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ORIGINAL RESEARCH PAPER

Production of H_2 from cellulose by rumen microorganisms: effects of inocula pre-treatment and enzymatic hydrolysis

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Abstract H_2 production from cellulose, using rumen fluid as the inoculum, has been investigated in batch experiments. Methanogenic archaea were inhibited by acid pre-treatment, which also inhibited cellulolytic microorganisms, and in consequence, the conversion of cellulose to H_2. Positive results were observed only with the addition of cellulase. H_2 yields were 18.5 and 9.6 mmol H_2 g cellulose^{-1} for reactors with 2 and 4 g cellulose l^{-1} and cellulase, respectively. H_2 was primarily generated by the butyric acid pathway and this was followed by formation of acetic acid, ethanol and *n*-butanol. In reactors using 4 g cellulose l^{-1} and cellulase, the accumulation of alcohols negatively affected the H_2 yield, which changed the fermentation pathways to solventogenesis. PCR–DGGE analysis showed changes in the microbial communities. The phylogenetic affiliations of the bands of DGGE were 99 % similar to *Clostridium* sp.

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Introduction

Cellulose is the most abundant renewable natural resource in the world and is a promising economical source for the production of biofuels. It is a polymer containing successive units of glucose in a complex arrangement and is associated with other polymers, such as hemicelluloses and lignin (Lynd et al. 2002; Lakshmidevi and Muthukumar 2010).

Effective hydrolysis is necessary to effectively produce biofuels from cellulosic materials. One possible approach to achieve such hydrolysis involves the delignification and conversion of polysaccharides into fermentable sugars, which can be further converted into other value-added products (Lynd et al. 2002).

Cellulosic materials can be enzymatically converted into reducing sugars by cellulase produced from various microbial species using a minimally energy-intensive process that offers high yields of glucose and other sugars at mild operating conditions (Lakshmidevi and Muthukumar 2010). Cellulase usually refers to a mixture of different enzymes involved in the hydrolysis of cellulose to glucose, which can

include endoglucanase, cellobiohydrolase, and β-glucosidase (Demain et al. 2005). A limitation of cellulase is that end products inhibit the reaction rates.

Although a large number of microorganisms can degrade cellulose by producing cellulase enzymes, only a few of these microorganisms produce this enzyme in significant amounts. Thus, the anaerobic digestion of cellulosic materials is expected to enhance the effectiveness of cellulose-degrading microbial communities. On the other hand, different inoculum sources, such as manure, digester sludge, landfill leachate, composts, or pure cultures isolated from these inoculum sources, have been used to start the reactors (Demain et al. 2005; Ho et al. 2011).

The rumen is a highly cellulolytic ecosystem with a complex microbial population of bacteria, archaea, protozoa, and fungi. This consortium hydrolyzes cellulosic materials to sugars and other hydrolysis products via anaerobic digestion. The glucose is subsequently fermented to short chain volatile fatty acids (VFA), and the end products of fermentation include acetate, H_2, CO_2, butyrate, and ethanol. H_2 and formate are produced by many microorganisms in the rumen; however, methanogens are also present in the rumen and convert H_2 and CO_2 to CH_4 (Nissila et al. 2011a).

H_2 can be produced by dark fermentation using either pure or mixed cultures. However, using a mixed culture to produce H_2 may encounter drawbacks. Specifically, substrates may compete with non-H_2-producing microbial populations, and the produced H_2 may be consumed by H_2-consuming bacteria in the mixed culture (Wang and Wan 2009). However, if the activity of H_2-consuming bacteria contained in mixed culture were inhibited, the mixed microflora would possess a significant capacity to transform cellulose into H_2 gas (Lay et al. 1999).

Mixed cultures can be pre-treated with many methods, such as acid treatment, base treatment, heat treatment and aerobic treatment (Wang et al. 2010). Of these, the most common and effective treatment has been the heat treatment of cultures at 100 °C for 15 min (Maintinguer et al. 2011). However, higher heat treatment temperatures used for pre-treatment resulted in lower H_2 yields according to Nissila et al. (2011b).

Although rumen fluid is a popular inoculum source for anaerobic digestion, we have obtained little information about the production of H_2 using this inoculum, pre-treatment methods, and the H_2 production potential of rumen fluid without and with the enzymatic hydrolysis of cellulose (Nissila et al. 2011a; Ho et al. 2011).

Therefore, the present study aimed to evaluate the production of H_2 using rumen fluid as the inoculum source. Batch experiments were performed to select the best pre-treatment method for rumen fluid. The effects of the cellulose concentration and enzyme loading on the production of H_2 were also analyzed.

