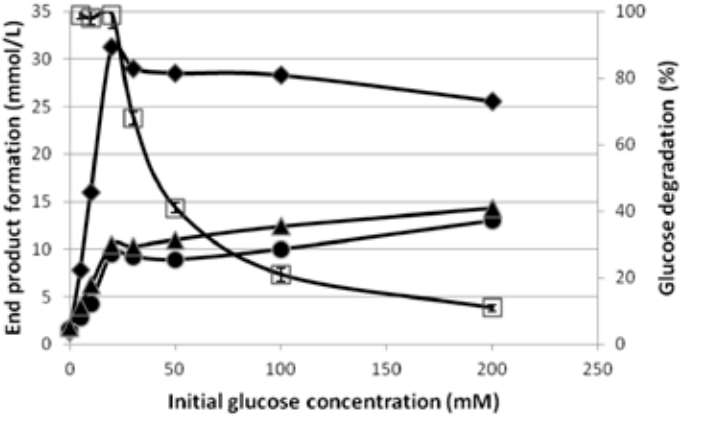

Figure 3. End product formation from different initial glucose concentrations. Also shown are the residual glucose concentrations (in percent) after fermentation. Hydrogen values are corrected to volume units. Glucose (□), Ethanol (♦), Acetate (●), Hydrogen (▲).

Using the fermentation data from the lowest and highest L-G ratios the following equations are ob-served:

1.0 Glucose → 1.20 Ethanol + 0.72 Acetate + 1.29 H_2 + 1.92 CO_2 (low L-G (0.05) ; (Eq.2)

1.0 Glucose → 1.72 Ethanol + 0.35 Acetate + 0.45 H_2 + 2.05 CO_2 (high L-G (3.27) ; (Eq.3)

These ethanol yields correspond to 6.67 and 9.44 mM ethanol g^{-1} glucose degraded at low and high L-G ratios, respectively.

End product formation from hydrolysates

Ethanol production from various lignocellu-losic biomasses was investigated for the strain.

Strain AK_1 produced between 37 to 38 mM of ethanol from hydrolysates made from cellulose (Table 1) and there was no difference in the end product formation with chemically pretreated cellulose (acid/alkali) compared to enzymatic pretreated cellulose. The yields on cellulose were between 7.3 to 7.4 mM g^{-1} dw, or slightly lower as compared to glucose degradation (see eq. 1). However, due to the high initial glucose concentrations in the cellulose (5 g cellulose corresponds to 30.8 mM glucose equivalents), the glucose was not completely degraded (Table 1), explaining the lower ethanol yields. Other end products (acetate, hydrogen) were also produced in similar or slightly lower proportions as observed on glucose.

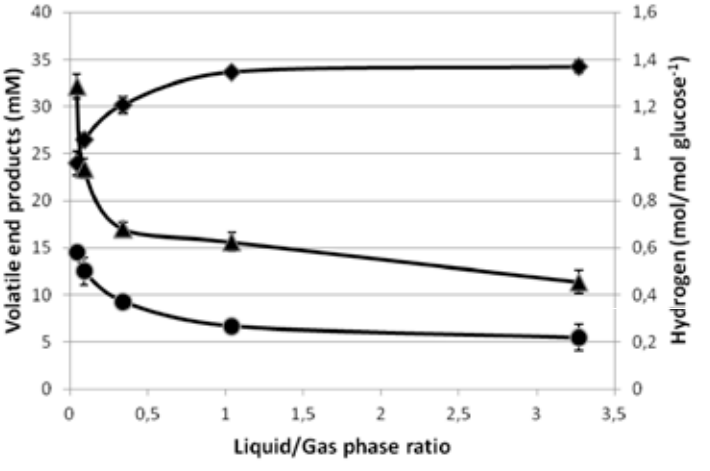

Figure 4. Effect of liquid/gas phase ratio on end product formation for strain AK_1. Standard deviations are shown as error bars. In some cases the error bars are smaller than the symbols. Ethanol (♦), Acetate (●), Hydrogen (▲).

