Effects of heat treatment on hydrogen production potential and microbial community of thermophilic compost enrichment cultures

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Abstract

Cellulosic plant and waste materials are potential resources for fermentative hydrogen production. In this study, hydrogen producing, cellulolytic cultures were enriched from compost material at 52, 60 and 70ºC. Highest cellulose degradation and highest H_2 yield were 57 % and 1.4 mol-H_2 mol-hexose^{-1} (2.4 mol-H_2 mol-hexose-degraded^{-1}), respectively, obtained at 52°C with the heat-treated (80°C for 20 min) enrichment culture. Heat-treatments as well as the sequential enrichment decreased the diversity of microbial communities. The enrichments contained mainly bacteria from families *Thermoanaerobacteriaceae* and *Clostridiaceae*, from which a bacterium closely related

to *Thermoanaerobium thermosaccharolyticum* was mainly responsible for hydrogen production and bacteria closely related to *Clostridium cellulosi* and *Clostridium stercorarium* were responsible for cellulose degradation.

Keywords: Dark fermentation, cellulose, mixed culture, thermophilic, *Thermoanaerobium thermosaccharolyticum*

1 Introduction

Increasing energy demand and global warming that is associated with the increasing use of fossil fuels increase the demand to produce clean, renewable energy. Hydrogen is considered as a good alternative to conventional fossil fuels because its energy content (122 MJ kg^{-1}) is high (Logan et al., 2002) and because hydrogen results in a cleaner combustion as the only side-product is water (Zhu et al., 2008). The ability to degrade cellulosic material is important since lignocellulosic biomass is the most abundant raw material in nature (Ren et al., 2009) and has low cost. Cellulose is the major constituent of plant biomass and is found in many waste streams, including agricultural and food industry wastes, brewery wastewaters, and waste sludges (Kapdan and Kargi, 2006). In dark fermentation, hydrogen can be produced either by simultaneous cellulose hydrolysis and hydrogen production or by separating these steps into different reactors (Saratale et al., 2008).

Pure cultures, including species of the genera *Enterobacter*, *Bacillus* and *Clostridiu*m, have been widely studied for hydrogen production (Hawkes et al., 2002). Simultaneous cellulose degradation and hydrogen production with pure cultures has also been

reported, for example, with *Clostridium acetobutylicum* (Wang et al., 2008) and *Clostridium thermocellum* (Levin et al., 2006). However, use of mixed cultures is preferred because they are easier to operate and control, they can treat non-sterilized feedstocks, their cellulose conversion rates are higher, and they consist of both hydrolytic and fermentative microorganisms (Levin et al., 2004; Hallenbeck and Ghosh, 2009; Ren et al., 2009). Hydrogen producing cellulolytic mixed cultures exist in environments such as anaerobic digester sludge and sludge compost (Ueno et al., 1995), forest soil (Ravindran et al., 2010), and hot springs (Koskinen et al., 2008a). Mixed cultures usually contain hydrogen consuming microorganisms, such as methanogens, homoacetogens or sulphate reducers, which have to be inhibited. This can be done with different pretreatments, from which the most common and effective has been heat-treating the cultures at 100°C for 15 min (Li and Fang, 2007). Furthermore, the use of elevated temperatures controls some hydrogen consuming bacteria (Ren et al., 2009).

In this study, fermentative hydrogen producers were enriched on cellulose at elevated temperatures (52, 60 and 70°C). Microflora for the experiments was derived from thermophilic compost. The effect of different heat treatments on hydrogen production potentials and microbial communities were delineated. To the authors knowledge the effect of heat pretreatment on microbial community composition of compost inoculum has not been studied.

2 Materials and Methods

2.1 Seed Microorganisms

Compost material from thermophilic (60-70°C) phase of pile compost obtained from a Solid Waste Management Site (Tarastenjärvi, Finland) was used as source material for the enrichments. In the first enrichment step, the ratio of inoculum (28.1 % VS) and substrate (w/w) was set to 1:2. Compost material was weighed prior to the addition.

2.2 Batch Enrichment of Thermophilic H_2-Producing Microorganisms

The microbial enrichments were incubated at 52, 60 and 70ºC. 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 and two control bottles without substrate were prepared for each temperature. The first enrichments were conducted in 25 mL anaerobic tubes with a working volume of 10 mL. The following two enrichment phases were done in 120 mL serum bottles with 50 mL working volume and with 2 % (v/v) inoculum. The enrichments at 52 and 60°C were incubated on a shaker (150 rpm), while the enrichments at 70°C were done under static conditions.

The enrichment medium was DSMZ 144 (German Collection of Microorganisms and Cell Cultures, 2008) with the following modifications: 5 g L-cellulose^{-1} (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. Cellulose was sterilized by autoclaving it at 121ºC for 20 min, while vitamin and $Na_2S \cdot 9\ H_2O$ solutions were sterile filtered (0.2 μm, Whatman FP 30). Gas production was monitored over time and volatile fatty acids (VFAs) and alcohols were analyzed in the end of the enrichments.

2.3 Chemical Analyses

The overpressure from the batch bottles was analyzed according to Owen et al. (1979). 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. (2002).

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 (1991). The cartridge was preconditioned with 2 mL methanol and 2 mL 0.01 M HCl (pH 2), and the VFAs and ethanol 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 (van Haandel and van der Lubbe, 2007).

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

2.4 Microbial Community Analyses

Bacterial communities were characterized 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. (2007), 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. (2007) 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. (2007). Sequence data were analyzed with Bioedit-software (version 7.0.5) and compared with sequences in GenBank (http://www.ncb.nlm.nih.gov/blast/).

