Hydrogen production from sugars and complex biomass by *Clostridium* species, AK_{14}, isolated from Icelandic hot spring

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ABSTRACT

The hydrogen production ability of strain AK_{14} a moderate thermophilic bacterium was studied. According to 16S rRNA gene sequence analysis the strain belongs to genus *Clostridium* but is most closely related to *Anaerobacter polyendosporus* (95.1% similarity). Growth of strain AK_{14} was observed at temperatures between 42 and 52°C with optimal growth at 50°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 acetate and butyrate (major) and ethanol (minor) as well as hydrogen and carbon dioxide. Effect of increased substrate (glucose) concentration was investigated and good correlation was observed between increased substrate loadings and end product formation up to 50 mM. At ≥ 50 mM glucose concentrations, end product formation levelled off and the substrate was not completely degraded. Also the pH at the end of fermentation dropped from 7.0 (control without glucose) to 5.3 (at ≥ 50 mM glucose). The ability to utilize various carbon substrates was tested, with positive growth observed on xylose, glucose, fructose, mannose, galactose, starch and xylan. The end products in all cases were the same as for glucose. By varying the liquid to gas phase during glucose fermentation under batch conditions a clear correlation was found between increased acetate, butyrate and hydrogen production at low gas-to-liquid conditions and less ethanol. Amount and identification of the end product formation from hydrolysates made of lignocellulosic (5.0 g L^{-1} [dw]) biomass cellulose (from Whatman paper), newspaper, grass (*Phleum pratense*), barley straw (*Hordeum vulgare*), and hemp (stem and leaves of *Cannabis sativa*), was investigated. The biomass was pretreated with either a weak acid (HCl) or a weak base (NaOH) as well as enzymes (Celluclast® and Novozyme 188). The strain produced the most hydrogen (8.5 mol H_2 g^{-1} VS) from cellulose hydrolysate but less from paper and lignocellulosic biomasses (between 0.26 to 3.60 mol H_2 g^{-1} VS). The hydrogen production from lignocellulosic biomass was however enhanced significantly by acid and base pretreatment with the highest production from grass (6.23 mol H_2 g^{-1} VS). Other end products were acetate, butyrate and ethanol.

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

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Framleiðsla á vetni úr sykrum og flóknum lífmassa með Clostridium tegund, AK_{14}, einangraðri úr íslenskum hver

Vetnisframleiðsluhæfileiki bakteríustofnsins AK_{14} sem er hitakær baktería einangruð úr íslenskum hver var rannsökuð. Skyldleikarannsókn með 16S rRNA greiningu leiddi í ljós að stofninn tilheyrir ættkvísl *Clostridium* en var skyldust *Anaerobacter polyendosporus* (95.1% skyldleiki). Stofninn vex við hitastig á milli 42 til 52°C en hámarksvöxtur var við 50°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 ediksýru og smjörsýru auk vetnis en einnig var smávægileg framleiðsla af etanóli. Áhrif mismunandi upphafsstyrks af glúkósa á móti framleiðslu lokaafurða var rannsakaður hjá bakteríunni. Við lágan upphafsstyrk glúkósa voru bein tengsl á milli aukins styrks hvarfefnis og myndefna, þ.e. aukning á glúkósa leiddi til línulegrar aukningar á lokaafurðum. Hins vegar þegar styrkur glúkósa náði 50 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 7.0 án glúkósa og fór niður í 5.3 við 50 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, sterkju og xýlan. Lokaafurðir við niðurbrot þessara hvarfefna var í öllum tilfellum ediksýra, smjörsýra, etanól, vetni og koltvísýringur. Með því að nota mismunandi hlutfall á milli gas og vökvafasa við niðurbrot á glúkósa kom í ljós að styrkur ediksýru, smjörsýru og vetnis var mestur þegar gasfasinn var hlutfallslega stór miðað við vökvaræktina en minna var framleitt af etanóli. Vöxtur var einnig athugaður í 0.5% (wv^{-1}) „hýdrólýsötum“ sem gerð voru 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. Mest vetni framleiddi stofninn úr sellulósa (8.5 mól H_2 g^{-1} VS) en mun minna úr öðrum lífmassa (0.26 to 3.60 mol H_2 g^{-1} VS). Hins vegar var hægt að auka þessa framleiðslu til muna með því að formeðhöndla lífmassann og hæsta gildið fékkst úr vallarfoxgrasi eða 6.23 mol H_2 g^{-1} VS. Aðrar lokaafurðir voru þær sömu og áður, þ.e. ediksýra, smjörsýra og etanól.