Table 1. Production of end products from different biomass hydrolysates (5 g L^{-1}). Values represent the mean of two replicates (± standard deviation). Hydrogen values are corrected to volume units. Nd = below detection limit (< 0.1 mM).

	Concentration (mmol L^{-1})				
	Ethanol	**Acetate**	**Hydrogen**	**Glucose (t = 0)**	**Glucose (t = final)**
Control	1.1 ± 0.1	1.5 ± 0.1	1.8 ± 0.1	Nd	Nd
Whatman	38.0 ± 0.1	9.2 ± 0.4	13.3 ± 0.6	30.3 ± 1.4	5.3 ± 0.3
Whatman acid	37.8 ± 0.1	9.3 ± 0.4	12.1 ± 0.4	31.3 ± 1.8	6.1 ± 0.2
Whatman alkali	37.5 ± 1.2	9.8 ± 0.6	13.9 ± 1.4	29.5 ± 0.4	5.9 ± 0.1
Paper	6.7 ± 0.3	6.4 ± 0.1	8.2 ± 0.3	8.6 ± 0.1	Nd
Paper acid	8.0 ± 0.2	7.8 ± 0.0	9.9 ± 0.1	10.2 ± 0.1	Nd
Paper alkali	8.0 ± 0.1	7.0 ± 0.1	7.7 ± 0.2	11.3 ± 0.1	Nd
Grass	11.2 ± 0.3	8.9 ± 0.1	10.3 ± 0.6	9.4 ± 0.1	Nd
Grass acid	16.4 ± 0.3	12.0 ± 0.2	15.2 ± 0.1	13.9 ± 0.1	Nd
Grass alkali	15.0 ± 0.3	11.0 ± 0.2	10.0 ± 0.5	14.2 ± 0.1	Nd
Hemp leaf	2.1 ± 0.0	4.9 ± 0.0	4.0 ± 0.2	2.5 ± 0.1	Nd
Hemp leaf acid	6.4 ± 0.1	8.8 ± 0.2	11.1± 1.2	5.7 ± 0.1	Nd
Hemp leaf alkali	4.7 ± 0.1	7.5 ± 0.1	8.8 ± 0.6	5.2 ± 0.1	Nd
Hemp stem	11.7 ± 0.9	9.8 ± 0.3	12.6 ± 0.5	9.6 ± 0.1	Nd
Hemp stem acid	18.3 ± 1.2	12.5 ± 0.8	13.9 ± 0.1	14.3 ± 0.1	Nd
Hemp stem alkali	18.5 ± 1.1	13.8 ± 0.7	16.0 ± 0.1	13.6 ± 0.1	Nd
Barley straw	4.7 ± 0.1	5.1 ± 0.2	7.5 ± 0.1	4.5 ± 0.1	Nd
Barley straw acid	7.0 ± 0.1	6.2 ± 0.2	5.7 ± 0.0	6.1 ± 0.1	Nd
Barley straw alkali	17.1 ± 0.8	12.7 ± 0.5	12.7 ± 0.1	13.5 ± 0.1	Nd

The highest ethanol yield on lignocellulosic biomass (without chemical pretreatment) was observed on hemp stem (2.1 mM g^{-1} dw) but lowest on hemp leaf (0.2 mM g^{-1} dw). Treatment with either acid or alkali increased yields substantially on all lignocellulosic biomass tested. The increase was most profound on hydrolysates from straw (3.42 times; alkali) and hemp leaves (3.05 times; acid).

DISCUSSION

Earlier experiments on phylogenetic analysis of the strain showed that the bacterium is actually far away from its nearest neighbours (Orlygsson & Baldursson, 2007). Results from 16S rRNA analysis of strain AK_1 showed that it is most closely related to species within *Clostridium* Cluster IV but with very low homology to any known species (< 90%). The most intriguing aspect concerning the physiology of the strain is the fact that the temperature growth range is very narrow or only about 15°C (from 40 to 55°C). This could be caused by the ecological niche of its origin but the temperature of the hot spring it was isolated from was 47.8°C (Orlygsson & Baldursson, 2007). Interestingly, another new *Clostridium* species, *Clostridium* AK_{14}, a powerful hydrogen producer isolated from the same area, seems to have a similar temperature optimum and narrow growth range characteristics (Orlygsson & Baldursson, 2007; Almarsdottir et al. 2010).