3 Results and Discussion

3.1 Enriching Compost Culture for Hydrogen Production

The original compost culture with or without heat treatment produced negligible amounts of hydrogen when incubated at 70°C and the enrichment of hydrogen producers was unsuccessful. Hydrogen production at temperatures of 70ºC or higher has rarely been reported especially with complex substrates, such as cellulose. There are, however, few exceptions; Koskinen et al. (2008a) reported H_2 production at 70 and 78ºC with different enrichment cultures from a hot spring and one of these enrichments utilized cellulose. In addition, Liu et al. (2008) used an inoculum from a glucose fermentation reactor to produce H_2 at 70ºC from a household solid waste and Yokoyama et al. (2007) reported H_2 productions from cow waste slurry at 60 and 75ºC.

Cumulative hydrogen production with compost enrichments at 52 and 60°C was studied and the results were as presented in Figure 1. The HT2 culture (heat-treated at 100ºC for 10 min) grown at 60ºC produced hydrogen only in the 1st enrichment phase. In addition, at 60°C with NHT (no heat-treatment) and HT1 (heat-treated at 80ºC for 20 min) cultures as well as at 52ºC with HT2 cultures the hydrogen production decreased considerably in the 3rd enrichment phase. The compost enrichment HT1 at 52°C was the only culture that continued increasing H_2 production in the 3rd enrichment phase resulted in the highest hydrogen yield, 1.4 mol-H_2 mol-hexose^{-1} (2.4 mol-H_2 mol-hexose-degraded^{-1}, of all the enrichments (Table 1). Without HT at 52ºC the H_2 production in replicate bottles differed considerably, the other enrichment produced no

H_2, while the other enrichment produced similar H_2 yields as the HT1 enrichment at 52ºC. Similar inconsistancy in parallel enrichments was seen in all H_2 producing enrichment phases, although the extent of the fluctuation was less significant. The highest H_2 yield with HT1 culture (52ºC) was 35 % of the maximum H_2 yield (4 mol-H_2 mol-hexose^{-1}, Eq. 2). However, it was in the range of 1 – 2 mol-H_2 mol-hexose^{-1} that has been reported during cellulose fermentation (Levin et al., 2009). The highest H_2 yields from cellulose was reported by Ueno et al. (1995), 2.4 mol-H_2 mol-hexose^{-1} (10 g L^{-1}, 58.0 % cellulose degradation), with a sludge compost inoculum.

Figure 1

Heat treatment is used to increase hydrogen production by inhibiting hydrogen consuming microorganisms and for enrichment of spore-forming hydrogen producers, such as *Clostridia*. However, in this experiment the higher heat treatment temperatures (100ºC against 80ºC) used for pretreatment resulted in lower H_2 yield. This has also reported in few other studies. For example, Baghchehsaraee et al. (2008) reported a 15 % decrease in the hydrogen production yields of activated sludge and anaerobic sludge when the heat treatment temperature was increased from 65 to 95ºC. In addition, Ravindran et al. (2010) studied hydrogen potentials from soil with different heat pretreatment (65 - 120ºC) and reported the highest hydrogen yields with HT temperatures between 95 ºC and 105ºC.

Methane was not produced in any of the enrichments and hydrogen production in the control bottles was negligible. The absence of methane production without HT was likely associated with the drop of pH below 6 during hydrogen production, since most

methanogens are active at the pH range from 6.8 to 7.5 (Zhu et al., 2008). Changes in pH also affect metabolic pathways (Wang and Wan 2009) as well as the hydrogen production potentials since low pH can inhibit iron containing hydrogenase enzymes (Dabrock et al. 1992). The pH was not adjusted during the batch experiments and thus, the decrease in pH during the incubations (from initial pH 7 to pH 5) most likely decreased the hydrogen production in the sequential enrichments. Levin et al. (2009) suggested that the pH should be maintained between 7.0 and 7.2 for continuous H_2 production during cellulose fermentation.

3.2 Fermentation Products and Cellulose Degradation

The VFA and alcohol concentrations were higher in the first enrichment phases of compost cultures than after subsequent transfers (Table 1 and 2) since the initial inoculum provided additional substrate. The main degradation products both at 52°C and 60ºC were acetate and ethanol, accompanied by butyrate. High hydrogen yields are associated with the production of acetate (Eq. 2) and/or butyrate (Eq. 3) with theoretical maximums of 4 and 2 mol-H_2 mol-hexose^{-1}, respectively (Levin et al., 2004). Formation of reduced end products, such as ethanol (Eq. 4), is usually associated with lower hydrogen yields (Levin et al., 2004). However, ethanol and acetate production can be coupled (Eq. 5) with a theoretical maximum of 2 mol-H_2 mol-hexose^{-1} (Ren et al., 2009). Ethanol was one of the main metabolites in all of the enrichment phases resulting in lower H_2 yields. On the other hand, simultaneous H_2 and ethanol could also be feasible (Koskinen et al., 2008b).