INTRODUCTION

Depletion of fossil fuel reserves together with global climate changes caused by increase of CO_2 in the atmosphere are leading to the search for alternative, renewable, carbon-free energy sources (Rittmann 2008). Biohydrogen has a great potential as a clean renewable energy carrier since it has a high energy content, producing water as the sole end product after combustion (Das & Veziroglu 2001). Additionally molecular hydrogen has a high energy yield (120 MJ kg^{-1}) which is almost three times higher than most hydrocarbon fuels (Kapdan & Kargi 2006). 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 an interesting alternative for the production of biohydrogen (Balat et al. 2008). Hydrogen can be produced microbiologically through fermentation from various starch- and sugar-based materials (Hawkes et al. 2002), including lignocellulosic biomass. The thermal pretreatment of lignocellulosic biomass most often results in two main streams of substrates, i.e. a solid fraction consisting of cellulose (hexose; glucose) (Klinke et al. 2002) and a liquid phase (hydrolysate) mainly consisting of hemicellulose (pentose; xylose and arabinose) (Bercier et al. 2007). Producing biofuels from lignocellulosic biomass economically requires that all the main carbohydrates are converted to high hydrogen production through fermentation routes (Olsson & Hahn-Hagerdahl 1996). Hydrogen production by fermentation has been receiving increased attention and high production rates have been achieved in bacterial fermentations (Wu et al. 2006, Koskinen et al. 2008a). Thermophiles have many advantages compared to mesophilic microorganisms in hydrogen production because of their high growth rates and their ability to degrade a broad range of substrates and their high hydro-

gen yields (Sommer et al. 2004, van Groenestijn et al. 2002, Zeidan & van Niel 2010). Additionally, high temperature favours the kinetics and stoichiometry of hydrogen-producing reactions (van Groenestijn et al. 2002, Wang & Wan 2009). With increasing temperatures, hydrogen-producing metabolism becomes thermodynamically more favourable (Stams 1994) and less affected by the partial pressure of hydrogen in the liquid phase (van Gooenestijn et al. 2002). Additionally, many thermophiles have a narrower spectrum of fermentation end products compared to mesophiles (van Niel et al. 2003).

Hydrogen production from carbohydrates is considered to be produced mainly through acetate and butyrate production:

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

$$C_6H_{12}O_6 + 2\,H_2O \rightarrow CH_3CH_2CH_2COO^- + 2H_2 + 2HCO_3^- + 3H^+ \quad (2)$$

Other end products common in fermentation are ethanol and lactate, which both result in no hydrogen production (Thauer et al. 1977, Wiegel 1980). Thus, the most feasable fermentation of carbohydrates is towards acetate and butyrate and not to reduced electron scavenging products like ethanol and lactate. The highest yields observed for hydrogen production are from the extreme thermophile *Caldicellulosiruptor saccharolyticus* under gas sparging (de Vrije et al. 2007). The strain produced only acetate and H_2 + CO_2 with hydrogen yields of 3.6 mol-H_2 mol-glucose^{-1}. *Thermotoga elfii* has been reported to produce 3.3 mol-H_2 mol-glucose^{-1} (van Niel et al. 2002) and an enrichment culture from Icelandic hot spring, 3.2 mol-H_2 mol-glucose^{-1} (Koskinen et al. 2008a).