Different experiments were done to investigate the ethanol production potential of strain AK_1. The strain produces about 1.5 mol EtOH mol $glucose^{-1}$ and is thus with a similar potential to that of many species within *Clostridium, Thermoanaerobacter* and *Thermoanaerobacterium* (Lovitt et al.1984; Lacis & Lawford

Table 2. Examples of high ethanol yields during fermentation of lignocellulosic biomass by thermophilic bacteria. * = continuous culture.

	Biomass	Substrate conc. ($g\ L^{-1}$)	Ethanol Yields ($mM\ g\ sugar^{-1}$)	Temp. (°C)	Reference
Thermoanaerbacter ethanolicus	Wood hydrolysate	8.0	3.3 – 4.5	70	Wiegel et al. (1983)
Clostridium thermocellum	Avicel	2.5	5.0	60	Lynd et al. (1989)
Clostridium thermocellum	Whatman paper	8.0	7.2 – 8.0	60	Rani et al. (1998)
Clostridium thermocellum	Paddy straw	8.0	6.1 – 8.0	60	Rani et al. (1998)
Clostridium thermocellum	Sorghum stover	8.0	4.8 – 8.1	60	Rani et al. (1998)
Thermoanaerobacterium saccharolyticum	Xylan	10.0	6.3	60	Ahring et al. (1996)
Thermoanaerobacter mathranii	Wheat straw	6.7	5.3	70	Klinke et al. (2001)
Thermoanaerobacter sp. 65-2	Beet molasses	19.5	7.3	70	Avci & Donmez. (2006)
Thermoanaerbacter ethanolicus	Beet molasses	19.5	4.8	65	Avci & Donmez. (2006)
Thermoanaerbacter BG1L1*	Corn stover	12.0 – 41.0	8.5 – 9.2	70	Georgieva and Ahring (2007)
Thermoanaerbacter BG1L1*	Wheat straw	11.0 – 40.0	8.5 – 9.2	70	Georgieva et al. (2008)
Thermoanaerobacterium AK17	Cellulose	5.0	8.6	60	Almarsdottir et al. (2012)
Thermoanaerobacterium AK17	Grass	5.0	5.5	60	Almarsdottir et al. (2012)
Clostridium AK1	Whatman paper	5.0	7.3 – 7.4	45	This study
Clostridium AK1	Hemp stem	5.0	3.5	45	This study
Clostridium AK1	Grass	5.0	3.1	45	This study

1988; Fardeau et al. 1996; Sveinsdottir et al. 2009; Almarsdottir et al. 2012). One of the major reasons for using thermophilic bacteria for ethanol production from lignocellulosic material is their ability to degrade a broad range of carbohydrates present in the biomass. The strain degraded all of the hexoses that were used as substrates as well as both disaccharides, but xylose was the only pentose used. Cellulose degradation is a common property of many species within the genus *Clostridium.* Strain AK_1 did not degrade pure cellulose, nor CMC or Avicel, but could degrade both pectine and xylan as well as starch.

High initial substrate concentration may play an important role in growth and end product formation rates and yields and this has been investigated for several thermophilic bacteria, e.g. *Clostridium* and *Thermoanaerobacterium* (Lacis & Lawford, 1988; Sommer et al. 2004; Almarsdottir et al. 2012). Increased substrate concentrations clearly affected the strain strongly, as shown by levelling off of end product formation with increased substrate loadings and only partial degradation of glucose. This inhibition could also have been caused by the lower pH observed in the experimental bottles with high glucose loadings. Partial pressure of hydrogen (pH_2) is also known to be of huge importance concerning the flow of carbon substrate to various end products. Thus, a high pH_2 may shift end product formation towards more reduced products, e.g. ethanol, lactate, butyrate, alanine, and away from acetate (and hydrogen) (van Niel et al. 2003; Soboh et al. 2004). This was indeed observed for strain AK_1, when the strain was cultivated under different L-G phase ratios. At a low L-G phase, the ratio between ethanol and acetate formation was 1.65 but increased to 6.27 at the highest L-G phase used. This was also reflected in much lower hydrogen yields at the high L-G phase ratio. Ethanol yields at high L-G phase were 1.7 mol ethanol mol $glucose^{-1}$, which is among the highest yields obtained by thermophilic bacteria.