Table 1

225 Table 2

226

227 $C_6H_{12}O_6 + 2\ H_2O \rightarrow 4\ H_2 + 2\ CH_3COOH + 2\ CO_2$ (2)

228 $C_6H_{12}O_6 \rightarrow 2\ H_2 + CH_3CH_2CH_2COOH + 2\ CO_2$ (3)

229 $C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2OH + 2\ CO_2$ (4)

230 $C_6H_{12}O_6 + H_2O \rightarrow CH_3CH_2OH + CH_3COOH + 2\ H_2 + 2\ CO_2$ (5)

231

232 Cellulose degradation (Figure 2) in the end of enrichment phases was calculated by
233 determining the initial and final COD concentrations based on the added cellulose and
234 produced hydrogen and degradation products. The cellulose degradations and hydrogen
235 production potentials were consistent; The highest hydrogen yields were obtained in the
236 enrichments with the highest cellulose degradation, 1.4 mol-H_2 mol-hexose^{-1} and 57 %,
237 respectively. Thus, the hydrogen yield in most of the enrichment phases was limited by
238 the cellulose hydrolysis. Degradation of cellulose has been reported to slow down the
239 hydrogen production also in other studies where cellulose is hydrolyzed and hydrogen is
240 produced simultaneously. Liu et al. (2003) reported a hydrogen yield of 102 mL-H_2 g-
241 cellulose^{-1} (18 % of the theoretical maximum) at 55ºC with cellulose (5 g L^{-1}) and
242 concluded that the low H_2 yield was likely due to partial hydrolysis of cellulose.
243 Nutrient requirements for cellulose hydrolysis include carbon and nitrogen as well as
244 trace metals, growth factors and essential vitamins (Ren et al., 2009) that were included
245 in the medium used in this study. The effect of nitrogen source on cellulose degradation
246 and H_2 production was reported by Ueno et al. (2001) showing that with inorganic
247 nitrogen source and with peptone the cellulose degradation and hydrogen yields were
248 72.5 and 90.4 % and 1.0 and 2.0 mol-H_2 mol-hexose^{-1}, respectively. Furthermore, an
249 increase in cellulose degradation might be possible by adjusting the pH. In the

250 experiments of Ueno et al. (2001) the cellulose decomposition increased from 49.9 to
251 90.4 % when the pH was adjusted to 6.4 during the run.
252
253 Figure 2
254
255 Simultaneous cellulase production, cellulose hydrolysis and H_2 production i.e.
256 consolidated bioprocessing (CBP) reported in this study is desirable since it provides
257 lower costs, simpler processing and lower energy inputs (Carere et al., 2008) than
258 separate hydrolysis and H_2 production. However, H_2 yields obtained with CBP remain
259 low (Levin et al., 2009). Thus, cellulase production and cellulose hydrolysis has also
260 been separated into unit processes in order to increase the hydrogen production rates in
261 the second reactor. This could be done by growing cellulolytic microorganisms, e.g.
262 fungi, in the first reactor, from where the effluents could be further used for hydrogen
263 production (Lakshmidevi and Muthukumar, 2010).
264
265 ***3.3 Microbial Communities in 52 and 60°C Enrichments***
266
267 Microbial communities of compost enrichments at 52 and 60°C were analyzed by PCR-
268 DGGE. The DGGE profiles of different pretreatments were as presented in Figure 3 and
269 the results of the sequence affiliation were as shown in Table 3. The enrichments
270 contained mainly bacteria from families *Thermoanaerobacteriaceae* and
271 *Clostridiaceae*. Non-heat-treated cultures contained more bands suggesting that heat
272 treatments enriched for spore formers and reduced species diversity. Furthermore, in
273 most of the enrichments the number of bands decreased during enrichment phases.
274 Microorganism closely related (100 % similarity) to *Thermoanaerobium*

thermosaccharolyticum (band 3) was present both at 52 and at 60°C in almost every enrichment phase. *T. thermosaccharolyticum* has optimal temperature and pH at 60°C and at 5 – 6, respectively, and hexose fermentation products include hydrogen, acetate and butyrate (O-Thong et al., 2009). It is a thermophilic, saccharolytic microorganism that does not degrade cellulose (Ueno et al., 2001). Hydrogen yields of 2.0 – 2.2 mol-H_2 mol-hexose^{-1} have been obtained with *T. thermosaccharolyticum* dominated cultures (Ueno et al., 2001; O-Thong et al., 2009).

Figure 3

Table 3

In addition to *T. thermosaccharolyticum*, enrichments contained other microorganisms capable of hydrogen production. These microorganisms were closely related to *Clostridium cellulosi*, *Clostridium stercorarium*, *Clostridium caenicola*, and *Coprothermobacter proteolyticus*. From these *C. cellulosi* and *C. stercorarium* were likely the two main species responsible for cellulose degradation. *C. cellulosi* is thermophilic and cellulolytic microorganism that produces hydrogen, ethanol and acetic acid as fermentation products. It is a spore former and grows optimally at temperatures 55 - 60°C and at pH 7.3 – 7.5 (Yanling et al., 1991). *C. stercorarium* is a thermophilic, spore-forming, cellulolytic bacterium, its fermentation products include hydrogen, ethanol, acetate and lactate, and its optimal temperature is 65ºC (Madden, 1983). Although *C. stercorarium* is a spore former, it was present only in non-heat-treated enrichments. The fermentation products of *C. caenicola* and *C. proteolyticus* include hydrogen, lactate, acetate and ethanol (Shiratori et al., 2009), and hydrogen and acetate (Ollivier et al., 1985), respectively. In NHT enrichment at 60°C two other hydrogen

producing bacteria, *Thermoanaerobacterium mathranii* (Larsen et al., 1997) and *Thermoanaerobacterium italicus* (Kozianowski et al., 1997), were detected. Although these microorganisms were the main hydrogen producers, the enrichments also contained numerous uncultured *Clostridia* species that may also have contributed to hydrogen production.