Hot springs are a potential source for hydrogen producing microorganisms (Wiegel & Ljungdahl 1981, Sommer et al. 2004, Orlygsson & Baldursson 2007, Koskinen et al. 2008b). In this study a moderate thermophilic fermentative bacterium efficient in hydrogen and acetate/butyrate production was studied. Hydrolysates from various types of complex lignocellulosic biomass were used to test the hydrogen production performance of the bacterium. Optimal conditions for hydrogen production in terms of initial substrate concentration and the partial pressure of hydrogen (ρH_2) were investigated as well as the main substrate spectrum capacity of the strain.

MATERIALS AND METHODS

Media, isolation and strain identification

The medium (BM medium) composition and preparation was done according to Orlygsson & Baldursson (2007) and Sveinsdóttir et al. (2009). Isolation and full 16S rRNA gene sequence analysis for the strain were described earlier (Orlygsson & Baldursson 2007).

Determination of growth

Cell concentration was determined by measuring absorbance at 600 nm by a Perkin Elmer 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 duplicates and average values are reported.

Determination of pH_{opt} and T_{opt}

To determine the strain's optimum pH for growth the pH was set to various levels in the range of 3.0 to 9.0 with increments of 0.5 pH units. The experimental bottles were supplemented with acid (HCl) or base (NaOH) to adjust the pH accordingly. To determine the optimum temperature for growth the incubation temperature varied from 30°C to 60°C. For the pH optimum determination the strain was cultivated at 50°C and for the temperature optimum determination the pH was 6.5. Control samples did not contain glucose. Optimal pH and temperature were thereafter used in all experiments performed.

Effect of substrate concentration

The effect of increased glucose concentration

was tested on strain AK_{14}. Initial glucose concentration varied between 5 to 400 mM. Control samples did not contain glucose. Optical density was measured at the beginning and at the end of the incubation period (5 days) to determine the growth. Glucose, hydrogen, volatile fatty acids (VFA) and ethanol were measured as well as the final pH in the experimental bottles. Experiments were done in 120 mL serum bottles with 50 mL of liquid medium.

Substrate utilization

The ability of strain AK_{14} to utilize different substrates was tested using the BM medium (Orlygsson & Baldursson 2007) 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 which was measured at the beginning and at the end of the incubation period (5 days). Where growth was detected, hydrogen, volatile fatty acids and ethanol were analysed. Experiments were done in 23 mL serum bottles with 10 mL of liquid medium.

Effect of gas-liquid volume ratio on hydrogen production

The influence of partial hydrogen pressure (ρH_2) on hydrogen production was investigated with different ratios of liquid and gas phases on glucose (20 mM). The liquid phase varied from 2 to 90 mL in serum bottles with a total volume of 120 mL; thus, the liquid/gas volume ratio varied from 0.017 to 3.00. After 5 days of incubation, glucose content and the end product formation (hydrogen, acetate, butyrate and ethanol) were measured.

Pretreatment of biomass and hydrolysate preparation

Hydrolysates (HL) were made from different biomasses: Whatman filter paper (cellulose), hemp (*Cannabis sativa*) - leaves and stem fibres (Fedora cultivar), newspaper with ink (NPi), barley straw (BS) (*Hordeum vulgare*) and grass (*Phleum pratense*). Whatman paper consists of 99% cellulose and was therefore used as a control sample. The preparation of the hydrolysates was according to Sveinsdottir et al (2009) yielding a final dry weight of 25 g L^{-1}. Chemical pretreatment in the form of acid (0.75% H_2SO_4) or base (0.75% 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® and Novozyme 188 (1 mL of each; 0.25% vol/vol), and incubated in a water bath at 45°C for 68h. After the enzyme treatment the pH was measured again and adjusted to the pH optimum of the strain. The solutions were then filtered into sterile bottles to collect the hydrolysates.

Fermentation of hydrolysates

Fermentation of carbohydrates present in hydrolysates by strain AK_{14} 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. The concentration of salts, vitamins and trace elements was kept the same as in the medium without any addition of hydrolysate.