One of the main aims of this study was to investigate the ethanol production potential from lignocellulosic biomass available in Iceland. Thus, various hydrolysates (HL) were made of agricultural residues and waste material, e.g. grass, hemp, paper and straw. The

concentration chosen was 5 g L^{-1} and cellulose was used as the control "biomass". The theoretical amount of glucose after enzymatic hydrolysis of cellulose is 30.9 mM. This is indeed very close to the concentration of glucose analysed after hydrolysis, indicating successful activity of both the cellulase and the β-glucosidase used (Table 1). After fermentation of cellulose HL, some glucose (5.3 to 6.1 mM) remained in the fermentation broth and incomplete glucose degradation occurred. This inability to degrade all glucose present in the hydrolysate is in good correlation with results on different initial glucose concentrations (Figure 3) where only 68% of the glucose was degraded when the initial glucose concentration was 30 mM. The strain produced 8.1 to 8.6 mM ethanol g^{-1} glucose equivalent degraded or 7.3 to 7.4 mM ethanol g^{-1} cellulose. Not surprisingly, chemical pretreatment did not enhance end product formation from cellulose HL since no hemicellulose and lignin were present in this type of substrate. To increase yields of ethanol from lignocellulosic biomass, various chemical pretreatment methods have been developed. Most commonly, either dilute acid or alkali pretreatments are used (Moiser et al. 2005). Clearly, the chemical pretreatment increased ethanol yields significantly on the various types of biomass used in the present investigation. The highest ethanol yields observed were on hemp stem (with acid and alkali), or 3.4 mM ethanol g^{-1} dw of HL. This was considerably higher than what was found for *Thermoanerobacterium* AK_{17}, which produced between 2.5 and 2.6 mM ethanol g^{-1} hemp stem (Almarsdottir et al. 2012). The highest ethanol yields reported from lignocellulosic biomass are by *Thermoanaerobacter* BG1L1 in continuous cultures on wet-exploited wheat straw hydrolysates (Georgieva et al. 2008) and corn stover pretreated with dilute sulphuric acid (Georgieva & Ahring 2007) or 8.5 to 9.2 mM g^{-1} sugar consumed. Other strains that show good ethanol yields are *Clostridium thermocellum* on paddy straw, sorghum stover and corn stubs (Rani et al. 1998) with 4.6 to 8.1 mM ethanol g^{-1} and *Thermoanerobacterium* AK_{17} on grass (Almarsdottir et al., 2012) with 5.5 mM ethanol g^{-1}.

CONCLUSION

Ethanol production capacity by strain AK_1 was studied. The strain produces maximally1.72 mol ethanol $glucose^{-1}$, and has a relatively broad substrate spectrum, degrading various hexoses, xylose, disaccharides and polymeric substrates. Ethanol production can be enhanced considerably by cultivating the organism under high partial pressure of hydrogen. The main drawback of the strain is its relatively low tolerance to substrate (glucose) concentrations. This limitation can be solved, however, by cultivating the strain in fed-batch or continuous culture. Finally, the strain produced up to 3.1 and 3.5 mM ethanol g^{-1} on chemically pretreated grass and hemp stem hydrolysates, respectively corresponding to about 200 L of ethanol from one ton of this type of biomasses.

ACKNOWLEDGEMENTS

This work was sponsored by RANNÍS, (Technology Development Fund, projects 081303408 (BioEthanol) and RAN091016-2376 (BioFuel), The Nordic Energy Fund (NER; project BioH2, 06-Hydr-C13), The Research Fund of the University of Akureyri and The KEA fund.