C. stercorarium and *C. cellulosi* were the only cellulolytic microorganisms and they were present in almost every enrichment phase with cellulose degradation and hydrogen production. The only exception was the 3rd enrichment of HT1 culture at 52°C that had the highest H_2 yield. This suggests the presence of cellylolytic microorganisms among the uncultured bacteria (for example bands 27, 33 or 35), which may have contributed cellulose degradation also during the other enrichment phases. In other enrichment phases that lacked cellulolytic microorganisms, cellulose degradation prevented hydrogen production. Previously, it was discussed that large pH drop might have decreased H_2 production in sequential enrichments. This may be associated with *C. cellulosi* that has an optimum pH between 7.3 and 7.5 and may have been inhibited by the large decrease in pH.

Heat pretreatment has been widely used to enrich hydrogen producers and to improve the hydrogen yields (Hawkes et al., 2002). However, in this study heat pretreatment did not increase the hydrogen yields considerably. There were a large number of bands in the enrichments at 52°C as well as in the NHT enrichment at 60°C. However, in the heat treated cultures at 60°C there were considerably less bands, which suggest that fewer spore formers were present that would tolerate higher growth temperatures. Consequently, the lowest H_2 yields were obtained with these enrichments.

4 Conclusions

Hydrogen can be produced from cellulose with microorganisms of compost origin. The heat treatments at 100°C do not enrich for H_2 producers and thus, higher H_2 yields are obtained at 52°C that at 60°C. The highest H_2 yield and the highest cellulose degradation, 1.4 mol-H_2 mol-hexose^{-1} (2.4 mol-H_2 mol-hexose-degraded^{-1}) and 57 %, respectively, are obtained at 52°C with 80°C 20 min heat treatment. The H_2 producing compost enrichments contain mainly bacteria from families *Thermoanaerobacteriaceae* and *Clostridiaceae*, from which bacterium closely related to *Thermoanaerobacterium thermosaccharolyticum* is mainly responsible for hydrogen production and bacteria closely related to *Clostridium cellulosi* and *Clostridium stercorarium* were responsible for cellulose degradation.

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References

Baghchehsaraee, B., Nakhla, G., Karamanev, D., Margaritis, A., Reid, G., 2008. The effect of heat pretreatment temperature on fermentative hydrogen production using mixed cultures. Int. J. Hydr. Energy 33, 4064-4073.

Carere, C.R., Sparling, R., Cicek, N., Levin, D.B., 2008. Third generation biofuels via direct cellulose fermentation. Int. J. Mol. Sci. 9, 1342-1360.

Dabrock, B., Bahl, H., Gottschalk, G., 1992. Parameters affecting solvent production by *Clostridium Pasteurium*. Appl. Environ. Microbiol. 58, 1233-1239.

Hallenbeck, P.C., Ghosh, D., 2009. Advances in fermentative biohydrogen production: the way forward? Trends Biotechnol. 27, 287-297.

Hawkes, F.R., Dinsdale, R., Hawkes, D.L., Hussy, I., 2002. Sustainable fermentative hydrogen production: challenges for process optimization. Int. J. Hydr. Energy 27, 1339-1347.

Horspool, L.J.I., McKellar, Q.A., 1991. Determination of short-chain fatty acids in equine caecal liquor by ion exchange high performance liquid chromatography after solid phase extraction. Biomed. Chrom. 5, 202-206.

Kapdan, I.K., Kargi, F., 2006. Bio-hydrogen production from waste materials. Enz. Microb. Technol. 38, 569-582.

Koskinen, P.E.P., Kaksonen, A.H., Puhakka, J.A., 2007. The relationship between instability of H_2 production and compositions of bacterial communities within a dark fermentation fluidized-bed bioreactor. Biotechnol. Bioeng. 97(4), 742-758.

Koskinen, P.E.P., Lay, C.H., Beck, S.R., Tolvanen, K.E.S., Kaksonen, A.H., Örlygsson, J., Lin, C.-Y., Puhakka, J.A., 2008a. Bioprospecting thermophilic microorganisms from Icelandic hot springs for hydrogen and ethanol production. Energy Fuels 22, 134-140.

Koskinen, P.E.P., Beck, S.R., Örlygsson, J., Puhakka, J.A., 2008b. Ethanol and hydrogen production by two thermophilic, anaerobic bacteria isolated from Icelandic geothermal areas. Biotechnol. Bioeng. 101(4), 679-690.

Kozianowski, G., Ganganella, F., Rainey, F.A., Hippe, H., Antranikian, G., 1997. Purification and characterization of thermostable pectate-lyases from a newly isolated thermophilic bacterium, *Thermoanaerobacter italicus* sp. nov. Extremophiles 1, 171-182.

Lakshmidevi, R., Muthukumar, K., 2010. Enzymatic saccharification and fermentation of paper and pulp industry effluent for biohydrogen production. Int. J. Hydr. Energy 35, 3389-3400.

Larsen, L., Nielsen, P., Ahring, B.K., 1997. *Thermoanaerobacter mathranii* sp. nov., an ethanol-producing, extremely thermophilic anaerobic bacterium from a hot spring in Iceland. Arch. Microbiol. 168, 114-119.

Levin, D.B., Pitt, L., Love, M., 2004. Biohydrogen production: prospects and limitations to practical application. Int. J. Hydr. Energy 29, 173-185.

Levin, D.B., Islam, R., Cicek, N., Sparling, R., 2006. Hydrogen production by *Clostridium thermocellum* 27405 from cellulosic biomass substrates. Int. J. Hydr. Energy 31, 1496-1503.

