Thermophilic hydrogen production from cellulose with rumen fluid enrichment cultures: effects of different heat treatments

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Abstract

Elevated temperatures (52, 60 and 65°C) were used to enrich hydrogen producers on cellulose from cow rumen fluid. Methanogens were inhibited with two different heat treatments. Hydrogen production was considerable at 60ºC with the highest H_2 yield of 0.44 mol-H_2 mol-hexose^{-1} (1.93 mol-H_2 mol-hexose-degraded^{-1}) as obtained without heat treatment and with acetate and ethanol as the main fermentation products. H_2 production rates and yields were controlled by cellulose degradation that was at the highest 21 %. The optimum temperature and pH for H_2 production of the rumen fluid enrichment culture were 62ºC and 7.3, respectively. The enrichments at 52 and 60ºC contained mainly bacteria from *Clostridia* family. At 52ºC, the bacterial diversity was

larger and was not affected by heat treatments. Bacterial diversity at 60ºC remained similar between heat treatments, but decreased during enrichment. At 60ºC, the dominant microorganism was *Clostridium stercorarium* subsp. *leptospartum.*

Keywords: Biohydrogen, cellulose, dark fermentation, mixed culture, thermophilic

1. Introduction

There is a need for clean, renewable energy, because of the increasing energy demand and global warming that is associated with the increasing use of fossil fuels. Renewable energy can be derived, for example, from biological production of hydrogen and methane gases. Hydrogen is considered a more valuable energy source since methane has only 42 % of the energy content of hydrogen [1] and the only side-product in the combustion of hydrogen is water [2]. Dark fermentative hydrogen production is beneficial since it results in high hydrogen production rates and it can utilize cellulosic wastes [3, 4]. Industry and agriculture generate huge amounts of cellulosic wastes and by-products that are potential energy resources [5, 6] and amenable to hydrogen production [2, 3, 7].

Anaerobic digestion is widely used for biogas production, but the same process can also be used for hydrogen production if the methanogenesis is inhibited. This can be done with different pretreatments (for a review, see [8]), from which the most common and effective pretreatment has been heat-treating the cultures at 100°C for 15 min [8]. Heat treatment kills or suppresses methanogens and selects for spore-forming, hydrogen-

producing bacteria such as *Clostridia* [3]. The composition of microorganisms can also be controlled by pH [9] and/or by temperature [10]. Hydrogen production from carbohydrates is usually associated with acetate or butyrate formation. The theoretical production maximum is 4 mol-H_2 mol-hexose^{-1} with acetate as the sole by-product, while with butyrate formation only 2 mol-H_2 mol-hexose^{-1} is produced [11]. In practice, hydrogen production through dark fermentation can only convert 30 % of the energy content of the substrate to hydrogen [7], whilst the rest is converted to volatile fatty acids (VFA) and alcohols.

Hydrogen production as well as cellulose degradation is affected by several factors, such as initial and final pH, temperature, feedback inhibition by end-products, and substrate type and concentration [12]. Decreases in pH can inhibit cellulose degradation through control of hydrogenase activity and the metabolic pathways [4]. Dark fermentation has been studied with mesophiles (30 - 40°C) and thermophiles (40 - 65°C). High temperatures have many advantages over lower ones: (i) gases have lower solubility at higher temperatures (Henry´s law), (ii) the hydrogen synthesis pathways are less affected by the partial pressure of hydrogen (pH_2) at higher temperatures [11]; (iii) the rates of chemical and enzymatic reactions are higher [13]; and (iv) high temperature waste waters could be directly used without cooling. On the other hand, elevated temperatures require higher energy input, which is acceptable if process heat is available [12] or if high temperature process wastewaters, such as forest industry wastewaters (for a review, see [6]) or food processing wastewaters [14], are used for hydrogen production.

High hydrogen yields have already been reported with thermophilic microorganisms obtained, for example, from hot spring [15], anaerobic digester sludge [16], wastewater treatment plant [17], and cow dung [18]. Cow rumen contains microorganisms that can degrade cellulose at high rate. The temperature and pH of cow rumen are 39ºC and near neutral, respectively, and the solid retention time is at the highest 72 h. Because of the low retention time methane fermentation from acetate does not occur, whilst some methane may be produced from hydrogen and carbon dioxide [19 in 20].

Our researchers have previously studied hydrogen production with thermophilic microorganisms derived from hot springs [21, 22] and from a geothermally active underground mine [23]. However, it would be more feasible to obtain hydrogen producers from local origins. The aim of this work was to enrich fermentative hydrogen producers from rumen fluid at elevated temperatures (52, 60 and 65°C). Three sequential inoculations to new medium were done to study the progress of enrichment of hydrogen producing and cellulose degrading microorganisms. Cellulose was used as substrate and the effects of different heat treatments of rumen fluid cultures on hydrogen production potential and microbial communities were examined. In addition, the effects of pH and temperature on hydrogen production potential were studied with the most efficient H_2 producing enrichment. To the authors knowledge the effect of heat pretreatment on H_2 producing microbial communities has only been reported few times [24, 25] and never for rumen fluid cultures.

2. Materials and Methods

2.1. Seed microorganisms

Rumen fluid inoculum obtained from a fistulated cow (MTT Agrofood Research Institute, Jokioinen, Finland) was enriched for fermentative hydrogen production. In the first enrichment step, the ratio of inoculum (5.7 % VS) and substrate (w/w) was set to 1:2. Prior to addition to the medium, the liquid part of rumen fluid was diluted with 1xPBS-solution (10xPBS-solution has 10.9 g L^{-1} Na_2HPO_4, 3.2 g L^{-1} $NaH_2PO_4 \cdot H_2O$, and 90 g L^{-1} NaCl).