REFERENCES

Ahring BK, Jensen K, Nielsen P, Bjerre AB & Schmidt AS 1996. Pretreatment of wheat straw and conversion of xylose and xylan to ethanol by thermophilic anaerobic bacteria. *Bioresource Technology* 58, 107-113.

Ahring BK, Licht D, Schmidt AS, Sommer P & Thomsen AB 1999. Production of ethanol from wet oxidised wheat straw by *Thermoanaerobacter mathranii. Bioresource Technology 68*, 3-9.

Almarsdottir AR, Gunnarson I, Tarazewicz A & Orlygsson J 2010. Hydrogen production by moderate thermophilic *Clostridium* bacterium, strain AK14 from sugars and lignocellulosic biomass. *Icelandic Agricultural Sciences* 23, 61-71.

Almarsdottir AR, Sigurbjornsdottir MA & Orlygsson J 2012. Effects of various factors on etha-

nol yields from lignocellulosic biomass by *Thermoanaerobacterium* AK17. *Biotechnology* and *Bioengineering* 109, 686-6694.

Avci A & Donmez S 2006. Effect of zinc on ethanol production by two *Thermoanaerobacter* strains. *Process Biochemistry* 41, 984-989.

Bai FW, Anderson WA & Moo-Young M 2008. Ethanol fermentation technologies from sugar and starch feedstocks. *Biotechnology Advances* 26, 89-105.

Ballesteros I, Negro MJ, Oliva JM, Cabanas A, Manzanares P & Ballesteros M. 2006. Ethanol production from steam-explosion pretreated wheat straw. *Applied Biochemistry and Biotechnology* 130, 496-508.

Ben-Bassat, A, Lamed R & Zeikus JG 1981. Ethanol-production by thermophilic bacteria – metabolic control of end product formation in *Thermoanaerobium brockii. Journal of Bacteriology* 146, 192-199.

Binod P, Sindhu R, Singhania RR, Vikram S, Devi L, Nagalakshmi S, Kurien N, Sukumaran RK & Pandev A 2010. Bioethanol production from rice straw: An overview. *Bioresource Technology* 101, 4767-4774.

Fardeau ML, Faudon C, Cayol JL, Magot M, Patel BKC & Ollivier B 1996. Effect of thiosulphate as electron acceptor on glucose and xylose oxidation by *Thermoanaerobacter finnii* and a *Thermoanaerobacter* sp isolated from oil field water. *Research in Microbiology* 147, 159-165.

Georgieva TI & Ahring BK 2007. Evaluation of continuous ethanol fermentation of dilute-acid corn stover hydrolysate using thermophilic anaerobic bacterium *Thermoanaerobacter* BG1L1. *Applied Microbiology and Biotechnology* 77, 61-68.

Georgieva TI, Mikkelsen MJ & Ahring BK 2008. Ethanol production from wet-exploded wheat straw hydrolysate by thermophilic anaerobic bacterium Thermoanaerobacter BG1L1 in a continuous immobilized reactor. *Applied Biochemistry and Biotechnology* 145, 99-110.

Hawkes FR, Dinsdale R, Hawkes DL & Hussy I 2002. Sustainable fermentative hydrogen production: Challenges for process optimisation. *International Journal of Hydrogen Energy* 27, 1339-1347.

Klinke HB, Thomsen AB & Ahring BK 2001. Potential inhibitors from wet oxidation of wheat straw and their effect on growth and ethanol production by *Thermoanaerobacter mathranii. Applied Microbiology and Biotechnology* 57, 631-638.

Lacis LS & Lawford HG 1988. Ethanol production from xylose by *Thermoanaerobacter ethanolicus* in batch and continuous culture. *Archives of Microbiology* 150, 48-55.

Lamed R & Zeikus JG 1980. Ethanol-production by thermophilic bacteria - Relationship between fermentation product yields of and catabolic enzyme-activities in *Clostridium thermocellum and Thermoanaerobium brockii. Journal of Bacteriology* 144, 569-578.