Levin, D.B., Carere, C.R., Cicek, N., Sparling, R., 2009. Challenges for biohydrogen production via direct lignocellulose fermentation. Int. J. Hydr. Energy 34, 7390-7403.

Li, C., Fang, H.H.P., 2007. Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. Crit. Rev. Environ. Sci. Technol. 37, 1-39.

Liu, H., Zhang, T., Fang, H.H.P., 2003. Thermophilic H_2 production from a cellulose-containing wastewater. Biotechnol. Letters 25, 365-369.

Liu, D.W., Zeng, R.J., Angelidaki, I., 2008. Enrichment and adaptation of extreme-thermophilic (70ºC) hydrogen producing bacteria to organic household solid waste by repeated batch cultivation. Int. J. Hydr. Energy 33, 6492-6497.

Logan, B.E., Oh, S.E., Kim, I.S., van Ginkel, S., 2002. Biological hydrogen production measured in batch anaerobic respirometers. Environ. Sci. Technol. 36, 2530-2535.

Madden, R.H., 1983. Isolation and characterization of *Clostridium stercorarium* sp. nov., cellulolytic thermophile. Int. J. Syst. Bacteriol. 33(4), 837-840.

Ollivier, B.M., Mah, R.A., Ferguson, T.J., Boone, D.R., Garcia, J.L., Robinson, R., 1985. Emendation of the genus *Thermobacteroides*: *Thermobacteroides proteolyticus* sp. nov., a proteolytic acetogen from a methanogenic enrichment. Int. J. Syst. Bacteriol. 35(4), 425-428.

O-Thong, S., Prasertsan, P., Birkeland, N.K., 2009. Evaluation of methods for preparing hydrogen-producing seed inocula under thermophilic condition by process performance and microbial community analysis. Biores. Technol. 100, 909-918.

436 Owen, W.F., Stuckey, D.C., Healy, J.B.Jr., Young, L.Y., McCarty, P.L., 1979. Bioassay
437 for monitoring methane potential and anaerobic toxicity. Water Res. 13, 485-492.
438
439 Ravindran, A., Adav, S., Yang, S.S., 2010. Effect of heat pre-treatment temperature on
440 isolation of hydrogen producing functional consortium from soil. Ren. Energy, doi:
441 10.1017/j.renene.2010.04.010.
442
443 Ren, N., Wang, A., Cao, G., Xu, J., Gao, L., 2009. Bioconversion of lignocellulosic
444 biomass to hydrogen: Potential and challenges. Biotechnol. Adv. 27, 1051-1060.
445
446 Saratale, G.D., Chen, S.D., Lo, Y.C., Saratale, R.G., Chang, J.S., 2008. Outlook of
447 biohydrogen production from lignocellulosic feedstock using dark fermentation – a
448 review. J. Sci. Ind. Res. 67, 962-979.
449
450 Shiratori, H., Sasaya, K., Ohiwa, H., Ikeno, H., Ayame, S., Kataoka, N., Miya, A.,
451 Beppu, T., Ueda, K., 2009. *Clostridium clariflavum* sp. nov. and *Clostridium caenicola*
452 sp. nov., moderately thermophilic, cellulose-/cellobiose-digesting bacteria isolated from
453 methanogenic sludge. Int. J. Syst. Evol. Microbiol. 59, 1764-1770.
454
455 Ueno, Y., Kawai, T., Sato, S., Otsuka, S., Morimoto, M., 1995. Biological production of
456 hydrogen from cellulose by natural anaerobic microflora. J. Ferm. Bioeng. 79, 395-397.
457
458 Ueno, Y., Haruta, S., Ishii, M., Igarashi, Y., 2001. Microbial community in anaerobic
459 hydrogen-producing microflora enriched from sludge compost. Appl. Microbiol.
460 Biotechnol. 57, 555-562.
461
462 van Haandel, A., van der Lubbe, J., 2007. Handbook biological waste water treatment.
463 Design and optimisation of activated sludge system. Quist Publishing, Leidschendam.
464
465 Wang, A.J., Ren, N.Q., Shi, Y.J., Lee, D.J., 2008. Bioaugmented hydrogen production
466 from microcrystalline cellulose using co-culture-*Clostridium acetobutylicum* X9 and
467 *Ethanoigenens harbinense* B49. Int. J. Hydr. Energy 33, 912-917.
468
469 Yanling, H., Youfang, D., Yanquan, L., 1991. Two cellulolytic Clostridium species:
470 *Clostridium cellulosi* sp. nov. and *Clostridium cellulofermentans* sp. nov. Int. J. Syst.
471 Bacteriol. 41(2), 306-309.
472
473 Yokoyama, H., Waki, M., Moriya, N., Yasuda, T., Tanaka, Y., Haga, K., 2007. Effect
474 of fermentation temperature on hydrogen production from cow waste slurry by using
475 anaerobic microflora within the slurry. Appl. Microbiol. Biotechnol. 74, 474-483.
476
477 Zhu, H., Stadnyk, A., Béland, M., Seto, P., 2008. Co-production of hydrogen and
478 methane from potato waste using a two-stage anaerobic digestion process. Bioresour.
479 Technol. 99, 5078-5084.

Figure captions:

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

Figure 2. Cellulose degradation at 52 and 60ºC with compost enrichment cultures based on formed COD after 14 (1.E, 2.E) and 23 (3.E) days of incubation.