2.2. Batch Enrichment of Thermophilic H_2-Producing Microorganisms

The microbial enrichments were incubated at 52, 60 and 65ºC. The microbial cultures were manipulated in the following ways: (i) no pretreatment (NHT), (ii) heat treatment at 80ºC for 20 minutes (HT1), and (iii) heat treatment at 100ºC for 10 minutes (HT2). Duplicate samples were pretreated and two control bottles without the addition of substrate were prepared for each temperature. The first enrichments were conducted in 25 mL anaerobic tubes with a working volume of 10 mL to achieve efficient heat treatment. The following two enrichment phases were done in 120 mL serum bottles with 50 mL working volume and with 2 % (v/v) inoculum from the previous enrichment phase. The enrichments at 52 and 60°C were incubated on a shaker (150 rpm), while the enrichments at 65°C were done under static conditions because of practical reasons.

The enrichment medium was DSMZ 144 (German Collection of Microorganisms and Cell Cultures, 2008) with the following modifications: 5 g L^{-1} cellulose (Sigmacell Cellulose, Type 20) was used instead of glucose, no tryptone was used and yeast extract concentration was decreased to 0.3 g L^{-1}. Headspace in the serum bottles was made anaerobic with N_2 and sterile cellulose, vitamin solution and $Na_2S \cdot 9\ H_2O$ were aseptically added to the medium from stock solutions after sterilization of the medium. Gas production was monitored regularly and volatile fatty acids (VFAs) and alcohols were analyzed in the end of the enrichments.

2.3. Temperature-Gradient Experiment

The temperature dependency of H_2 production by the enriched rumen fluid culture (NHT, 60ºC) was determined with a temperature-gradient incubator (Test Tube Oscillator, Terratec). The temperature gradient was set from 45 to 75ºC and duplicate samples were used. The culture was grown in 25 mL anaerobic tubes with 10 mL working volume and 10 % (v/v) inoculum. The cellulose supplementation was 2.5 g L^{-1} and mixing was at 60 oscillations min^{-1}. Gas production was measured daily and end-point volatile fatty acids and alcohols were analyzed.

2.4. Effect of pH on Hydrogen Production

The effect of pH on hydrogen production by the rumen fluid enrichment (NHT, 60°C) was studied with initial pH from 5.2 to 7.3 The pH experiment was done in triplicate in 60 mL serum bottles with 25 mL working volume and 10 % (v/v) inoculum. The pH of the medium was first adjusted with K_2HPO_4 and KH_2PO_4, and after purging the medium

with nitrogen the pH was adjusted to the desired value with HCl or NaOH. The concentration of cellulose was kept low at 1.0 g L^{-1} to prevent high acid production and the bottles were mixed (150 rpm). Gas production was analyzed five times a week, pH three times a week from one of the triplicate bottles and VFA and alcohol concentrations were determined from the end-points.

2.5. Chemical Analyses

The overpressure from the batch bottles was analyzed according to Owen et al. [26]. The gas composition (H_2, CH_4 and CO_2) in the headspace was analyzed with a Shimadzu gas chromatograph GC-2014 equipped with Porapak N column (80/100 mesh) and a thermal conductivity detector (TCD). The temperatures of oven, injector and detector were 80, 110 and 110ºC, respectively. Nitrogen was used as carrier gas at a flow rate of 20 mL min^{-1}. The gas volumes were corrected to a standard pressure (760 mm Hg) and temperature (0°C), and cumulative H_2 production was calculated according to Logan et al. [1].

The concentrations of volatile fatty acids and alcohols were analyzed with Shimadzu High Performance Liquid Chromatography (HPLC) with a Shodex Sugar SH1011 column (Showa Denko K.K., Japan) and a refraction index detector (Shimadzu). Mobile phase was 5 mM H_2SO_4 and flow rate 0.7 mL min^{-1}. Samples for HPLC were pretreated with a solid phase extraction (SPE) method modified from Horspool and McKellar [27]. The cartridge was preconditioned with 2 mL methanol and 2 mL 0.01 M HCl (pH 2), and the VFAs and alcohols were eluted from the sorbent with 1.75 mL 0.05 M PBS. Cellulose degradation was calculated based on formed COD in the end of each

enrichment phase. COD was calculated as a sum of degradation products and produced hydrogen according to Equation 1 [28].

$$COD = 8 * (4x + y – 2z) / (12x + y + 16z) \text{ g COD / g } C_xH_yO_z \quad (1)$$

2.6. Microbial Community Analyses

Bacterial communities were determined using DNA extraction and Polymerase Chain Reaction – Denaturing Gradient Gel Electrophoresis (PCR-DGGE) of partial 16S rRNA genes followed by their sequencing. Duplicate samples were taken at the end of each enrichment phase and stored at -20°C. DNA was extracted with a VIOGENE Blood and Tissue Genomic DNA kit (Proteogenix SA, Fegersheim, France). Partial bacterial 16S rRNA genes were amplified using a primer pair GC-BacV3f and 907r as previously described by Koskinen et al. [29], only exception being that PCR was done with T3000 Thermocycler (Biometra).

DGGE was performed with INGENYphorU2 x 2 –system (Ingeny International BV, GP Goes, The Netherlands) as described by Koskinen et al. [29] with following exceptions: denaturing gradient from 30 % to 70 % was used, gels were run with 100 V for 22.5 h, and dominant bands were eluted in 20 µL of sterile H_2O. The re-amplification of bands for sequencing was done as described by Koskinen et al. [29]. Sequence data were analyzed with Bioedit-software (version 7.0.5) and compared with sequences in GenBank (http://www.ncb.nlm.nih.gov/blast/).