Analytical methods

Hydrogen, ethanol and volatile fatty acids were measured by gas chromatograph as previously described (Orlygsson & Baldursson 2007). Glucose was determined by injecting 20 µL samples filtered through a 0.2 µm PTFE filter onto a Shimidzu HPLC equipped with a Thermo Hypersil Gold aQ reverse phase

column (4.6 x 250 mm), RID-10A Refractive Index Detector, a SPD-20A UV-Vis detector at 190 nm and a LC-20AD solvent pump delivering 50 mM NaH_2PO_4 at a flow rate of 1 mL min^{-1}. Glucose was also analysed by 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 AND DISCUSSION

Hydrogen- and ethanol-producing bacteria have gained much attention in recent years because of increased interest in renewable energy sources. High yields of hydrogen from sugar fermentation have been reported using the extremophiles *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii*, e.g. 3.3 to 3.6 mol-H_2 mol-hexose^{-1} (van Niel et al. 2002, de Vrije et al. 2007) and an enrichment culture from Icelandic hot springs, 3.2 mol-H_2 mol-glucose^{-1} in a semi-continuous batch reactor (Koskinen et al. 2008a).

Identification and phenotypic characteristics

The isolates had a rod shape, with a length from 0.5 to 2.5 µm. The cells had a rounded end and did not produce spores. They occurred singly or in pairs, though rarely in chains. The cells stained gram-positive. 16S rRNA gene sequence analysis revealed that strain AK_{14} is a member of the genus *Clostridium* Cluster I (Orlygsson & Baldursson 2007). The closest phylogenetic relative was *Anaerobacter polyendosporus* with 95.1% similarity of the 1425 bp analysed. Other species that were most closely related were *Clostridium bovipelis* (93.7%), *Clostridium tertium* (93.0%), *Clostridium sartagoforme* (92.9%), *Clostridium putrefaciens* (92.2%) and *Clostridium disporicum* (92.0%). All these species are anaerobic mesophiles fermenting sugars and/or amino acids. *Anaerobacter polyendosporus* and *Clostridium disporicum* produce five and two spores, respectively (Duda et al. 1987, Horn 1987), but no spores have been detected from strain AK_{14} under any of the growth conditions tested. The most closely thermophilic bacterium related to AK_{14} is *Clostridium thermobutyricum* (89.9%) isolated from horse manure and which produces butyrate from sugar fermentation as the main end product (Wiegel et al. 1989).

The strain was isolated from Grensdalur in SW-Iceland (Orlygsson & Baldursson 2007). The temperature of the hot spring it originated from was 51.0°C and the pH 7.8. The strain grows in a very narrow temperature range (42 to 52°C), with optimal growth at 50°C (generation time 0.54h). Optimum pH for growth was pH 6.5 (generation time; 0.56h), though growth was observed from pH 4.5 to 7.5.

End product formation from glucose and xylose

The phenotypic properties of AK_{14} were consistent with those of many saccharolytic *Clostridium* species capable of degrading various carbohydrates to volatile fatty acids, ethanol and H_2 and CO_2. End product formation from glucose and xylose (20 mM each) resulted in the production of acetate and butyrate (as main products) and ethanol (minor product) together with H_2 and CO_2:

20 mM Glucose → 2.8 mM EtOH + 7.5 mM Acetate + 10.7 mM Butyrate + 30.5 mM H_2 + 31.7 mM CO_2

20 mM Xylose → 0.0 mM EtOH + 5.9 mM Acetate + 10.9 mM Butyratre + 35.3 mM H_2 + 27.7 mM CO_2

The carbon recovery on glucose and xylose were 79.3 and 83.1%, respectively. Lactate was not analysed in this study but has been shown to be a minor product from glucose fermentation by AK_{14} (Orlygsson & Baldursson 2007). Additionally, some of the substrate is converted into biomass but Kim et al. (2006) have reported that this value is approximately 11%.

All species in the *Clostridium* cluster I (*Clostridium* sensu stricto) produce butyrate (Collins et al. 1994, Andreesen et al. 1989).