Materials and methods

Inoculum and pretreatment conditions

Rumen fluid was collected from fistulated cows maintained on a forage diet at the Canchin Farm–EMBRAPA Southeast (São Carlos, SP, Brazil). After sampling, the rumen contents were squeezed through four layers of cotton cloth to remove coarse solids, and they were stored in a thermic jar and purged with N_2/CO_2 (70/30 %) to ensure anaerobic conditions.

To select H_2-producing microorganisms and inhibit methanogenic archaea, the ruminal fluid was pre-treated by different methods: aeration, acid and heat pretreatments (Wang and Wan 2009; Wang et al. 2010).

Each pre-treated rumen fluid sample (10 % v/v) was used to inoculate 50 ml culture medium containing 0.5 g cellulose/l in 100 ml vials. The pH of the mixture was adjusted to 7.0 with 1 M NaOH. To ensure anaerobic conditions, the vials were purged with N_2 (100 %) for 10 min before they were sealed and incubated at 37 °C. The biogas accumulating in the headspace of the vials was sampled and measured periodically for H_2 and methane analyses.

Culture medium

The culture medium used in the experiments consisted of nutrient solutions A, B, C and D, as well as urea (40 mg l^{-1}) and yeast extract (1,000 mg l^{-1}). The vitamin solution was composed of *p*-aminobenzoic acid (40 mg l^{-1}) and biotin (10 mg l^{-1}) (Maintinguer et al. 2011; Ratti et al. 2013). The cellulose used as the sole carbon source was a microcrystalline powder with a particle size of 20 μm (Sigma-Aldrich).

H_2 production in batch fermentation

H_2 production experiments were conducted in triplicate in anaerobic batch reactors with a total volume of 5 l. The working volume was of 1.5 l, and the solution in the reactors was composed of culture medium, microbial enrichment of the best pre-treatment method for the rumen fluid (10 % v/v), cellulose (2 g l^{-1} or 4 g l^{-1}), and cellulase (10 ml) for the enzymatic hydrolysis of cellulose.

The cellulase used for these experiments was a commercially-available enzyme solution named Celluclast-1.5 L produced by *Trichoderma reesei* (Ratti et al. 2013).

Prior to the operation of the reactors, the pH was adjusted to 7.0 by 1 M NaOH, the reactors were purged with N_2 (100 %), and they were then incubated at 37 °C without agitation. The biogas content, volatile fatty acids, alcohols and cellulose consumption analyses were sampled and measured periodically throughout the experiments. The experiments were deemed complete when no biogas production was observed for at least 24 h. A liquid sample was taken at the beginning and end of the experiments to analyze the pH.

Chemical and chromatographic analysis

The H_2 content in the biogas, the volatile fatty acids and alcohol concentrations were determined by GC 2010 according to Maintinguer et al. (2011).

The final pH values were determined according to the Standard Methods (APHA 1998). The cellulose concentration was determined using the phenol–sulfuric acid method for sugars with glucose as the standard (Dubois et al. 1965).

Data analysis

The cumulative volume of H_2 production in the batch experiments followed the modified Gompertz equation (Lay et al. 1999; Ratti et al. 2013):

$$H = P\exp\left\{-\exp\left[\frac{R_m e}{P}(\lambda - t) = 1\right]\right\}, \quad (1)$$

where H is the cumulative H_2 production (mmol), P is the H_2 production potential (mmol), R_m is the maximum H_2 production rate (mmol h^{-1}), λ is the lag phase time (h), and e is 2.71. The values of P, R_m, and λ for each batch experiment were estimated using the nonlinear estimation function in the Statistica 8.0 software.

Microbiology analysis

The morphological characteristics of the microorganisms were monitored by phase-contrast microscopy (Olympus BX60-FLA) according to Maintinguer et al. (2011).

The bacterial communities were analyzed by the denaturing gradient gel electrophoresis (PCR-DGGE) of partial 16S rRNA genes utilizing primers for the bacteria domains 968FGC and 1392R (Nielsen et al. 1999). The total genomic DNA was obtained with glass beads and phenol/chloroform extraction after cell lysis, as described by Maintinguer et al. (2011). The conditions of PCR-DGGE were carried out in accordance to Maintinguer et al. (2011). The dendrogram was built using the Bionumerics software version 2.5 (Applied Maths, Belgium). The dendrogram was based on UPGMA (Unweighted Pair Group Method with Arithmetic Averages) and similarity coefficients using Pearson correlations from the patterns of DGGE bands. The DGGE bands were re-amplified with the same primer set (without the GC clamp). The nucleotide sequence was performed in an ABI Prism 310 Genetic Analyzer (Applied Biosystems) sequencer. The phylogenetic affiliations of the obtained sequences were determined by using the BLAST search program at the NCBI (National Center for Biotechnology Information). The 16S rRNA gene sequence obtained in this study was deposited in GenBank with the accession number KF494802.

