Hydrogenic and methanogenic fermentation of birch and conifer pulps

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Abstract

Conifer and birch pulp fermentation to hydrogen and methane was studied using dry and wet pulps with a compost enrichment culture at a pH range from 6 to 9. Hydrogen was produced at each pH, whilst methane was produced at all other pH values except pH 6 with dry conifer pulp and pH 9. Hydrogen and methane yields were generally higher with birch than with conifer pulp and the overall energy yields were higher with wet than dry pulp. The highest hydrogen and methane yields were 560 mL H_2/g TS with wet birch pulp at pH 6 and 4800 mL CH_4/g TS with wet conifer pulp at pH 7, respectively. Fermentation of dry pulps at pH 6 resulted in 160 mL H_2/g TS. Hydrogenic bacteria belonging to phyla Bacteroidetes, Firmicutes and Proteobacteria were present in the cultures. Hydrogen was also produced from chemically hydrolyzed pulps. The highest hydrogen yield from dry conifer pulp hydrolysate was 63 mL H_2/g TS. In summary, hydrogen and energy (calculated as H_2) yields were higher with direct fermentation than from chemically hydrolyzed pulps. However, chemical hydrolysis followed by hydrogen production required less than 10 days compared to 28 days required for direct pulp fermentation to hydrogen.

Keywords: Biohydrogen; methane; dark fermentation; bacterial hydrolysis; chemical hydrolysis.

1. Introduction

Lignocellulosic biomass is the most abundant raw material in nature [1] and has low cost [2]. Lignocellulose consists of lignin, cellulose and hemicellulose, from which cellulose and hemicellulose can be fermented to hydrogen and/or methane. Dark fermentative

hydrogen production has high hydrogen production rates, simple operation, and is independent of sunlight [3,4]. In nature, fermentation proceeds to methanogenesis. Renewable cellulosic materials, such as agricultural crops, pulp and paper industry wastewaters, food processing wastewaters, or algae, are amenable to hydrogenic and methanogenic fermentation (for a review, see [5]). Hydrogen fermentation of agricultural wastes and different waste materials have resulted in yield of 1-209 mL H_2/g VS and 0.7-389 mL H_2/g VSS/h (reviewed by [6] and [7]), respectively. Methane yields from agricultural resources and energy crops have been in the range of 30-590 mL CH_4/g VS and 290-420 mL CH_4/g TS (for reviews, see [8],[9]), respectively. Fermentation is inexpensive, has low energy requirements, is easy to operate, and proceeds in mild environmental conditions [5]. However, the rate of polymer hydrolysis and sugar yields may be low and the enrichment of suitable microbial community may be difficult [5,10].

Hydrogen gas has a high-energy content (122 MJ/kg) [11] and produces only water when combusted [12]. The enrichment procedure for hydrogen producing communities often involves pretreatment, such as heat, acid or base, to inhibit methanogens [13-16]. However, some pretreatments may also inhibit some cellulolytic and hydrogen producing bacteria [14,15,17]. Thus, with complex substrates pretreatments cannot always be used [18]. High hydrogen yields have also been reported with non-pretreated cultures [16,19]. Fermentation of food waste (initial concentration 8 g VS/L/d) resulted in 90 % cellulose degradation and a hydrogen yield of 2.2 mol H_2/mol hexose [18]. In our previous study, an enriched compost culture degraded 57 % of the cellulose (initial concentration 5 g/L) and produced hydrogen with a yield of 2.4 mol H_2/mol $hexose_{degraded}$ [16].

Temperature and pH are important factors in direct cellulose fermentation to hydrogen/methane. The pH affects cellulose hydrolysis, metabolic pathways as well as

hydrogen and methane production [19-22]. The pH influences microbial community composition and may thus be used to select for hydrogen producers and inhibit methanogens [21,22]. Hydrogen production from cellulosic substrates has been reported at pH values between 6-8.5 [19] and 4-10 [21]. Hu et al. [23] reported that pH below 6.0 inhibited ruminal, cellulolytic bacteria. The optimal pH for methanogenic fermentation is between 6.8 and 7.2, and pH below 6.0 or above 8.5 inhibits methanogens [22]. Temperature affects enzymatic reaction rates, hydrogen and methane production, metabolite distribution, and hydrogen partial pressure that influences hydrogen production [22,24-26]. Majority of hydrogen and methane production studies have been done with mesophiles i.e. between 30 and 40ºC (for reviews, see [9,27]).