198 # 3. Results and discussion

199

200 ### *3.1. Enrichment of Rumen Fluid Culture*

201

202 Enrichment of hydrogen producers from rumen fluid culture was successful at 60°C
203 (Figure 1). At 52ºC hydrogen was produced only in the first enrichment phases (Figure
204 1), while no hydrogen was produced at 65°C. No hydrogen was produced at 65ºC. The
205 incubation under static conditions was not a likely reason for the absence of the
206 hydrogen production. In control bottles (without substrate) hydrogen was produced only
207 in the first enrichment phase, and these H_2 yields were subtracted from the yields
208 obtained with cellulose. No methane was produced in any of the enrichment phase even
209 in the NHT cultivations, which was most likely due to the decrease in pH (below 6.5)
210 during the enrichments. Most methanogens remain active at pH between 6.8 and 7.5 [2].
211 At 60°C, the cumulative hydrogen production with HT2 decreased in the second and
212 third enrichment phases. The highest hydrogen yields at 60°C with NHT and HT1
213 enrichments were 71 and 53 mL-H_2 g-cellulose^{-1} corresponding to hydrogen yields of
214 0.44 and 0.32 mol-H_2 mol-hexose^{-1} and 1.93 and 1.91 mol-H_2 mol-hexose-degraded^{-1},
215 respectively. The highest hydrogen yields were similar to the results reported for cow
216 dung microflora from cellulose at 55°C (0.45 mol-H_2 mol-hexose^{-1}) [30]. The results
217 show that the rumen fluid culture without heat treatment resulted in higher hydrogen
218 yields and hydrogen production rates than the heat treated cultures. Lin and Hung [30]
219 reported two times higher H_2 production without HT than with HT, while in this study
220 the hydrogen production was 1.3 times higher without HT.

221

222 Figure 1

The VFA and alcohol concentrations at the end of the enrichment phases (at 52 and 60°C) were as presented in Tables 1 and 2, respectively. The VFA and alcohol concentrations in control bottles (1st enrichment phase) were subtracted from the results obtained with cellulose since the rumen fluid inoculum contained also soluble cellulose degradation products. The production of total VFAs was lower at 52 than at 60ºC. During sequential enrichments at 52 and 60ºC, the total VFAs decreased and increased, respectively. In rumen fluid cultures at 52°C the soluble degradation products remained low after the 1st enrichment phase. In the 1st enrichment phase at 52ºC, the main degradation products were butyrate and lactate with NHT and HT1 cultures, and butyrate, acetate and lactate with HT2 cultivation. The low soluble metabolite concentrations and low H_2 yields likely resulted from poor degradation of cellulose (Figure 2). At 60°C, the main soluble metabolites of the NHT and HT1 enrichments were acetate and ethanol, with smaller amounts lactate. However, lactate was the main metabolite in the HT2 enrichment (60°C). Lactate production is associated with low H_2 yields [11] and therefore it was a likely reason for the lower hydrogen yields obtained with the HT2 culture.

High hydrogen yields are usually associated with a mixture of acetate and butyrate production [11]. However, negligible amounts of butyrate were present in the end of rumen fluid cultivations. In addition to acetate, ethanol was the main degradation product in NHT and HT1 enrichments (60°C). Alcohol production is also associated with lower H_2 yields since it consumes protons from hydrogen production [12]. However, ethanol is a high energy product that could be produced simultaneously with hydrogen [31, 32]. Lin and Hung [30] also reported high ethanol production with cow

dung culture at 55ºC associated with high acetate and butyrate production. Non-heat-treated culture was shown to produce more ethanol than culture with HT [30] which was in agreement with our results.

Table 1

Table 2

Cellulose degradation (Figure 2) in the end of enrichment phases was calculated by determining the initial and final COD concentrations based on the added cellulose and produced hydrogen and soluble degradation products. Cellulose degradation at 52ºC was highest in the 1st enrichment phase, after which it decreased considerably, whereas at 60ºC the cellulose degradation increased during sequential enrichment phases. The highest cellulose degradation (20.8 %) was obtained at 60ºC with NHT enrichment. However, cellulose degradation remained low in all the enrichments leading to low H_2 yields. During the enrichment cultivations the pH in the batch bottles was not adjusted and it dropped to below 6.5, which might have slowed down or inhibited cellulose degrading micoorganisms. Hu et al. [33] studied the effect of pH on cellulose degradation and reported that the highest cellulose degradation (75 %) was obtained at pH values between 6.8 and 7.3, while at lower pHs the cellulose degradation decreased considerably.

Figure 2

Furthermore, the solubilization of cellulose by rumen microorganisms has been shown to depend on the presence of sterilized, clarified rumen fluid [5, 34, 35] that provides

nutrients. When sterilized rumen fluid (20 % v/v) was added to the medium the cellulose solubilization was 97 %, while it was 78 % without the rumen fluid [35]. In addition, Caldwell and Bryant [34] tested a medium without added rumen fluid for isolation of rumen bacteria and reported that without trypticase, yeast extract and VFA mixture (that was used instead of the rumen fluid) the colony counts reduced considerably. Thus, the low hydrogen yields were likely due to the lack of some essential nutrients resulting in low solubilization of cellulose. However, it would not be feasible to add sterilized rumen fluid in large scale applications. If rumen fluid microorganisms were used for hydrogen production at 60°C, it would be feasible to degrade cellulosic substrate before feeding it into the H_2 reactor. This could be done, e.g., by using cellulolytic microorganisms in a separate reactor [36].