Results and discussion

Pretreatment tests

Aeration, acid and heat pre-treatments were performed with the purpose of inactivating H_2-consuming bacteria and obtaining the best pre-treatment method to enrich H_2-producing bacteria from rumen fluid. According to Zhu and Béland (2006), the physiological differences between H_2-producing bacteria (also referred to as acidogenic bacteria) and H_2-uptake bacteria (methanogenic archaea) form the fundamental

basis of the development of the various methods proposed to prepare H_2-producing seeds.

A detailed analysis of biogas composition shows that aeration and heat pre-treatments were not effective to completely repress methanogenic activity because methane was detected in biogas after 144 and 48 h of incubation for aeration and heat pre-treatment, respectively. However, acid pre-treatment successfully enriched H_2 from rumen fluid. Similar results were obtained by Cheong and Hansen (2006) from cattle manure sludge. According to Wang and Wan (2009), the optimal pre-treatment method to enrich H_2-producing bacteria from mixed cultures has not yet been agreed upon. This lack of consensus can possibly be attributed to differences in the type of inoculum, pretreatment method studied, specific conditions of each pre-treatment method and the type of substrates.

Acid pre-treatment did not produce methane over the 144 h incubation period. The H_2 concentration changed after 120 h, when the H_2 volume gradually increased to 0.15 mmol H_2 at 144 h. Thus, the mesophilic H_2 production was very low and limited by the cellulose hydrolysis. Nevertheless, this finding was consistent with other studies in which cellulose was hydrolyzed and H_2 produced simultaneously (Nissila et al. 2011b). Levin et al. (2006) reported H_2 yields of 0.17 mmol H_2 g cellulose^{-1}. They concluded that the low H_2 yield was likely due to the partial hydrolysis of cellulose. Moreover, the solubilization of cellulose by rumen microorganisms has been shown to depend on the presence of sterilized, clarified rumen fluid that provides nutrients (O'Sullivan and Burrell 2007; Nissila et al. 2011a).

Datar et al. (2007) demonstrated that mesophilic anaerobic bacteria cannot utilize cellulose efficiently. Thus, the culture medium used in this study contained the carbon and nitrogen required for the involved microorganisms, as well as trace metals, growth factors, and vitamins (Ren et al. 2010; Nissila et al. 2011b). The H_2 production by anaerobic bacteria could be enhanced further by the addition of cellulolytic enzymes to hydrolyze cellulose (Lakshmidevi and Muthukumar 2010).

H_2 production tests

As previously mentioned, the fermentation of cellulose (2 and 4 g l^{-1}) was further enhanced by the addition of cellulase. The cumulative H_2 production profiles from cellulose during the cultivation of the rumen fluid with acid pretreatment are shown Fig. 1.

Table 1 summarizes the estimated kinetic parameters, P, R_m and λ, for cellulose concentrations of 2 and 4 g l^{-1} with cellulase. All cumulative H_2 production data fit Eq. (1), and the high R^2 values (>0.981) indicated that the parameters were statistically significant.

As shown in Fig. 1 and Table 1, H_2 was generated after 12 h for 2 g cellulose l^{-1}, and its volume increased to 54.5 mmol at 47 h. For 4 g cellulose l^{-1}, H_2 was generated after 9.58 h, and the H_2 volume increased gradually to 45 mmol at 24 h and to 57.6 mmol at 122 h. At the end of fermentation, ~78.5 and 90 % of the cellulose had degraded for the 2 and 4 g l^{-1} experiments, respectively. The generated gas consisted of H_2 (average 47 %) and CO_2 (average 53 %) for both cellulose concentrations Fig. 2.

The lag phase time for both experiments containing cellulase was ~11 h, which was shorter than the lag phase time of 120 h for the 0.5 g l^{-1} experiment without cellulase. The shorter lag phase times observed are related to the addition of cellulase, which promoted the rapid hydrolysis of cellulose into readily fermentable soluble carbohydrates, allowing faster biomass growth.