Chemical hydrolysis can be used for hydrolyzing cellulosic materials into sugars [28-30] prior to hydrogen fermentation. Chu et al. [31] hydrolyzed cotton cellulose with 55 % H_2SO_4 with a sugar yield of 64-73 % (g reduced sugar/g cotton cellulose) and reported a 0.99 mol H_2/mol reduced sugar yield. Lo et al. [6] hydrolyzed sugarcane bagasse either with phosphoric acid (at 50ºC for 30 min) or sodium hydroxide (autoclaved at 120ºC for 20 min). The resulting reduced sugar concentrations were 1.34 and 1.30 g/L for NaOH- and H_3PO_4-treated bagasse, respectively, with the corresponding hydrogen yields of 125 and 147 mL H_2/g reduced sugar.

In this study, cellulosic substrates (dry and wet birch and conifer pulps obtained from a paper factory) were fermented for hydrogen and methane production. Microbial communities were enriched from a compost sample at 37ºC, at four different pH values (6,7,8,9) to select for hydrogen producing cultures. In the second enrichment step, methanogens were inhibited with 2-bromoethanesulfonic acid (BESA) to determine the hydrogen production potential at pH 7. The microbial communities responsible for direct

pulp fermentation to hydrogen were characterized using polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE). The effect of acid hydrolysis (55 % H_2SO_4 at 37ºC) of pulp on hydrogen fermentation was determined.

2. Materials and Methods

2.1 Direct pulp fermentation to hydrogen and methane

The wet and dry pulp materials made from birch or conifer were obtained from a paper factory (UPM-Kymmene Oyj, Jämsänkoski, Finland). Dry pulps were delivered as paper sheets that were cut to pieces (1-2 cm), while wet pulp was delivered as wet mass and was added according to the total solid (TS) weight. Microbial communities were enriched from compost sample obtained from a Solid Waste Management Site (Tarastenjärvi, Tampere, Finland). The enrichment was done without pretreatment in 500 mL batch bottles with a working volume of 150 mL. One liter medium contained 10.7 g NaH_2PO_4, 3.2 g Na_2HPO_4, 0.6 g NH_4Cl, 0.125 g KH_2PO_4, 0.11 g $CaCl_2 \cdot 2H_2O$, 0.1 g $MgCl_2 \cdot 6H_2O$, 4 g $NaHCO_3$, 2.18 g $FeCl_2 \cdot 4H_2O$, 50 µg H_3BO_3, 50 µg $ZnCl_2$, 38 µg $CuCl_2 \cdot 2H_2O$, 41 µg $MnCl_2 \cdot 2H_2O$, 50 µg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 50 µg $AlCl_3$, 50 µg $CoCl_2 \cdot 6H_2O$, 50 µg $NiCl_2 \cdot 6H_2O$, 0.5 g EDTA, 2 g yeast extract, 0.013 g $Na_2S_2O_4$ and vitamin solution (DSMZ medium No141, German Collection of Microorganisms and Cell Cultures). Batch bottles were incubated at 37ºC with a mechanical mixing of 150 rpm (dry pulps) or without mixing (wet pulps). The initial concentrations of dry pulps, wet birch and wet conifer pulps were 5, 1.1 and 1.2 g TS/L. Growth on each substrate was tested in triplicate at four different pH values (6, 7, 8 and 9). The fine compost material was separated from the non-degradable and larger pieces and 20 g/L of the fine compost sample was added to the batch bottles as inoculum. Gas and liquid samples were taken

two or three times a week and pH was adjusted (± 0.1) at the same time.