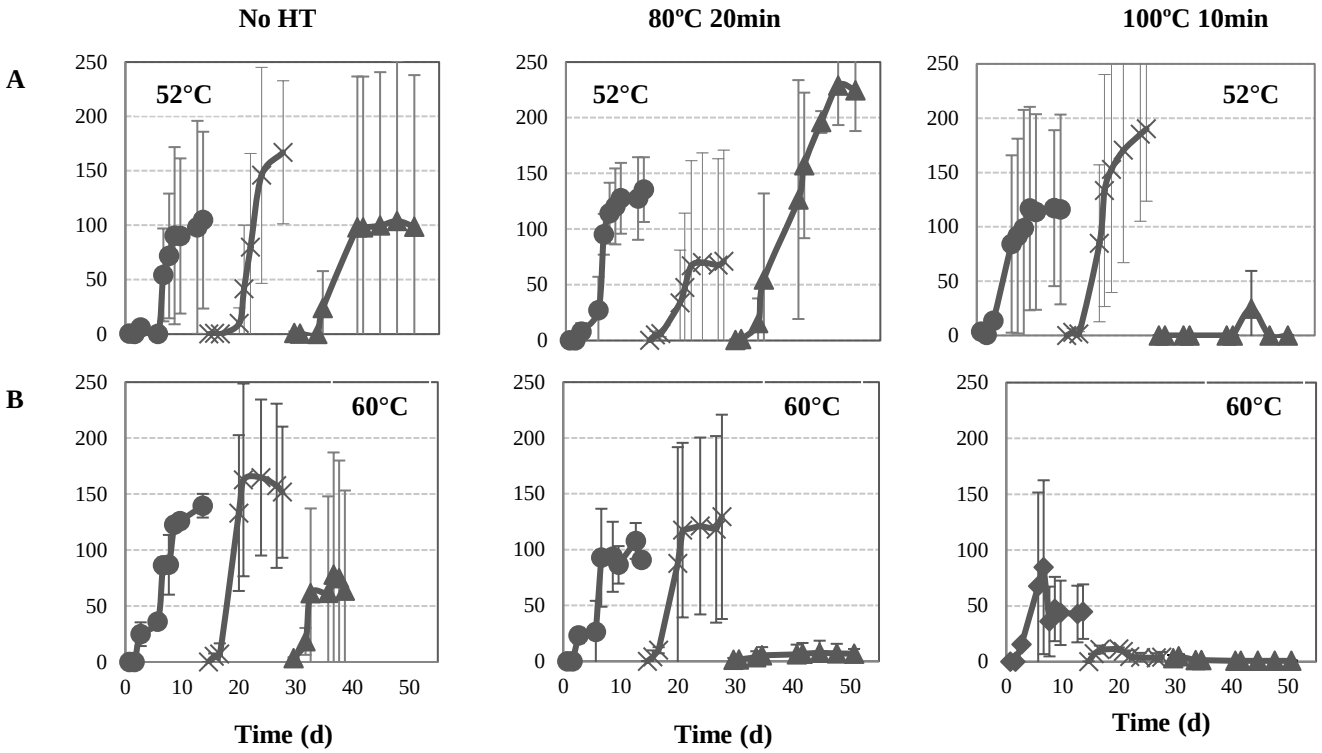
Figure 3. Bacterial community profile determined with PCR-DGGE of partial 16S rRNA genes of compost enrichments at 52 and 60°C without heat treatment (NHT) and with heat treatments at 80ºC for 20 min (HT1) and at 100ºC for 10 min (HT2). See Table 3 for the labeled bands.

Table captions:

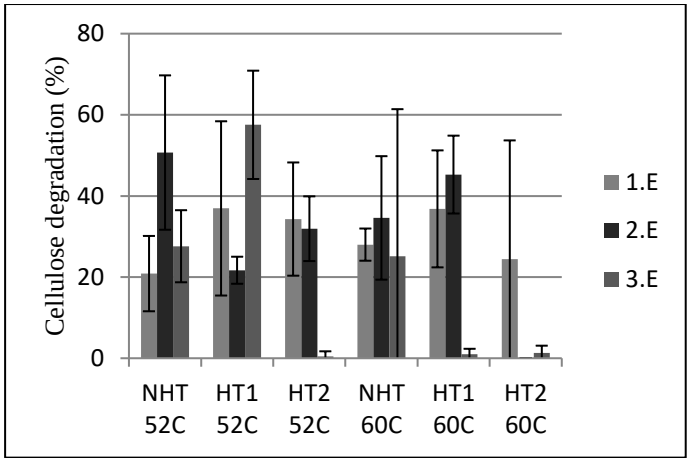
Table 1. Hydrogen yields and degradation products at 52ºC from cellulose by compost enrichment cultures (standard deviations in parenthesis), n.d. = not detected.

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

Table 3. Affiliations of DGGE fragments determined by their 16S rDNA sequence from compost enrichment cultures.



505 Figure 1. Hydrogen production (mL-H_2 g-cellulose^{-1}) from cellulose with compost
506 inoculum at 52°C (A) and at 60°C (B). Sphere (●): the 1st enrichment, cross (x): the 2nd
507 enrichment, and triangle (▲): the 3rd enrichment phase. HT = heat-treatment.
508



509
510
511 Figure 2. Cellulose degradation at 52 and 60ºC with compost enrichment cultures based on

512 formed COD after 14 (1.E, 2.E) and 23 (3.E) days of incubation.