The values of kinetic parameters P and R_m increased from 54.5 to 57.6 mmol and from 2.2 to 3.6 mmol h^{-1} respectively, when the cellulose concentration rose from 2 to 4 g l^{-1}, suggesting that more of the substrate was available for biomass growth and H_2 production. The results of this work are somewhat similar to those of Ren et al. (2010), who evaluated the effects of cellulose concentration on the values of P and R_m using cow dung compost-enriched cultures without adding cellulase. According to these authors, the P and R_m values increased from 37 mmol H_2 to 55.8 mmol H_2 and from 0.42 to 0.65 mmol l^{-1} h^{-1}, when the concentration of cellulose rose from 5 to 10 g l^{-1}, respectively, although, the degradation of cellulose decreased from 61 to 57 %.

In this study, the pH in the batch bottles was not adjusted and dropped from 7 to below 4, which might have inhibited cellulose-degrading microorganisms (Nissila et al. 2011b). Furthermore, Wang and Wan (2009) and Nissila et al. (2011b) noted that changes in

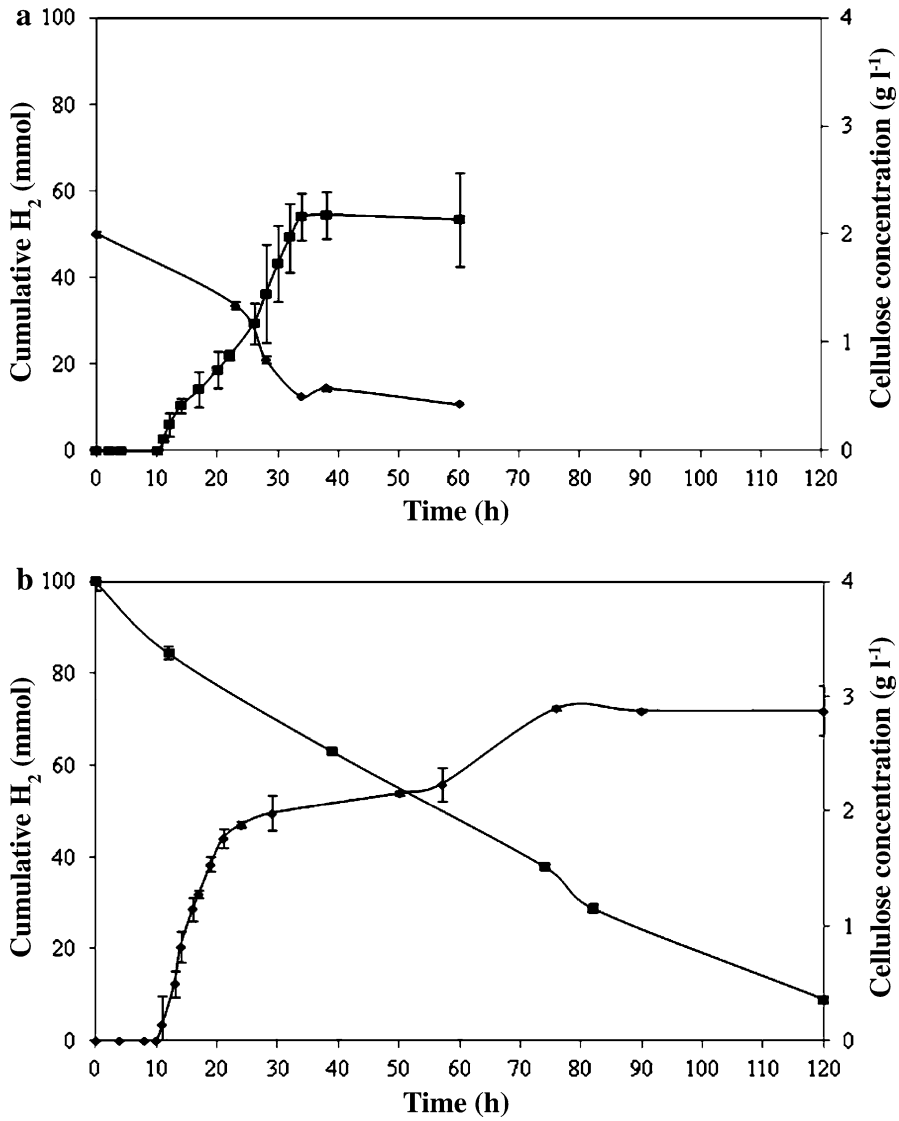

Fig. 1 Cumulative H_2 production (*square*) and cellulose concentration (*diamond*). **a** reactor fed with 2 g cellulose l^{-1} and cellulase; **b** reactor fed with 4 g cellulose l^{-1} and cellulase

Table 1 Kinetic parameters obtained from the modified Gompertz equation

Parameter	2 g cellulose l^{-1} with cellulase	4 g cellulose l^{-1} with cellulase
P (mmol)	54.47	57.59
R_m (mmol h^{-1})	2.24	3.63
λ (h)	12.13	9.58
R^2	0.990	0.981

pH also affect the metabolic pathways and H_2 production potential because low pH values can inhibit iron-containing H_2ase enzymes.