Laurentin A & Edwards CA 2003. A microtiter modification of the anthrone-sulfuric acid colorimetric assay for glucose-based carbohydrates. *Analytical Biochemistry* 315, 143-145.

Lin C-W, Wu C-H, Tran D-T, Shih M-C, Li W-H & Wu C-F 2010. Mixed culture fermentation from lignocellulosic materials using thermophilic lignocellulose-degrading anaerobes. *Process Biochemistry* 46, 489-493.

Lovitt RW, Longin R & Zeikus JG 1984. Ethanol production by thermophilic bacteria: Physiological comparison of solvent effects on parent and alcohol-tolerant strains of *Clostridium thermohydrosulfuricum. Applied and Environmental Microbiology* 48, 171-177.

Lynd LR, Grethlein HE & Wolkin RH 1989. Fermentation of cellulosic substrates in batch and continuous culture by *Clostridium thermocellum. Applied and Environmental Microbiology* 55, 3131-3139.

Moiser N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M & Ladisch M 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology* 96, 673-686.

Orlygsson J & Baldursson SRB 2007. Phylogenetic and physiological studies of four hydrogen-producing thermoanaerobes from Icelandic geothermal areas. *Icelandic Agricultural Sciences* 20, 93-106.

Rani KS, Swamy MV & Seenayya G 1998. Production of ethanol from various pure and natural cellulosic biomass by *Clostridium thermocellum* strains SS21 and SS22. *Process Biochemistry* 33, 435-440.

Renewable Fuels Association. 2011. Choose Ethanol. Accessed 26.04.2012 at: http://chooseethanol.com/what-is-ethanol/entry/ethanol-at-a-glance/

Sarkar N, Gosh SK, Bannerjee S & Aikat K. 2012. Bioethanol production from agricultural

waste: An overview. *Renewable Energy* 37, 19-27.

Sigurbjornsdottir MA & Orlygsson J 2012. Combined hydrogen and ethanol production from sugars and lignocellulosic biomass by *Thermoanaerobacterium* AK54, isolated from hot spring. *Applied Energy*. Doi: 10/1016/j.apenergy.2011.11.035.

Soboh B, Linder D & Hedderich RA 2004. A multisubunit membrane-bound (NiFe) hydrogenase and an NADH-dependent Fe-only hydrogenase in the fermenting bacterium *Thermoanaerobacter tengcongensis*. *Microbiology* 150, 2451-63.

Sommer P, Georgieva T & Ahring BK 2004. Potential for using thermophilic anaerobic bacteria for bioethanol production from hemicellulose. *Biochemical Society Transactions* 32, 283-289.

Sveinsdottir M, Beck SRB & Orlygsson J 2009. Ethanol production from monosugars and lignocellulosic biomass by thermophilic bacteria isolated from Icelandic hot springs. *Icelandic Agricultural Sciences* 22, 45-58.

Taylor MP, Eley KL, Martin S, Tuffin MI, Burton SG & Cowan DA 2009. Thermophilic ethanologenesis: future prospects for second-generation bioethanol production. *Trends in Biotechnology* 27, 398-405.

van Niel EW, Claassen PA & Stams AJM 2003. Substrate and product inhibition of hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus. Biotechnology and Bioengineering* 81, 255-262.

Wiegel J & Ljungdahl LG 1981. *Thermoanaerobacter ethanolicus* gen. nov., spec. nov., a new, extreme thermophilic, anaerobic bacterium. *Archives of Microbiology* 128, 343-348.

Wiegel J, Carreira LH, Mothershed CP & Puls J 1983. Production of ethanol from bio-polymers by anaerobic, thermophilic, and extreme thermophilic bacteria. II. *Thermoanaerobacter ethanolicus* JW200 and its mutants in batch cultures and resting cell experiments. *Biotechnology and Bioengineering* 13: 193-205.

Zaldivar J, Nielsen J & Olsson L 2001. Fuel ethanol production from lignocellulose: A challenge for metabolic engineering and process integration. *Applied Microbiology and Biotechnology* 56, 17-34.

Manuscript received 5 March 2012
Accepted 2 May 2012