Butyrate production is however relatively uncommon among thermophilic bacteria and has only been reported as the main product for *Clostridium thermobutyricum* (Wiegel et al. 1989, Canganella et al. 2002). The fact that AK_{14} grows at temperatures that are on the boundary between mesophilic and thermophilic perhaps explains this property of the strain. Indeed, enrichment studies from various geothermal areas in Iceland, ranging in temperatures from 50 to 75°C, revealed that butyrate production was common only from the lower (50°C) temperatures enrichments (Orlygsson et al. 2010).

Carbon source utilization pattern

One of the major reasons for using thermophilic bacteria for hydrogen production from lignocellulosic material is their ability to degrade a broad range of carbohydrates present in the biomass. AK_{14} grew on various types of carbohydrates as the sole carbon and energy source (Table 1). Of the three tested pentoses this strain only degraded xylose but all four hexoses tested (glucose, frucose, galactose, mannose) were degraded as well as the disaccharide sucrose thought not lactose. Additionally, the strain degraded starch and xylan. All of the other substrates tested were not degraded. The end products formed on all substrates were, as for glucose and xylose: acetate, butyrate, H_2 + CO_2 (major) and ethanol (minor).

Effect of environmental parameters on fermentation

Effect of partial pressure of hydrogen

The change in the partial hydrogen pressure (ρH_2) is known to affect the end product formation by anaerobic bacteria such that at a high ρH_2 more reduced products like ethanol and lactate are formed rather than acetate, butyrate and H_2 (Nath & Das 2004, Hawkes et al. 2002). This was indeed observed for strain AK_{14} in a previous study (Orlygsson & Baldursson 2007) where the theoretical yield of hydrogen increased from 38 to 55% when the liquid/gas volume ratio was changed from 3 to 0.02 (Figure 1). 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 (Nandi & Sengupta 1998, 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 1.6 to 2.6 mol-H_2 mol glucose^{-1} (40 to 65% of theoretical yield). As expected, lower H_2 yields followed the decrease in acetate and butyrate formation as against an increase in ethanol production (Figure 1).

Table 1. End product formation from various substrates by strain AK_{14}. Values represent means of two replicates.

Substrates	**End product formation (mmol L^{-1})**			
	Ethanol	**Acetate**	**Butyrate**	**Hydrogen**
Yeast extract	1.6	1.8	1.2	1.2
Xylose	0.7	6.7	11.2	51.1
Glucose	3.2	9.2	10.8	44.8
Fructose	2.3	8.2	11.8	39.0
Galactose	1.0	9.5	13.1	31.2
Mannose	0.6	8.9	14.1	28.2
Sucrose	3.1	10.8	16.2	46.0
Starch	1.6	5.5	8.6	22.3
Xylan	1.0	2.8	2.3	3.9

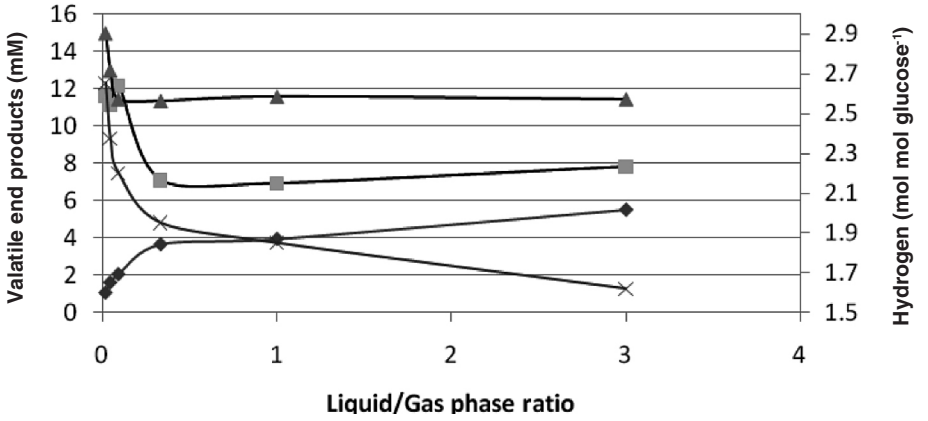

Figure 1. Effect of liquid/gas volume ratio on end product formation for strain AK_{14}. Ethanol (◆), Acetate (■), Butyrate (▲) and Hydrogen (×).