In the experiments of Ueno et al. (2001), the cellulose decomposition increased from 49.9 to 90.4 % when the pH was adjusted to 6.4 during the run. Hu et al. (2004) 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 the cellulose degradation decreased considerably at lower pH values. According to Ren et al. (2010) a decrease in the pH and an increase in the volatile organic acid content were observed in parallel with H_2 production from cellulose by cow dung compost-enriched cultures, and the optimum pH value to produce H_2 ranged from 5.7 to 4.5. Lay et al. (1999) indicated that a shift

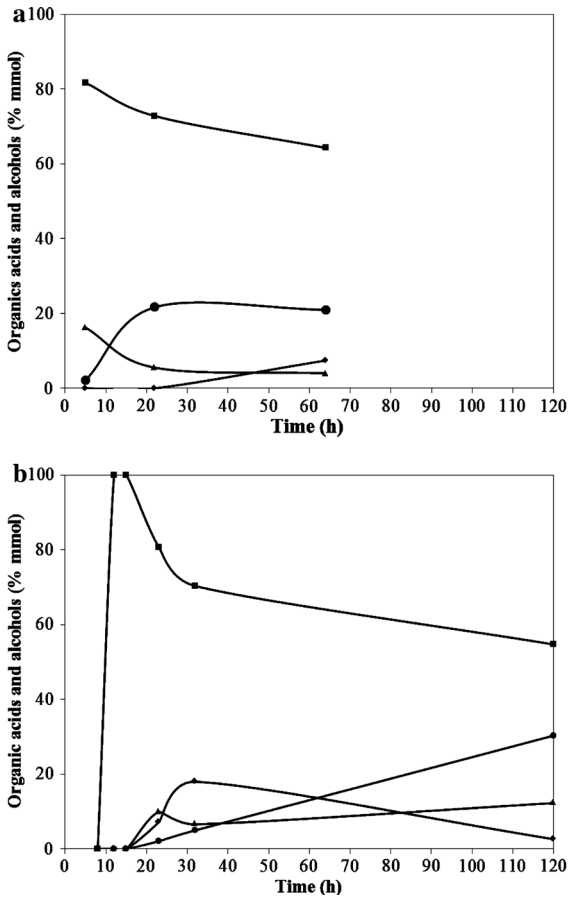

Fig. 2 Soluble microbial product (SMP) profiles for cellulose concentrations of 2 and 4 g l^{-1}. (—●—) Acetic acid; (—■—) Butyric acid; (—▲—) Ethanol; (—●—) *n*-Butanol

from H_2/VFA production to solventogenesis occurs at approximately pH 5.6.

Butyric acid was the major metabolite from 2 g cellulose l^{-1}, and its concentration decreased from 81.7 % (t = 5 h) to 64.3 % (t = 64 h). Conversely, the butanol concentration increased from 2.1 % (at t = 5 h) to 21 % (t = 64 h). The behavior of ethanol was similar to that of butyric acid, i.e., its concentration decreased from 16.2 % (t = 5 h) to 4 % (at t = 64 h). Acetic acid (7.4 %) was observed only at 64 h. The total volatile fatty acid (TVFA) concentration (corresponding to the sum of the concentrations of butyric acid and acetic acid) was higher than the alcohol concentration (corresponding to the sum of the concentrations of ethanol and butanol). The TVFA/SMP ratio ranged from 72.8 to 81.7 %, and a predominantly butyric fermentation prevailed for a concentration of 2 g cellulose l^{-1}.