The second enrichment step was done at pH 7 with substrate concentration of 5 g TS/L. The total and working volumes of batch bottles were 500 and 250 mL and they were inoculated in triplicate from the first enrichment cultures (10 % v/v) at pH 7. Methanogens were inhibited by adding 20 mM BESA. Batch bottles were incubated at 37ºC with mechanical mixing of 150 rpm. Sampling was done as in the first enrichment step.

2.2 Chemical hydrolysis

The four different pulp materials were chemically hydrolyzed with 55 % H_2SO_4 at 37ºC with mechanical mixing of 150 rpm. First, the optimal time (from 15 to 180 min) for hydrolysis was determined with initial substrate concentrations of 50, 11 and 12 g/L for dry pulps, wet birch pulp and wet conifer pulp, respectively. Sulfate ions were removed from the solution by adding calcium oxide (41.35 g CaO/100 mL 55 % H_2SO_4) and MQ-water (4:5 v/v) to the reaction mix, which also stopped acid hydrolysis due to neutralization. The reaction mix was cooled down to room temperature and the solids were removed by filtration (0.45 μm, Whatmann GC/C-filters), after which the concentrations of total sugars were analyzed.

2.3 Hydrogen production from acid hydrolysate

The sugars obtained from acid hydrolysis of dry pulps were tested for hydrogen production in batch assays. Hydrogen production was studied with a hot spring culture enriched for hydrogen production [26] and known to ferment many hexoses and pentoses

(results not published). Batch experiments were conducted in triplicate in 120 mL flasks with a working volume of 50 mL. The medium was the same used in direct fermentation experiment. In the beginning, 4 % inoculum (v/v) and 10 g total sugars/L were added to the medium and pH was adjusted to 7.0 ± 0.1. Hydrogen was produced at 37ºC for 5 days with mechanical mixing of 150 rpm. The biogas and liquid samples were taken and analyzed every second day.

The effect of initial pH (5, 6, 7, 8 and 9) and temperature (from 25 to 43°C) on hydrogen production from dry conifer pulp hydrolysate was determined. The pH experiment was done in triplicate at 37ºC in 120 mL bottles with a working volume of 50 mL, substrate concentration of 10 g total sugars/L and 4 % (v/v) inoculum that was used from the previous experiment. The temperature experiment was performed in a temperature-gradient incubator (Test Tube Oscillator, Terratec) with duplicate samples. The cultures were grown in 25 mL anaerobic tubes with 10 mL working volume, 4 % (v/v) inoculum and initial concentration of 10 g total sugars/L. Mixing was kept at 60 oscillations/min.

2.4 Chemical analysis

The amount of total sugars were analyzed with phenol-sulphuric acid method modified from Dubois et al. [32], where the volumes of sample, 5 % phenol and H_2SO_4 were 1, 0.5 and 2.5 mL, respectively. The total solids were analyzed according to the standard SFS 3008 [33], where substrate was added to a weighed cup. The cup and substrate were heated at 105°C overnight and weighed after cooling.

The overpressure from the batch bottles and the gas composition (H_2, CH_4 and CO_2) were analyzed according to Nissilä et al. [16]. The concentrations of different sugars (glucose, xylose, L-arabinose, maltose, sucrose, lactose and cellobiose), volatile fatty acids (VFAs) and alcohols (lactate, formate, propionate, acetate, ethanol and butyrate) were analyzed with a Shimadzu High Performance Liquid Chromatography (HPLC) with a Rezex RHM-Monosaccharide column (Phenomenex) and a refraction index detector (Shimadzu). Mobile phase was 5 mM H_2SO_4 with a flow rate of 0.5 mL/min. Samples were filtered (0.2 μm) and diluted with MQ-water before analysis. The hydrogen yield from hydrolysate was calculated according to Chu et al. [31]. The energy yields from hydrogen and methane were calculated based on their lower heating values, 120 MJ/kg and 50 MJ/kg, respectively.