Using the fermentation data from the lowest and highest L/G ratios the following equations are observed:

1.0 Glucose → 0.06 EtOH + 0.59 Acetate + 0.75 Butyrate + 2.60 H_2 + 2.15 CO_2
(low L/G (0.02) ; Eq.1)

1.0 Glucose → 0.28 EtOH + 0.39 Acetate + 0.58 Butyrate + 1.60 H_2 + 1.63 CO_2
(high L/G (3.0) ; Eq.2)

It has been reported that butyrate to acetate (B/A) ratios are directly proportional to H_2 yields (Kim et al. 2006). The B/A ratios observed in batch cultures with different L/G ratios ranged from 1.3 to 1.5 on a molar basis. Therefore, in this study, 1.4 was used to characterize the stoichiometric reaction for the production of H_2 from glucose. This would result in a theoretical hydrogen yield of 2.36 mol-H_2 mol-glucose^{-1}. This is in good agreement with the obtained result and fits between the results obtained in eq. 1 and eq. 2.

Effect of initial substrate concentrations

High initial substrate concentration may play an important role in hydrogen production rates and yields (Kumar & Das 2001, Lacis & Lawford 1988, van Ginkel & Sung 2001, Sommer et al. 2004). This has been investigated for many bacteria, e.g. *Citrobacter* species where glucose loadings of 1, 5 and 25 g L^{-1} resulted in 2.5, 1.2 and 0.8 mol-H_2 mol-glucose^{-1} degraded (Oh et al. 2003). However, fermenting microorganisms can also have limited tolerance to increased substrate loadings (van Ginkel & Logan 2005, Olsson & Hahn-Hägerdal 1996). This was clearly observed in the present study when strain AK_{14} was cultivated at different initial concentrations of glucose varying from 5 to 400 mM (Table 2). More than 94% of the glucose was degraded at low (5, 10 and 30 mM) concentrations but much less at higher (≥ 50 mM) concentrations. This is 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 7.0) to high (≥ 50mM) loadings (pH 5.2 to 5.3). This, together with the fact that end product formation levels off at high substrate loadings, indicates that this inhibition is more likely to be caused by the low pH rather than the high substrate loadings.

End product formation from hydrolysates

Hydrogen production from lignocellulosic bio-

Table 2. End product formation from different initial glucose concentrations. Also shown are glucose conentrations after fermentation and pH values. Values represent means of two replicates.

Initial glucose (mM)	End product formation (mmol L^{-1})			End glucose (mM)	End pH
	Ethanol	Acetate	Butyrate		
0	1.6	2.4	1.0	0.0	7.0
5	3.1	3.8	1.0	0.3	6.6
10	5.0	9.8	3.1	0.3	6.4
30	6.6	12.9	5.5	0.4	5.9
50	6.7	11.5	15.8	10.3	5.2
100	5.9	10.6	20.0	55.9	5.3
200	6.3	11.3	17.2	159.5	5.3
400	7.0	14.1	16.6	357.5	5.3

Table 3. Production of end products from hydrolysates made from different biomass. Values represent mean of two replicates.

Biomass and pretreatement	End product formation (mmol L^{-1})			
	Ethanol	Acetate	Butyrate	Hydrogen
Cellulose	0.6	8.6	11.9	33.0
Cellulose - acid	1.2	6.6	10.9	29.7
Cellulose - base	0.6	8.7	11.8	32.8
Hemp Stem	0.9	4.1	4.8	13.5
Hemp Stem - acid	0.8	4.5	7.1	17.8
Hemp Stem - base	0.3	5.0	6.2	16.7
Hemp Leaf	0.1	0.2	0.7	0.0
Hemp Leaf - acid	0.4	2.8	3.3	10.0
Hemp Leaf - base	0.2	1.8	2.3	6.4
Grass	1.0	2.9	4.4	10.9
Grass - acid	1.1	5.1	8.0	22.6
Grass - base	1.2	5.9	8.5	24.2
Paper	0.1	2.5	3.2	7.2
Paper- acid	0.4	3.0	4.0	10.7
Paper - base	0.1	0.5	1.0	2.6
Straw	1.0	2.0	3.0	6.5
Straw - acid	0.9	4.8	7.2	19.6
Straw - base	1.2	5.8	8.6	20.9