Butyric acid was also the major metabolite at 4 g cellulose l^{-1}, and its concentration decreased from 100 % (at 12 h) to 55 % (at 120 h). Importantly, only butyric acid was produced for the first 15 h of fermentation. Conversely, the butanol concentration increased from 2.1 % (at t = 23 h) to 30.3 % (t = 120 h). The behavior of ethanol was different from that of butyric acid, as the concentration decreased from 9.9 % (t = 23 h) to 6.6 % (reaching a minimum value at t = 32 h), but increased again to 12.3 % at t = 120 h. The behavior of acetic acid was opposite to that of ethanol. Specifically, the concentration increased from 7.3 % (at 23 h) to 18 % (reaching a maximum value at 32 h), but decreased again to 2.6 % at 120 h. The production of acid is also predominant at 4 g cellulose l^{-1} for the first 32 h of fermentation because the TVFA/SMP ratio ranged from 88 to 88.4 %, and the butyric acid fermentation dominated for the first 32 h of cultivation. However, TVFA/SMP decreased from 88.4 to 57.4 % between 32 and 120 h due to the sharp increase in the concentrations of butanol and ethanol and the decreased production of butyric and acetic acid. Thus, the metabolic pathway changed from acidogenesis to solventogenesis between 32 and 120 h.

Liu et al. (2003) studied the effect of pH (5.5–8.5) on the production of H_2 from synthetic wastewater containing 5 g cellulose l^{-1} at 55 °C, and they reported that acetate (46–55.6 %) and butyrate (15.3–29.3 %) were the two main products of fermentation in all batches, followed by ethanol, methanol and propionate.

In the experiments of Ren et al. (2010), H_2 production from cellulose by cow dung compost-enriched cultures and the decrease of pH were accompanied by the formation of volatile fatty acids and ethanol throughout the cellulose fermentation. Among the fermentation end products, the lower fatty acids, mainly 1,280 mg acetate l^{-1} and 1,010 mg butyrate l^{-1}, constituted more than 79 % of the total soluble metabolites.

Nissila et al. (2011a) studied the effect of increasing the initial pH from 5.2 to 7.3 on H_2 production by rumen fluid enrichment at 60 °C. The amount of SMP increased with increasing pH, and the VFA and alcohol profiles changed considerably with the pH. At pH 6.0 and below, the production of ethanol was predominant, and some acetate and butyrate was also produced. At initial pH values of 6.9 and 7.3, acetate

was the main degradation product with relatively high ethanol concentrations.

However, in a study of the effects of heat treatment on H_2 production from cellulose by thermophilic compost enrichment cultures, Nissila et al. (2011b) verified that the main degradation products at both 52 and 60 °C were acetate and ethanol, accompanied by butyrate. The authors observed that ethanol and acetate production can be coupled, despite the fact that high production rates of H_2 are generally associated with the production of acetate and/or butyrate and ethanol is generally associated with lower H_2 yields. On the other hand, Barros and Silva (2012) verified that simultaneous H_2 and ethanol production from the fermentation of glucose was also feasible.

The final SMP values obtained in this study indicate that the major metabolites of the fermentation of 2 g cellulose l^{-1} were: butyric acid (66.7 %), butanol (21.8 %), acetic acid (7.4 %) and ethanol (4.1 %). For 4 g cellulose l^{-1}, butyric acid was still prevalent (54.7 %) followed by butanol (30.3 %), ethanol (12.4 %) and acetic acid (2.6 %). When examining the final values of SMP, we can conclude that even at 2 g cellulose l^{-1}, total acid content formed in relation to the SMP (TVFA/SMP) was 74.1 %, whereas the TVFA/SMP ratio decreased to 57.4 % at 4 g cellulose l^{-1} due to the higher production of alcohols. Therefore, the presence of butyric acid, acetic acid, and ethanol as well as a decrease in pH has been reported for the production of H_2 from cellulose.

Table 2 compares the H_2 yields with previous studies using different fermentation temperatures, H_2 producer microorganism sources, pH and culture conditions.

With 0.5 g cellulose l^{-1}, the H_2 yield obtained in this study without the addition of cellulase was 0.3 mmol H_2 g cellulose^{-1}, a value similar to that obtained by Levin et al. (2006) for a cellulose concentration of 10 g l^{-1}. In this study, the H_2 yields obtained for cellulose concentrations of 2 g l^{-1} (18.5 mmol H_2 g cellulose^{-1}) and 4 g l^{-1} (9.6 mmol H_2 g cellulose^{-1}) with the addition of cellulase at a mesophilic temperature were comparable to the H_2 yields obtained in other reports at thermophilic and hyper-thermophilic temperatures. This behavior indicates a change in the metabolic pathway at a cellulose concentration of 4 g l^{-1}, and the higher butanol and ethanol production can justify the lower H_2 yield.