2.5 Microbial community analysis

Bacterial communities were analyzed with DNA extraction and PCR-DGGE of partial 16S rRNA genes followed by their sequencing. Duplicate samples were taken from the end points of bacterial hydrolysis experiment and stored at -20ºC. DNA was extracted with a PowerSoil™ DNA isolation kit (MoBio Laboratories, Inc.). PCR-DGGE analysis, the re-amplification of the bands as well as analysis of the sequence data was done as described by Nissilä et al. [16].

3. Results and Discussion

3.1 Direct pulp fermentation to hydrogen and methane

Direct pulp fermentation to hydrogen and methane was studied with four different pulp materials at 37ºC and the effect of pH on hydrogen and methane production potentials was determined. The first enrichment step was without culture pretreatment, since pretreatments may inhibit cellulolytic or hydrogen producing bacteria [14,15,17] and are costly in large scale. Methane was produced in the first enrichment step and thus, methanogens were inhibited with BESA in the second enrichment step.

3.1.1 Enrichment of hydrogenic and methanogenic communities

Compost sample was used for enriching cellulolytic, hydrogen and methane producing communities. Simultaneous cellulose hydrolysis and hydrogen production from pure cellulose with a high hydrogen yield of 2.4 mol H_2/mol $hexose_{degraded}$ was reported earlier with a culture enriched from the same compost [16]. Hydrogen was produced at pH values from 6 to 9, while methane was produced in incubations expect at pH 6 from dry conifer pulp and at pH 9 with all substrates (Fig. 1). This result is in line with known optimum pH range of 6.5 to 8.2 [34] and inhibitory pH above 8.5 [22] for methanogens. Reports on hydrogen production at pH 9 are scarce in mixed cultures. However, hydrogen production at pH 9 was reported from cassava stillage with a yield of 39.0 ± 1.9 mL H_2/g VS [35] and from starch with a yield of 49 mL H_2/g starch [36]. Our results show that hydrogen production from dry conifer pulp would be feasible at pH 6, since rather high hydrogen yield of 160 ± 10 mL H_2/g TS was obtained with no methane production. Shin and Youn [18] and Zhu et al. [37] reported hydrogen yields of 125 and 209 mL H_2/g VS

from food waste and liquid swine manure, respectively.

Fig. 1

With dry pulps at pH 7 and 8, higher hydrogen yields were obtained from conifer pulp while methane yields were higher from birch pulp (Figure 1). Higher overall energy yields (calculated from hydrogen and methane yields) were obtained from dry birch than dry conifer pulp. The highest overall H_2 and CH_4 energy yields were obtained at pH 7 and 8 and they were 8.6 ± 1.9 and 14.0 ± 1.2 kJ/g TS from dry conifer pulp and 15.4 ± 0.4 and 14.7 ± 1.4 kJ/g TS from dry birch pulp, respectively. The methane yield from wet conifer pulp at pH 7 differed considerably from the other batch cultures resulting in the highest energy yield of 163 ± 11 kJ/g TS. High methane yield was obtained as acetate and butyrate were effectively used for methane production (Fig. 2.B). Methane yields of 356 and 369 mL CH_4/g VS have been reported from cellulose [38] and from office paper [39], respectively. The energy yields at pH 6, 7 and 8 were 30 ± 6, 163 ± 11 and 39 ± 4 kJ/g TS from wet conifer pulp and 41 ± 16, 35 ± 5 and 42 ± 6 kJ/g TS from wet birch pulp, respectively. The hydrogen and methane yields (as mL/g TS) were considerably higher with wet than dry pulps (Fig. 1). This was likely due to lower initial total solid concentration and higher surface area of wet pulp material [40].

Fig. 2

The total sugars were monitored throughout the experiment and their amount remained between 0.06 and 0.48 g/L, and consisted mainly of glucose (results not shown). The low sugar concentrations show that sugars obtained from cellulose hydrolysis were effectively used for dark fermentation. The metabolite distribution of wet conifer pulp culture at pH

7 differed considerably from other assays and consisted mainly of propionate due to efficient use of acetate and butyrate for methane production. Methane production from propionate was reported to be more difficult than that from acetate or butyrate [41]. In other batch bottles, acetate was the main soluble metabolite and constituted 56-83 % of the total soluble metabolites (Figure 2). At pH 6, butyrate was the other main soluble metabolite, while at other pH values acetate production was followed by propionate production. The soluble metabolites distribution and concentrations at pH 9 were similar to pH values 7 and 8 despite the lower hydrogen production yields at pH 9.