513

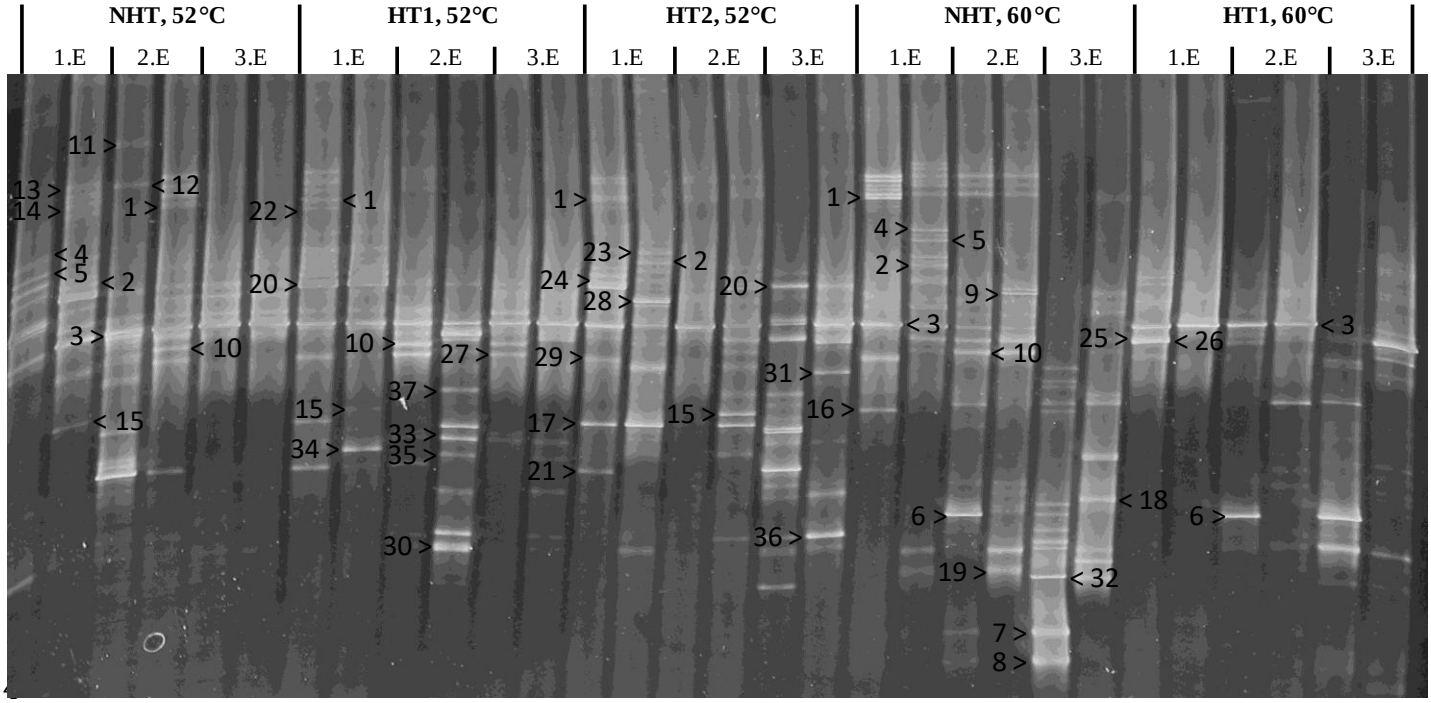


Figure 3. Bacterial community profile determined with PCR-DGGE of partial 16S rRNA genes of compost enrichments at 52 and 60°C without heat treatment (NHT) and with heat treatments at 80ºC for 20 min (HT1) and at 100ºC for 10 min (HT2). See Table 3 for the labeled bands.

521 Table 1. Hydrogen yields and degradation products at 52ºC from cellulose by compost

522 enrichment cultures (standard deviations in parenthesis), n.d. = not detected.

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})	0.66 (0.51)	1.05 (0.41)	0.62 (0.87)	0.85 (0.18)	0.45 (0.63)	1.41 (0.23)	0.73 (0.55)	1.19 (0.42)	n.d.
Lactate (mM)	n.d.	7.96 (8.57)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Acetate (mM)	13.86 (8.03)	8.66 (2.69)	7.22 (2.32)	20.57 (3.35)	3.79 (1.46)	13.37 (2.92)	17.73 (5.26)	9.61 (3.65)	n.d.
Butyrate (mM)	3.24 (2.81)	2.55 (0.69)	4.77 (2.03)	2.93 (0.01)	1.09 (0.07)	7.11 (3.24)	3.64 (1.69)	3.58 (0.44)	0.30 (0.42)
Ethanol (mM)	10.32 (5.15)	10.13 (0.84)	3.48 (0.00)	16.76 (9.68)	7.68 (0.76)	12.08 (0.06)	16.01 (7.74)	5.33 (1.26)	n.d.
Total VFAs (mM)	27.24	29.3	15.47	40.26	12.56	32.56	37.38	18.52	0.30

523

524

525 Table 2. Hydrogen yields and degradation products at 60ºC from cellulose by compost

526 enrichment cultures (standard deviations in parenthesis), n.d. = not detected.