3.2. Microbial Communities

The effect of different heat treatments on microbial communities grown at 52 and 60°C were analyzed by PCR-DGGE followed by band sequencing (Figure 3, Table 3). The main bacteria were related to *Clostridium* sp., including *Clostridium stercorarium* subsp. *leptospartum*, *C. stercorarium* subsp. *thermolacticum*, and *Clostridium caenicola*. In addition, some uncultured *Clostridium* species, uncultured *Symbiobacterium* sp. and *Symbiobacterium thermophilum* were present both at 52 and 60°C. *Both C. stercorarium* subsp. *leptospartum* and *C. stercorarium* subsp. *thermolacticum* are thermophilic, cellulolytic microorganisms that produce acetate and ethanol as their main fermentation products. *C. stercorarium* subsp. *leptospartum* grows optimally at 60°C and at pH 7.5, while the optimal temperature and pH of *C. stercorarium* subsp. *thermolacticum* are 60 - 65°C and 7.0, respectively [37].

Furthermore, *C. stercorarium* subsp. *thermolacticum* produces hydrogen from lactose [38]. *C. caenicola* is a thermophile that produces hydrogen, lactate, acetate and ethanol from cellobiose. The optimum growth conditions of *C. caenicola* are 60°C and pH 6.5 [39].

Both 52 and 60°C enrichments had some similar bacterial species. However, hydrogen production at 52°C was negligible, while hydrogen was produced at 60°C with every heat treatment and every enrichment. At both temperatures, strong bands (2, 3, 4 and 6) representing *C. stercorarium* were present. However, the main degradation products of *C. stercorarium subsp. leptospartum*, acetate and ethanol, were only present at 60ºC suggesting reduced activity of *C. stercorarium* at 52ºC. Furthermore, *Bacillus thermozeamaize* (band 13) and *C. caenicola* (band 8) dominate at 60 and 52ºC, respectively. At 52°C some cellulose was degraded, but H_2 yields remained negligible. *B. thermozeamaize* that was present at 60°C has been isolated from light corn steep liquor. It remains unclear whether *B. thermozeamaize* was associated with cellulose degradation and/or hydrogen production.

The presence of thermophiles has been reported before in cow waste slurry [7] and undiluted cow dung [18]. Yokoyama et al. [7] reported that at 60ºC the two main hydrogen producers were *C. stercorarium* and *Clostridium thermocellum*. At 75ºC and 60ºC with undiluted cow dung hydrogen production was associated with *Caldanaerobacter subterraneus* and cellulolytic *Clostridium cellulosi*, respectively [18].

The 52°C enrichments had considerably more bands than those of 60°C. The heat treatments of 52°C cultures decreased number of bands, while at 60°C they were similar

regardless of the applied heat treatment. At 60°C, the number of bands decreased during the enrichments, which was less significant in the 52°C enrichments. Yokoyama et al. [7] suggested that thermophiles in cow waste slurry cultivated at 60 and 75ºC survived in cow rumen at 39ºC by forming spores. Thus, our results suggest that microorganisms in 60ºC enrichments consisted mainly of spore formers. This would also explain the small differences in microbial communities between different heat treatments. However, the highest H_2 yield was obtained at 60ºC with no-heat-treatment. This could be explained by metabolic differences as seen in the VFA profiles (Table 2). In NHT enrichments, acetate and butyrate were the main degradation products with small amounts of lactate. In HT1 enrichments the relative amount of lactate increased, while in the 2nd enrichment of HT2 culture lactate was the main fermentation product. Thus, the different VFA profiles suggest that although the same microorganisms were present, different species predominated after different heat treatments.

3.3. Effect of Temperature on Hydrogen Production

The effect of temperature on hydrogen production by the rumen fluid enrichment (NHT, 60ºC) was studied in the temperature range from 45 to 75°C (Figure 4). At temperatures < 50°C and > 70°C the rumen fluid enrichment did not produce hydrogen. The hydrogen production rate was highest at 62°C (0.23 mL-H_2 d^{-1}), while the highest hydrogen yield was obtained at 60ºC (0.14 mol-H_2 mol-hexose^{-1}) with a longer lag time. At 66.4°C, hydrogen production had a lag period of 5 days, while at other temperatures where hydrogen was produced the lag time was below two days. The optimum temperatures of diluted and undiluted cow dung for H_2 production have been reported to be 60 and 75ºC [7] and 60ºC [18], respectively. Both studies concluded that hydrogen

348 production significantly increased at temperatures over 50°C as it did in our study,
349 which was associated with methane production at temperatures under and at 50ºC.
350 However, no methane was produced during our experiments. Bacterial community
351 results indicate that *C. stercorarium* subsp. *leptospartum* was the main cellulose
352 degrader and H_2 producer in the 60ºC cultivations. The optimum temperature of *C.*
353 *stercorarium* subsp. *leptospartum* is 60ºC, which is very close to the optimum
354 temperature for H_2 production reported here.
355
356 Figure 4
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358 ***3.4. Effect of pH on Hydrogen Production***
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360 The effect of pH (initial pH from 5.2 to 7.3) on hydrogen production by the rumen
361 fluid enrichment was analyzed with the 60ºC NHT rumen fluid culture as it had the
362 highest H_2 yields. H_2 production was negligible at pH 6.0 and below (Table 4). At pH
363 6.4 and 6.9 the H_2 production rates were low when compared to the highest
364 H_2 production rate of 9.5 mL-H_2 d^{-1} at initial pH 7.3. In addition, the total amount of
365 soluble degradation products was highest at the highest initial pH of 7.3 showing higher
366 cellulose degradation. The amount of soluble degradation products increased with
367 increasing pH and the VFA and alcohol profiles changed considerably with the pH. At
368 pH 6.0 and below, ethanol production was predominant with some acetate and butyrate
369 production. At initial pH of 6.9 and 7.3, acetate was the main degradation product with
370 relatively high ethanol concentrations.
371
372 Table 4

The H_2 production rate was considerably higher at the highest pH. During the enrichments, H_2 yields could have been increased by adjusting the pH during the experiments since it dropped below 6.5 during the incubations. For example, Yokoyama et al. [7] reported the neutral pH optimal for hydrogen production with cow waste slurry at 60 or 75ºC. In addition, Lin and Hung [30] studied cellulose utilization at pH 7.5 with a cow dung enrichment and reported a peak hydrogen production (0.50 mol-H_2 mol-hexose^{-1}). High H_2 production in their experiment was accompanied with high ethanol production (1.0 mol-EtOH mol-hexose^{-1}). This was in disagreement with this study, where the ethanol concentrations usually decreased with increasing H_2 yields. However, during the enrichments hydrogen production was accompanied with high ethanol yields. Lin and Hung [30] also concluded that even a pH change of 0.5 units from the optimal pH decreased the hydrogen production with 20 %. In our experiments a small pH change from 7.5 to 7.0 decreased the H_2 yields by over 80 %.