Methanogens were inhibited with BESA in the second enrichment step at pH 7 with all pulp materials (Fig. S1). The hydrogen yields with dry birch, dry conifer, wet birch and wet conifer were 68 ± 6, 76 ± 4, 66 ± 10 and 68 ± 7 mL H_2/g TS, respectively. The hydrogen production yields were lower than in the first enrichment step, which may have been due to BESA inhibition of some Clostridia [17]. Acetate was the main soluble metabolite, while propionate concentration was lower than in the first enrichment culture.

3.1.3 Bacterial communities responsible for direct fermentation of pulp to hydrogen

Bacterial communities were analyzed with PCR-DGGE followed by band sequencing (Table 1, Fig. 4). The samples were taken from batch assays at the end points of direct conifer or birch pulp fermentation to hydrogen at each pH. The bacterial communities belonged mainly to phyla Bacteroidetes, Firmicutes and Proteobacteria. Bacterium closely related to *Bacteroides* sp., a bacterium isolated from a landfill leachate bioreactor, was present in all samples. Bacteroidetes are obligately anaerobic and saccharolytic bacteria [42]. Furthermore, two Spirochaetes were detected at pH 6, 7 and 8 with both conifer and birch pulps. Spirochaetes are saccharolytic bacteria that ferment

carbohydrates to acetate, lactate, CO_2 and H_2 [43].

Fig. 4, Table 1

Bacteria detected with both substrates at pH 7 and 8 included bacteria closely related to *Ruminofilibacter xylanolyticum*, *Comamonas denitrificans*, a denitrigying bacterium isolated from activated sludge [44], and bacteria distantly related to *Clostridium populeti* (94.9 % similarity) and *Parabacteroides goldsteinii* (90.3-95.3 %). *Ruminofilibacter xylanolyticum* is a rumen bacterium that is involved in the digestion of xylan. It has previously been detected in biogas plant fed with maize silage, green rye and liquid manure [45] and during the production of biogas from grass silage [46]. *C. populeti* is a cellulolytic bacterium that grows optimally at pH 7 with metabolic products of acetate, butyrate, lactate, CO_2 and H_2 [47]. *P. goldsteinii* ferments e.g. cellobiose, glucose and xylose to various acids [48]. Furthermore, *Ruminobacillus xylanolyticum* was present at pH 8. *Ruminobacillus xylanolyticum* is a xylanolytic species isolated from rumen and has been earlier detected in a thermophilic anaerobic reactor co-treating organic fraction of municipal solid wastes and fat, oil and grease wastes [49].

Bacteria closely related to *Alcaligenes faecalis* and *Escherichia coli* were present at pH 6 and 9 with both birch and conifer pulps. E. *coli* is a facultatively anaerobic bacterium that carries out mixed acid fermentation to hydrogen [50]. The hydrogen yields obtained with *E. coli* include 0.84 mol H_2/mol glucose [51] and 0.54 mol H_2/mol glucose [50]. However, *E. coli* has also two hydrogenases that can consume hydrogen [52]. *A. faecalis* reduces nitrite anaerobically and can utilize acetate or caprate as carbon sources [53]. Furthermore, at pH 9 bacteria closely related to *Pseudomonas pertucinogena* and *Citrobacter sedlakii* as well as a bacterium distantly related to *Clostridium ultunense*

(95.4 %) were detected. *P. pertucinogena* grows on few organic acids and on L-alanine [54], while *C. sedlakii* grows on e.g. cellobiose, lactose and glycerol [55]. *C. ultunense* grows optimally at 37ºC, between pH 5 and 10 and produces acetate, formate, H_2 and CO_2 as the metabolites from glucose [56]. *C. ultunense* has been found from thermophilic co-digester treating organic fraction of municipal solid wastes and fat, oil and grease wastes [49].