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 (mol-H_2 mol-hexose^{-1})	0.85 (0.07)	0.93 (0.36)	0.39 (0.55)	0.56 (0.01)	0.79 (0.56)	0.04 (0.03)	0.27 (0.15)	0.02 (0.03)	n.d.
Lactate (mM)	n.d.	3.36 (3.40)	n.d.	n.d.	1.94 (0.55)	n.d.	n.d.	n.d.	n.d.
Acetate (mM)	12.95 (0.02)	5.77 (3.10)	4.51 (6.37)	15.18 (0.64)	6.13 (2.47)	0.80 (1.14)	13.12 (9.18)	n.d.	1.07 (1.51)
Butyrate (mM)	3.71 (0.61)	3.68 (0.66)	3.24 (4.30)	4.84 (2.81)	4.11 (1.45)	0.10 (0.14)	2.98 (4.21)	n.d.	0.16 (0.23)
Ethanol (mM)	5.96 (1.17)	5.84 (1.88)	6.17 (8.72)	7.48 (2.89)	12.23 (0.71)	n.d.	5.09 (9.21)	n.d.	n.d.
Total VFAs (mM)	22.62	18.65	13.92	27.50	24.41	0.90	21.19	0.00	1.23

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528

529 Table 3. Affiliations of DGGE fragments determined by their 16S rDNA sequence from

530 compost enrichment cultures.

BL[a]	Family[b]	Affiliation (acc)[c]	Sim (%)[d]	SL[e]
1	*Thermoanaerobacteriaceae*	*T. thermosaccharolyticum* DSM 571 (CP002171)	97.9	442
2	*Thermoanaerobacteriaceae*	*T. thermosaccharolyticum* DSM 571 (CP002171)	99.8	487
3	*Thermoanaerobacteriaceae*	*T. thermosaccharolyticum* DSM 571 (CP002171)	100.0	485
4	*Thermoanaerobacteriaceae*	*T. thermosaccharolyticum* DSM 571 (CP002171)	99.6	497
5	*Thermoanaerobacteriaceae*	T. *thermosaccharolyticum* DSM 571 (CP002171)	99.8	495
6	*Thermoanaerobacteriaceae*	*Thermoanaerobacter* sp. WC12 (HM585224)	99.8	500
7	*Thermoanaerobacteriaceae*	*T. mathranii* subsp. *mathranii* str. A3 (CP002032)	99.4	476
8	*Thermoanaerobacteriaceae*	*T. italicus* Ab9 (CP001936)	100.0	479
9	*Clostridiaceae*	*C. stercorarium* (NCIMB 11754) (LO9174)	94.3	485
10	*Clostridiaceae*	*Clostridiaceae* bacterium 37-7-1C1 (FJ907163)	99.6	487
11	*Clostridiaceae*	*C. thermopalmarium* (AF286862)	90.7	414
12	*Clostridiaceae*	*C. thermopalmarium* strain BVP (NR_026112)	95.0	437
13	*Clostridiaceae*	*C .thermoamylolyticum*, strain DSM 2335 (X76743)	93.8	348
14	*Clostridiaceae*	*C. thermoamylolyticum*, strain DSM 2335 (X76743)	92.3	433
15	*Clostridiaceae*	*C. cellulosi* strain D3 (FJ465164)	99.6	529
16	*Clostridiaceae*	*C. cellulosi* strain D3 (FJ465164)	95.1	491
17	*Clostridiaceae*	*C. cellulosi* strain D3 (FJ465164)	95.7	492
18	*Thermodesulfobiaceae*	*Coprothermobacter proteolyticus* strain IT3 (GU363592)	99.6	510
19	*Thermodesulfobiaceae*	*Coprothermobacter proteolyticus* DSM 5265 (CP001145)	100.0	480
20	*Bacillaceae*	Uncultured *Bacillus* sp. clone 126 (EU250947)	99.4	491
21	*Bacillaceae*	*Bacillus coagulans* strain BL174 (GU904695)	99.4	553
22	*Ruminococcaceae*	Uncultured bacterium clone VKW-TB-3.3 (GQ849504)	99.4	470
23	*Ruminococcaceae*	Uncultured bacterium clone VKW-TB-3.3 (GQ849504)	98.4	490
24	*Ruminococcaceae*	Uncultured bacterium clone VKW-TB-3.3 (GQ849504)	98.7	482
25	*Clostridiaceae*	Clostridium caenicola (AB221372)	95.4	494
26	*Ruminococcaceae*	Uncultured bacterium, clone HAW-R60-B-727d-AG (FN436074)	81.6	488
27	*Ruminococcaceae*	Uncultured bacterium clone HAW-RM37-2-B-877d-A6 (FN563212)	91.7	471
28	*Lachnospiraceae*	Uncultured bacterium clone VKW-TB-7 (GQ849508)	99.8	498
29	*Thermoanaerobacteraceae*	Uncultured bacterium clone HG6 (GU296468)	98.3	485
30	*Thermoanaerobacteraceae*	*Firmicutes* bacterium enrichment culture clone 0-6F07 (FJ796691)	91.4	482
31	*Clostridiaceae*	Uncultured bacterium clone: clone-38 (AB375714)	99.6	524
32	*Clostridiaceae*	Uncultured compost bacterium clone 1B07 (DQ346486)	100.0	481
33	*Clostridiaceae*	Uncultured bacterium clone E82 (FJ205847)	98.2	513
34	*Clostridiaceae*	Thermophilic anaerobic bacterium K1L1 (AY728365)	99.4	509
35	*Clostridiaceae*	Uncultured bacterium clone E82 (FJ205847)	98.2	516
36	*Incertae Sedis XVIII*	*Symbiobacterium* sp. KY38 (AB361629)	99.8	524
37	*Insertae Sedis XI*	Uncultured bacterium clone thermophilic_alkaline-116 (GU455356)	98.8	493

531 [a] Band label in Figure 3, [b] Family according to Ribosomal Database Project II, [c] Closest species in the
532 GenBank database with an accession number, [d] Similarity (%), [e] Sequence length (bp).
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