We obtained considerably higher H_2 yields in the pH experiments than during enrichments. This may be because the relative amount of inoculae (10 %) was higher than during the enrichments (2 %). In addition, cellulose concentration was low (1 g L^{-1}) in pH experiments to decrease the pH changes caused by VFA production. During the enrichments the used cellulose concentration was considerably higher (5 g L^{-1}). Levin et al. [40] reported before that with increasing cellulose concentrations the H_2 yields decreased. Thus, low cellulose concentration seems to increase the H_2 yields by the rumen fluid enrichment, which might be associated with lower decrease in pH.

4. Conclusions

Thermophilic, cellulolytic, hydrogen producing microorganisms can be enriched from rumen fluid. Heat pretreatments of rumen fluid enrichments do not increase hydrogen production. The highest H_2 yields are obtained at 60ºC without heat treatment of the enrichments, while at 52ºC hydrogen production is negligible. The optimum temperature and pH of the best rumen fluid enrichment are 62ºC and 7.3, respectively. Production of soluble metabolites is highly affected by pH. At pH 6.0 and below ethanol production dominates and no hydrogen is produced, while at higher pH of 7.3 hydrogen yields are the highest with simultaneous acetate and ethanol production. Hydrogen production is mainly associated with *Clostridium* species. At 52ºC, the bacterial diversity is considerably higher than at 60ºC and is decreased with heat treatments. At 60ºC, the bacterial diversity is not dependent on heat treatments, but it decreases during the enrichments. Bacteria closely related to *C. stercorarium* subsp. *leptospartum* are mainly associated with cellulose degradation and hydrogen production at 60ºC.

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Figure captions:

Figure 1. Hydrogen production (mL-H_2 g-cellulose^{-1}) from cellulose by rumen fluid at 52°C (A) and at 60°C (B). Sphere (●): the 1st enrichment, cross (x): the 2nd enrichment, and triangle (▲): the 3rd enrichment phase.

Figure 2. Cellulose degradation at 52 and 60ºC based on formed COD after 15 (1.E), 20 (2.E) and 40 (3.E) days of incubation.

Figure 3. Bacterial community profile determined with PCR-DGGE of partial 16S rRNA genes of rumen fluid enrichments at 52 and 60°C without heat treatment (NHT) and with heat treatments at 80ºC for 20 min (HT1) and at 100ºC for 10 min (HT2). See Table 3 for the labeled bands.

Figure 4. The effect of temperature on H_2 production rate by rumen fluid enrichment culture.

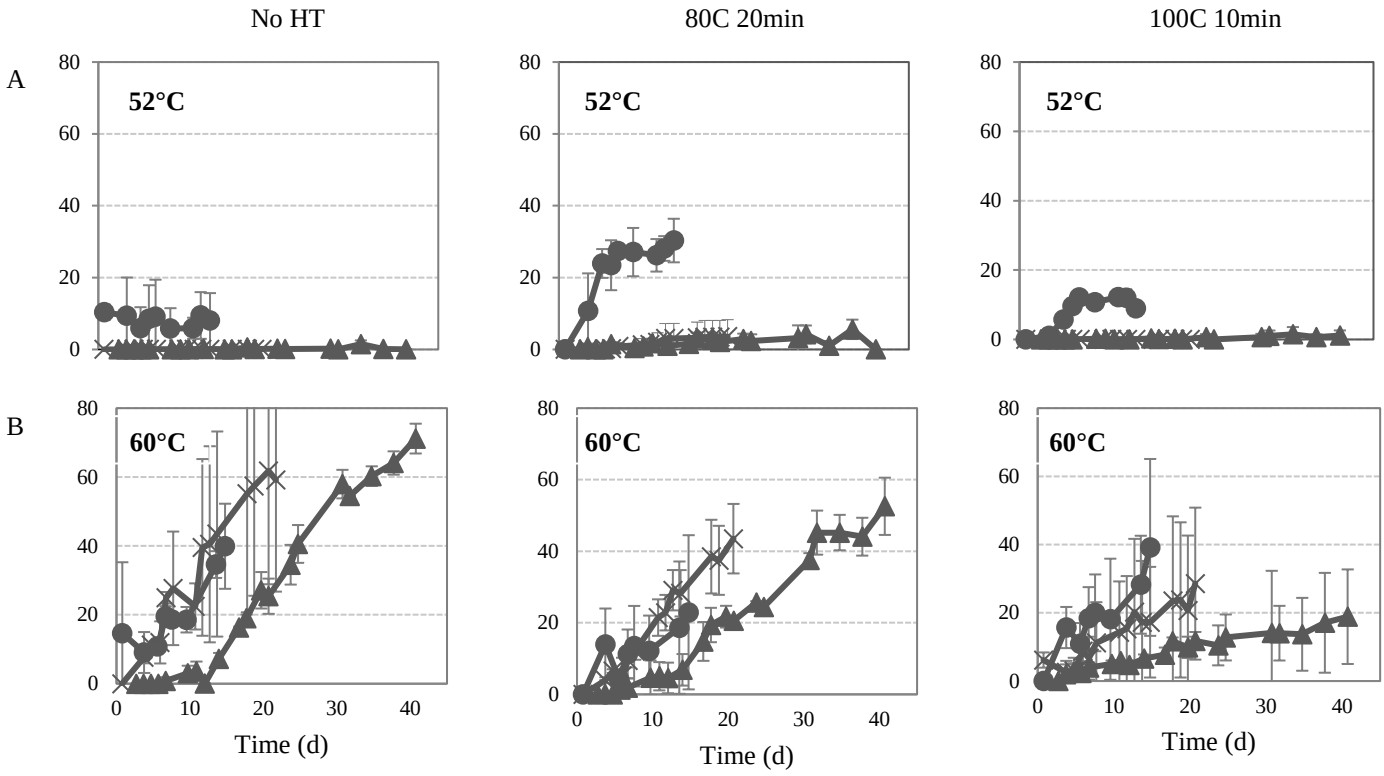
Table captions:

Table 1. Hydrogen yields and degradation products at 52ºC from cellulose by rumen fluid enrichment cultures (standard deviations in parenthesis), n.d. = not detected.

Table 2. Hydrogen yields and degradation products at 60ºC from cellulose by rumen fluid enrichment cultures (standard deviations in parenthesis), n.d. = not detected.

Table 3. Affiliation of DGGE fragments determined by their 16S rDNA sequences from rumen fluid enrichment cultures.

Table 4. VFA, alcohol and hydrogen production rates from cellulose with different initial pHs by rumen fluid enrichment cultures (standard deviations in parenthesis), n.d. = not detected.



608
609 Figure 1. Hydrogen production (mL-H_2 g-cellulose^{-1}) from cellulose by rumen fluid at 52°C
610 (A) and at 60°C (B). Sphere (●): the 1st enrichment, cross (x): the 2nd enrichment, and triangle
611 (▲): the 3rd enrichment phase.
612

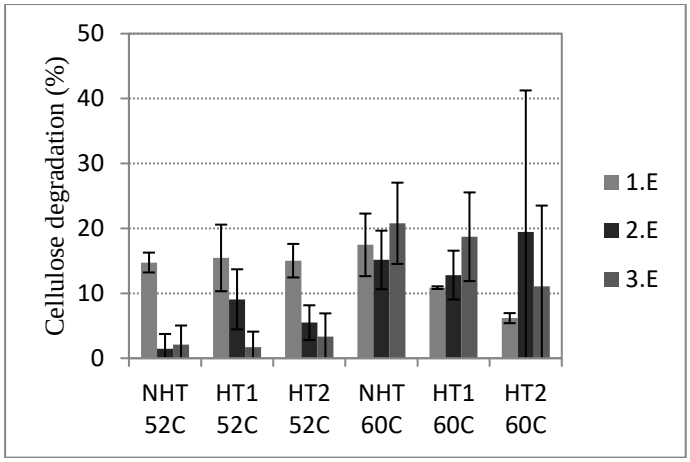


Figure 2. Cellulose degradation at 52 and 60ºC based on formed COD after 15 (1.E), 20 (2.E) and 40 (3.E) days of incubation.