Our results indicate that bacteria responsible for cellulose fermentation were different at different pH values, e.g. *C. populati* likely hydrolyzed cellulose at pH 7 and 8. Three saccharolytic bacteria were present at pH values from 6 to 9. Hydrogenic bacteria, on the other hand, were also dependent on pH, and different bacteria likely produced hydrogen at pH 9, e.g. *C. ultunense*, than at other pH values.

3.2 Chemical hydrolysis followed by H_2 production

3.2.1 Optimizing chemical hydrolysis

Acid hydrolysis was done with 55 % sulphuric acid at 37ºC. The optimal time for sulphuric acid hydrolysis was determined with all four pulp materials from 15 to 180 min and the results were as shown in Fig. 4. The highest sugar yields with dry birch and conifer pulps were obtained at 180 min and were 84.3 and 69.6 %, respectively. The highest sugar yields with wet birch and conifer pulps were obtained at 90 min and were 36.8 and 32.8 %, respectively. Dry conifer and dry birch pulps were selected for hydrogen production tests because higher sugar yields were obtained with them. Dry birch and conifer pulp hydrolysates consisted of 30 % cellobiose, 38 % glucose and 32 % xylose and of 37 % cellobiose, 44 % glucose and 19 % xylose, respectively.

Fig. 4

Acid hydrolysis is often done at increased temperature and pressure. For example, acid hydrolysis with sulfuric, nitric or hydrochloric acid has been performed by autoclaving the samples at 150ºC for one hour [30] and sulphuric acid hydrolysis by autoclaving the samples at 121°C and 1.5 kg/cm^2 pressure for 60 min [28]. However, using increased temperature and pressure is energy intensive. Acid hydrolysis at milder conditions would be more feasible. In addition to temperature and pressure, one of the main parameters for acid hydrolysis is the treatment time. If the acid hydrolysis time is too long, the sugars are further converted to other compounds, such as furfural, that are potential inhibitors of dark fermentation [57]. Short treatment time, on the other hand, results in low sugar yields.

3.2.2 Hydrogen production from acid hydrolysates

Hydrogen production was studied from dry birch and conifer hydrolysates with a hot spring culture [26,58] enriched for hydrogen production. This inoculum was used since it ferments various hexoses and pentoses present in hydrolysates (results not published). The microbial community of the hot spring culture has been characterized earlier at different temperatures [26] and pH values [58]. 84 ± 1 and 81 ± 2 % of the initial sugars were degraded from dry conifer and birch hydrolysates, respectively. Hydrogen yields per total sugars added to the medium were 0.40 ± 0.05 mol H_2/mol glucose and 46 ± 5 mL H_2/g total sugar from dry conifer and 0.40 ± 0.31 mol H_2/mol glucose and 45 ± 34 mL H_2/g total sugar from dry birch hydrolysates, respectively. If the efficiency of hydrolysis is taken into account (sugar yields from dry birch and conifer pulps were 84 and 70 %,

respectively), the hydrogen yields were 0.28 ± 0.03 mol H_2/mol sugar and 32 ± 4 mL H_2/g total sugar from dry conifer and 0.34 ± 0.26 mol H_2/mol sugar and 38 ± 29 mL H_2/g total sugar from dry birch pulps, respectively. Dry conifer pulp was selected for further studies, since lower standard deviations and higher sugar degradations were obtained with it.