Microbiological analysis

Microscopy analysis showed the predominance of both Gram-positive and Gram-negative rods, as well as rods with endospores (data not shown). These results suggest that endospore-forming bacteria were dominant in the H_2-producing mixed culture (Chong et al. 2009). *Clostridium*, for example, can resist high temperatures and acidic conditions because it forms endospores. Thus, the pre-treatment method applied to the inoculum to enrich the acidogenic consortium might have influenced the dominance of this group (Maintinguer et al. 2011). Microorganisms that do not form spores (coccus Gram-positive) were eliminated from the consortium.

The rumen microflora are composed of cellulolytic strains, such as *Ruminococcus albus* (Morrison and Miron 2000) and *R. succinogenes* (Schwarz 2001), and fermentative strains, such as *Clostridium* sp. and methanogens (Schwarz 2001). Therefore, the rumen is a highly cellulolytic ecosystem with a complex microbial population, and it has long been known for its active hydrolysis of cellulose (Hu et al. 2004).

Ruminococcus albus, R. flauefaciens, and *Fibrobacter succinogenes* were the predominant cellulolytic bacteria in the rumen. These species are non-spore-forming cocci and do not withstand adverse conditions (Levin et al. 2006). Several factors could alter the rumen microbial ecology, such as variations in pH, because cellulolytic bacteria are inhibited when the pH is lower than 6. Mccullough (1968) observed that a low ruminal pH reduced the activity and number of cellulolytic microorganisms. Cellulolytic cocci-shaped bacteria and methanogens were likely eliminated after the acid pre-treatment, and *Clostridium*-like bacteria were primarily favored due to their endospore-producing capacity. This result is consistent with other investigations of H_2 production by mixed cultures, which showed that only a few bacterial species survived the pre-treatments (Nissila et al. 2011b; Ratti et al. 2013). In addition, cocci-shaped bacteria, e.g., *Ruminococcus*, were not microscopically visible in this study.

PCR-DGGE analysis showed a difference in the number and pattern of bands among the biomass samples from H_2 production reactors (with and without cellulase) and the original inoculum before the pre-treatment. The higher number of bands (Fig. 3) in the original inoculum compared to the

Table 2 Comparison of the H_2 yield with previous studies

H_2 producer	Cellulosic substrate (g l^{-1})	Temp. (°C)	pH	H_2 yield (mmolH_2 g cellulose^{-1})	Culture condition	Reference
Mesophilic condition						
Digested sludge	Microcrystalline cellulose, 25	37	7.0	1.60	Batch	Lay et al. 1999
Digested sludge	Microcrystalline cellulose, 12.5	37	7.0	2.18	Batch	Lay et al. 1999
Heat-shocked mixed cultures	Cellulose, 4.7	26	6.0	0.02	Batch	Logan et al. 2002
Clostridium acetobutylicum	Cellulose, 10	37	7.0	0.17	Batch	Levin et al. 2006
Mixed culture from cow dung	Microcrystalline cellulose, 50	37	6.8	2.09	Batch	Ren et al. 2010
Rumen cellulose-degrading bacterial consortium	Avicel cellulose, 10	38	6.0	1.53	Batch	Wang et al. 2010
Acid-treated mixed culture from ruminal fluid	Microcrystalline cellulose without cellulase, 0.5	37	7.0	18.5	Batch	This study
Acid-treated mixed culture from ruminal fluid	Microcrystalline cellulose with cellulase, 2	37	7.0	18.5	Batch	This study
Acid-treated mixed culture from ruminal fluid	Microcrystalline cellulose, 4	37	7.0	9.6	Batch	This study
Thermophilic condition						
Sludge compost	Cellulose, 10	60	6.4	2.0	Batch	Ueno et al. 2001
Mixed culture from cow dung	Cellulose, 10	55	7.5	2.8	Batch	Lin and Hung 2008
Mixed culture from rumen	Cellulose, 5	60	7.3	1.93	Batch	Nissila et al. 2011a
Hyperthermophilic condition						
Mixed culture	Cellulose, 5	80	5.7	19.01	Continuous/ CSTR	Gadow et al. 2012

* *Temp.* temperature

biomass samples from reactors was due to the application of the acid pre-treatment to the rumen, which selected only a few bacteria. Moreover, the predominant bands present in the biomass of reactors with cellulase (arrow) could be distinguished from those of biomass from reactors without cellulase (arrow-head), in which cellulose consumption and H_2 production were not detected. The addition of cellulase to the reactors promoted the hydrolysis of cellulose to fermentable soluble sugars in the medium, allowing fermentative bacteria to growth and H_2 to be produced. In contrast, the bands found in samples from reactors without cellulase probably refer to bacteria not capable of degrading cellulose that were still maintained in the reactors by the presence of yeast extract and vitamins in the culture medium. Similar results were observed by Datar et al. (2007), who described that H_2 production was only observed after the addition of cellulase. The results obtained in the present study, and those by Datar et al. (2007), suggest that the inability of the consortium to produce H_2 from cellulose was due to the lack of cellulolytic microorganisms, which may have not been present initially or were inactivated during the pre-treatment.