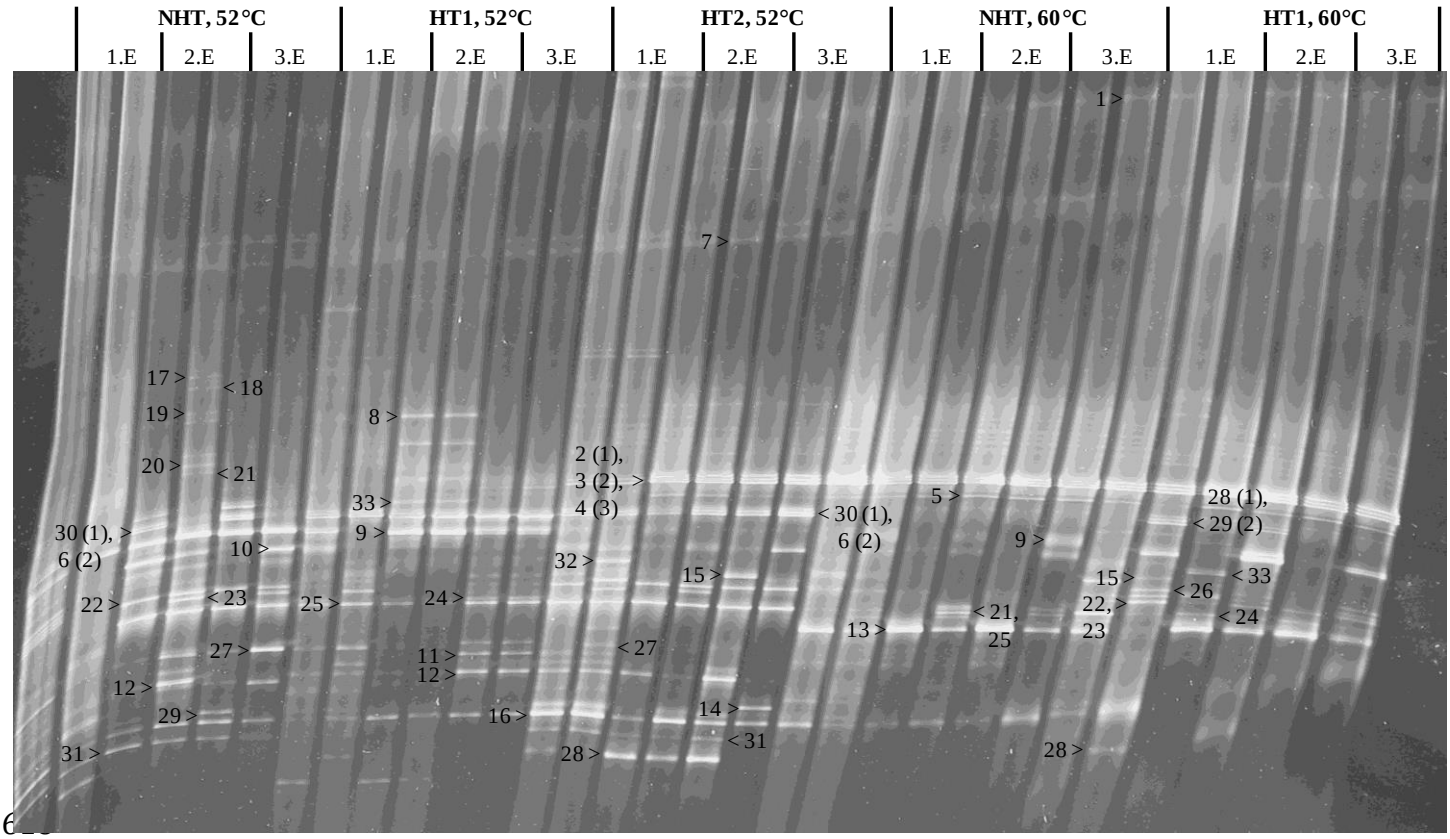


Figure 3. Bacterial community profile determined with PCR-DGGE of partial 16S rRNA genes of rumen fluid enrichments at 52 and 60°C without heat-treatment (NHT) and with heat-treatments at 80ºC for 20 min (HT1) and at 100ºC for 10 min (HT2). See Table 3 for the labeled bands.

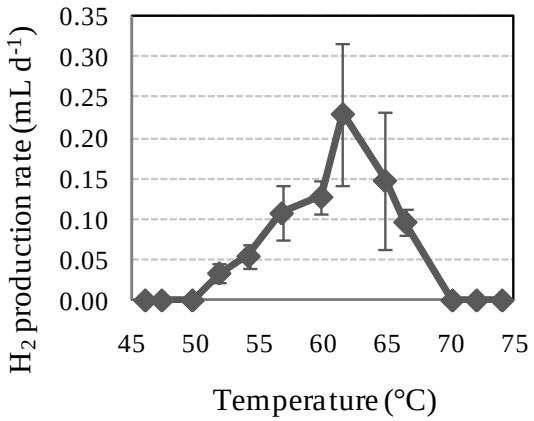


Figure 4. The effect of temperature on H_2 production rate by rumen fluid enrichment culture.

629 Table 1. Hydrogen yields and degradation products at 52ºC from cellulose by rumen fluid
630 enrichment cultures (standard deviations in parenthesis), n.d. = not detected.
631

Heat treatment	**NHT, 52°C**			**HT1, 52°C**			**HT2, 52°C**		
Enrichment phase	**1.E**	**2.E**	**3.E**	**1.E**	**2.E**	**3.E**	**1.E**	**2.E**	**3.E**
H_2 yield (mol-H_2 mol-hexose^{-1})	0.05 (0.06)	n.d.	n.d.	0.22 (0.04)	0.02 (0.03)	0.04 (0.02)	0.06 (0.03)	n.d.	n.d.
Lactate (mM)	4.54 (0.53)	0.33 (0.47)	0.39 (0.55)	3.73 (0.18)	0.67 (0.26)	0.19 (0.27)	3.29 (1.20)	0.05 (0.07)	0.86 (0.63)
Acetate (mM)	n.d.	0.35 (0.49)	1.07 (1.52)	0.33 (0.00)	1.90 (1.60)	0.97 (1.38)	2.06 (1.75)	4.04 (1.47)	1.18 (1.59)
Butyrate (mM)	2.19 (0.19)	0.15 (0.28)	0.03 (0.05)	2.98 (1.60)	0.90 (0.54)	0.06 (0.09)	1.84 (0.14)	0.18 (0.26)	0.12 (0.18)
Ethanol (mM)	n.d.	n.d.	n.d.	n.d.	1.60 (0.34)	n.d.	n.d.	n.d.	n.d.
Total VFAs (mM)	6.93	0.83	1.49	7.41	5.07	1.22	7.41	4.27	2.16

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634 Table 2. Hydrogen yields and degradation products at 60ºC from cellulose by rumen fluid
635 enrichment cultures (standard deviations in parenthesis), n.d. = not detected.
636

Heat treatment	**NHT, 60°C**			**HT1, 60°C**			**HT2, 60°C**		
Enrichment phase	**1.E**	**2.E**	**3.E**	**1.E**	**2.E**	**3.E**	**1.E**	**2.E**	**3.E**
H_2 yield	0.29	0.38	0.44	0.17	0.27	0.32	0.30	0.17	0.12
(mol-H_2 mol-hexose^{-1})	(0.03)	(0.20)	(0.03)	(0.14)	(0.06)	(0.05)	(0.22)	(0.14)	(0.09)
Lactate (mM)	0.73	1.24	1.52	3.17	1.37	2.28	2.41	8.90	0.87
	(0.00)	(0.16)	(0.85)	(0.00)	(0.34)	(0.39)	(0.43)	(10.52)	(0.00)
Acetate (mM)	4.49	4.88	6.36	1.67	5.34	4.22	n.d.	1.74	4.15
	(1.69)	(2.01)	(2.45)	(0.17)	(1.82)	(2.05)		(0.83)	(5.17)
Butyrate (mM)	3.58	0.13	n.d.	1.05	0.16	0.97	0.61	0.16	0.68
	(0.93)	(0.18)		(0.00)	(0.23)	(0.41)	(0.00)	(0.23)	(0.96)
Ethanol (mM)	n.d.	3.69	5.77	n.d.	1.91	3.67	n.d.	0.46	1.37
		(0.70)	(0.99)		(0.15)	(1.35)		(0.65)	(1.86)
Total VFAs (mM)	9.47	9.94	13.65	6.44	8.78	11.14	3.70	11.26	7.07