mass has recently received increased attention. Several studies on thermophilic bacteria growing on untreated wastewater cellulose have shown yields between 0.82 and 1.24 moles-H_2 mol-glucose^{-1} equivalents (Magnusson et al. 2008, Liu et al. 2003). Co-culture studies of *Clostridium thermocellum* and *Thermoanaerobacterium saccharolyticum* on hydrogen production from microcrystalline cellulose resulted in 1.8 mol-H_2 mol-glucose^{-1} equivalents (Liu et al. 2008). Other studies on pretreated hydrolysates from lignocellulosic biomass have shown higher yields. Lalaurette et al. (2009) showed hydrogen yields of 1.64 mol-H_2 mol-glucose^{-1} equivalent from corn stover hydrolysates (pretreated with dilute sulphuric acid) by *Clostridium thermocellum*. Mixed culture studies (35 and 50°C) on similar biomass pretreated with steam explosion and dilute sulphuric acid resulted in 2.84 mol-H_2 mol-glucose^{-1} equivalents (Datar et al. 2007).

When strain AK_{14} was grown on different hydrolysates of biomass (cellulose, hemp leaves, hemp stem fibres, newspaper, barley straw and grass) the highest hydrogen production resulted on cellulose (33.0 mmol L^{-1}), hemp stem (13.5 mmol L^{-1}) and grass (10.9 mmol L^{-1}) when no chemical pretreatment was used (Table 3). This represents 8.5, 3.5 and 2.9 mol H_2 g VS^{-1} of the corresponding biomass. Fermentation of hydrolysates from other biomass types resulted in lower hydrogen production. The volatile end products were, as observed on monosugars, acetate and butyrate (major end products) and ethanol (minor). Acid and base pretreatment did not enhance end product formation for cellulose hydrolysates, as was observed on other hydrolysate biomass types. This was mostly profound for the lignocellulosic biomass but less for paper. The hydrogen production from grass and straw was two and three times higher, respectively, with chemical pretreatment as compared with the untreated sample. A similar increase was observed on hemp leaves but less on hemp stems. In general there was little difference between using a base or an acid as pretreatment. Base pretreatment of grass and straw gave a slightly higher production but less for hemp and paper.

Glucose was analysed in the cellulose hydrolysate (without chemical treatment) after fermentation and measured 3.5 mM. Assuming that all glucose is released from pure cellulose during hydrolysis, an initial glucose concentration of 30.9 mM would be available for fermentation. Thus, assuming that 27.4 mM of glucose were degraded the end product formation can be compared to the pure glucose fermentation spectrum described earlier. The hydrolysate experiment was done with an L/G ratio of 0.76 compared to an L/G ratio of 1.00 (data from Figure 1). The stochiometry for pure glucose and the cellulose hydrolysate (HL) experiments are:

1.00 Glucose → 0.20 EtOH + 0.35 Acetate + 0.58 Butyrate + 1.60 H_2 + 1.84 CO_2 (glucose)

1.00 Glucose → 0.09 EtOH + 0.36 Acetate + 0.45 Butyrate + 1.78 H_2 + 1.35 CO_2 (HL)

The end product formation in the cellulose hydrolysate experiment was slightly higher except for ethanol and carbon recovery was 80%. The hydrogen yield on cellulose hydrolysate was 1.39 mol-H_2 mol-glucose^{-1} equivalent. This was higher than the results in many of the above mentioned studies (Liu et al. 2008, Lalaurette et al. 2009), which makes strain AK_{14} an interesting alternative as a hydrogen producer from lignocellulosic biomass. The lower hydrogen values obtained on other hydrolysates can be explained by the lignin content (not measured) or by inefficient pentose fermentation that was released from the hydrolysis of the hemicellulose fraction of the lignocelluloses.

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