According to the Jaccard coefficient of similarity, the natural microbial community of the ruminal fluid was 53 % similar to the community present in samples from reactors without cellulase and 30 % similar to those from reactors with cellulase. The coefficient was 79 % between the samples from reactors without

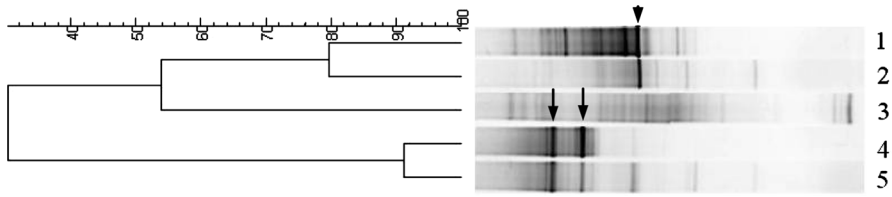

Fig. 3 Dendogram based on UPGMA and similarity coefficient using a Pearson correlation from the pattern of DGGE bands with a primer set to a bacteria domain (968FGC–1392R) (*1*) Control reactor fed with 4 g cellulose l^{-1}; (*2*) Control reactor fed with 2 g cellulose l^{-1}; (*3*) Inoculum ruminal fluid; (*4*) Reactor fed with 4 g cellulose l^{-1} and cellulase; (*5*) Reactor fed with 2 g cellulose $l^{-1}$and cellulase

cellulase and 91 % between the samples from reactors with cellulase (Fig. 3). Therefore, the different concentrations of cellulose in both reactors did not influence the structure of the microbial community. However, the acid pre-treatment and addition of cellulase apparently determined the structure of microbial community.

The sequence retrieved from a band was 99 % similar to that of an uncultured bacterium (Access number GU907815) deposited by Abreu et al. (2011). These authors also applied pre-treatments to suppress H_2-consuming microorganisms in the granular sludge and described that the predominant bacterial ribotypes present in the treated sludge were closely related to *Clostridium* species. This result strongly reinforces that *Clostridium* species were present in the pre-treated mixed culture in this study.

Ho et al. (2011) also used rumen fluid as the inoculums to produce H_2 and obtained a purified culture predominant in *Clostridium* sp. after repetitive batch cultures with napiergrass. Despite detecting a cellulolytic strain (*Ruminococcus* sp.) in the purified culture, the authors attributed the H_2 production to the *Clostridium* strains.

Saccharolytic *Clostridium* strains are widely studied because of their ability to form endospores and their potential to ferment carbohydrates to high levels of H_2 (Schwarz 2001; Ratti et al. 2013). Several species of *Clostridium* are acidogenic and ferment carbohydrates into butyrare, acetate, carbon dioxide and molecular H_2 (Maintinguer et al. 2011; Ratti et al. 2013). The typical metabolic route of *Clostridium* in an anaerobic process includes the production of H_2 and organic acids during the main phase of growth. However, when the population reaches the stationary growth phase, the metabolic route shifts from acid production to solvent production without H_2 generation. This shift usually occurs when the environmental pH drops to 5 or below (Lynd et al. 2002). The results of H_2 production, gradual reduction of pH and production of acids and alcohols observed in this study correspond to the metabolic characteristics of *Clostridium*. Thus, this group was probably the responsible for the generation of H_2, acids and solvents in the reactors.

In conclusion, the mixed culture obtained from ruminal fluid is a favorable source for the production of H_2 using cellulose. The acid treatment of the inoculum was considered to be the most suitable pre-treatment method because methane was not detected. However, this treatment may have inhibited cellulolytic bacteria because cellulose hydrolysis was verified only in the presence of cellulase. Increasing the cellulose concentration favored solvetogenesis, which was verified by a lower H_2 yield. H_2 was produced from cellulose primarily through the fermentation of butyric acid, a route typical of *Clostridium* species.

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