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639 Table 3. Affiliation of DGGE fragments determined by their 16S rDNA sequences from
640 rumen fluid enrichment cultures.
641

BL[a]	Family[b]	Affiliation (acc)[c]	Sim (%)[d]	SL (bp)[e]
1	*Clostridiaceae*	*C. stercorarium* subsp. *leptospartum* (AB572913)	97.3	488
2	*Clostridiaceae*	*C. stercorarium* subsp. *leptospartum* (AB572913)	98.1	486
3	*Clostridiaceae*	*C. stercorarium* subsp. *leptospartum* (AB572913)	100.0	436
4	*Clostridiaceae*	*C. stercorarium* subsp. *leptospartum* (AB572913)	99.4	479
5	*Clostridiaceae*	*C. stercorarium* subsp. *leptospartum* (AB572913)	98.9	447
6	*Clostridiaceae*	*C. stercorarium* subsp. *leptospartum* (AB572913)	95.6	462
7	*Clostridiaceae*	*C. stercorarium* subsp. *thermolacticum* (CLORG16SAF)	91.0	504
8	*Clostridiaceae*	*C. caenicola* (AB221372)	87.5	436
9	*Clostridiaceae*	*C. caenicola* (AB221372)	99.0	515
10	*Clostridiaceae*	*C. isatidis* strain WV6 (NR_026347)	99.8	424
11	*Bacillaceae*	*B. thermoamylovorans* strain 3LF 21P (FN666891)	99.8	454
12	*Bacillaceae*	*B. thermoamylovorans* strain 3LF 21P (FN666891)	99.6	530
13	*Bacillaceae*	*B. thermozeamaize* (AY288912)	99.4	474
14	*Thermoanaerobacteriaceae*	*T. wiegelii* strain B5 (DQ681066)	99.2	477
15	*Paenibacillaceae*	*Paenibacillus barengoltzii* strain THWCSN13 (GQ284361)	98.1	519
16	*Incertae Sedis XVIII*	*Symbiobacterium* sp. KY38 (AN361629)	100.0	503
		Symbiobacterium thermophilum IAM 14863 (AP006840)	99.0	
17	*Clostridiaceae*	*Tepidimicrobium* sp. HUT8119 (AB332034)	91.5	487
18	*Clostridiaceae*	*Tepidimicrobium* sp. HUT8119 (AB332034)	93.4	443
19	*Clostridiaceae*	*Tepidimicrobium* sp. HUT8119 (AB332034)	91.4	431
20	*Clostridiaceae*	*Tepidimicrobium* sp. HUT8119 (AB332034)	90.6	435
21	*Clostridiaceae*	*Tepidimicrobium* sp. HUT8119 (AB332034)	86.2	445
22	*Clostridiaceae*	*Tepidimicrobium* sp. HUT8119 (AB332034)	92.6	476
23	*Clostridiaceae*	*Tepidimicrobium* sp. HUT8119 (AB332034)	99.4	476
24	*Clostridiaceae*	*Clostridium* sp. enrichment culture clone 21B07 (FJ796698)	90.5	426
25	*Clostridiaceae*	*Clostridium* sp. enrichment culture clone 21B07 (FJ796698)	99.8	501
26	*Clostridiaceae*	*Clostridiales* bacterium NS5-3 (AY466716)	99.1	452
27	*Incertae Sedis XVIII*	*Symbiobacterium* sp. KY46 (AB455238)	99.3	455
28	*Incertae Sedis XVIII*	*Symbiobacterium* sp. KY38 (AB361629)	93.8	521
29	*Incertae Sedis XVIII*	Uncultured *Symbiobacterium* sp. clone MO313 (AB052391)	97.6	494
30	*Ruminococcaceae*	Uncultured bacterium, clone: OUT-B9 (AB428533)	91.7	429
31	*Incertae Sedis XVIII*	Uncultured compost bacterium clone PS2388 (FN667307)	99.4	528
32	*Incertae Sedis XI*	Uncultured bacterium clone thermophilic_alkaline-116 (GU455356)	98.4	430
33	*Lachnospiraceae*	Uncultured bacterium clone OUT-EBR-02E-0436 (AB221356)	99.7	397

642 [a] Band label in Figure 3, [b] Family according to Ribosomal Database Project II, [c] Closest species in the
643 GenBank database with an accession number, [d] Similarity (%), [e] Sequence length (bp).
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Table 4. VFA, alcohol and hydrogen production rates from cellulose with different initial pHs by rumen fluid enrichment cultures (standard deviations in parenthesis), n.d. = not detected.

Initial pH	**5.2**	**5.6**	**6.0**	**6.4**	**6.9**	**7.3**
H_2 production (mL d^{-1})	n.d.	n.d.	0.05 (0.08)	0.22 (0.14)	0.39 (0.33)	10.42 (0.73)
Lactate (mM)	0.26 (0.14)	0.31 (0.21)	0.25 (0.04)	1.68 (0.39)	0.44 (0.28)	0.92 (0.25)
Acetate (mM)	0.93 (0.09)	0.96 (0.02)	1.03 (0.77)	1.70 (1.78)	2.50 (1.79)	6.12 (2.34)
Butyrate (mM)	0.54 (0.28)	0.51 (0.27)	0.77 (0.35)	0.70 (0.38)	1.12 (0.97)	0.51 (0.35)
Ethanol (mM)	1.82 (0.07	1.93 (1.71)	1.96 (0.11)	1.91 (0.05)	1.92 (0.05)	3.14 (1.75)
Total (mM)	3.55	3.72	4.01	5.98	5.98	10.69