3.2.3 Optimizing hydrogen production from dry conifer pulp hydrolysate

The effect of initial pH (from 5 to 9) on hydrogen production was studied with dry conifer pulp hydrolysate and the results were as shown in Table 2. At initial pH 5 hydrogen production was negligible and the metabolism was directed towards lactate production. The highest hydrogen yield and total sugar utilization efficiency was obtained at initial pH 6 with lactate, acetate and ethanol as the main soluble metabolites. The hydrogen yield at initial pH 6 was 91 ± 1 mL H_2/g total sugar, which corresponds to 63 ± 1 mL H_2/g total sugar and 0.54 ± 0.01 mol H_2/mol sugar when the efficiency of acid hydrolysis is taken into account. Further increases in the initial pH decreased the hydrogen yields and increased lactate production. The optimal pH for hydrogen production depends on the microbial community and the substrate used. Optimal initial pH for hydrogen production from cotton cellulose hydrolysate with a mixed culture was 8.2 with maximum hydrogen yield of 0.95 mol H_2/mol reduced sugar [31]. Pattra et al. [28] reported optimal initial pH of 5.5 for hydrogen production from sugarcane bagasse hydrolysate with *Clostridium butyricum* with a hydrogen yield of 1.73 mol H_2/mol sugar.

Table 2

The effect of temperature (from 25 to 43ºC) on hydrogen production from dry conifer

pulp hydrolysate was studied at the initial pH 6 and the results were as shown in Table 3. The hydrogen production yields were the highest at 28 and 34ºC being 0.24 and 0.21 mol H_2/mol sugar, respectively. The highest total sugar removal of 78.5 % was obtained at 34ºC. The soluble metabolite profiles were different at the two optimal temperatures. At 28ºC butyrate was the main metabolite followed by lactate, ethanol and acetate, while at 34ºC lactate production dominated and was followed by butyrate, ethanol and acetate production. The 28°C was considered as the optimal temperature, since lactate producing bacteria may excrete bacteriocins that can inhibit hydrogen production [59].

Table 3

3.3 Comparison of bacterial and chemical hydrolysis

Hydrogen was produced from dry conifer pulp by direct fermentation and after chemical hydrolysis. Concentrated sulphuric acid hydrolysis of dry conifer pulp at 37°C for 180 min resulted in total sugar yield of 69.6 % (g total sugar/g TS). The highest hydrogen yield from the hydrolysate was 63 mL H_2/g TS corresponding to energy yield of 0.6 kJ/g TS. Direct pulp fermentation to hydrogen and methane was done with a compost enriched culture. In the first enrichment step, the maximum hydrogen yield at pH 8 was 120 mL H_2/g TS with simultaneous production of methane, 390 mL CH_4/g TS, corresponding to energy yields of 1.2 and 12.8 kJ/g TS, respectively. In the second enrichment step methanogens were inhibited, which resulted in hydrogen yield of 76 mL H_2/g TS corresponding to energy yield of 0.75 kJ/g TS.

These results show that higher hydrogen yields were obtained with direct bacterial fermentation than after chemical hydrolysis. However, direct pulp fermentation to

hydrogen and methane required 28 days, while chemical hydrolysis followed by hydrogen production required less than 10 days. Lower hydrogen yield from acid hydrolysate may be due to the presence of sulphate ions that were not totally removed after acid hydrolysis. Sulphate may increase the growth of sulphate-reducing bacteria [60]. In addition, formation of inhibitory compounds, such as furfural and 5-hydroxymethyl-furfural, during acid hydrolysis may reduce hydrogen yields from acid hydrolysates [28,31,57].

The hydrogen yield obtained with pulp fermentation to hydrogen was comparable to other studies with cellulosic materials. Hydrogen yields from fodder maize and wilted perennial ryegrass were 62.4 ± 6.1 and 75.6 ± 8.8 mL H_2/g dry matter, respectively [61]. The maximum hydrogen yield of 0.77 mol H_2/mol sugar obtained from acid hydrolysate was also in the range obtained in other studies. The hydrogen yield from waste ground wheat hydrolysate (10 g total sugar/L) was 1.46 mol H_2/mol glucose [62], while cotton cellulose hydrolysate (15 g reduced sugar/L) yielded 0.99 mol H_2/mol reduced sugar [31].

4. Conclusions

Conifer and birch pulps were amenable to direct fermentation to hydrogen and methane. The following conclusions can be drawn from this study:

1) Direct pulp fermentation resulted in the highest yields of 130 mL H_2/g TS from dry birch pulp at pH 6 and 4800 mL CH_4/g TS from wet conifer pulp at pH 7, respectively.
2) Bacteria responsible for direct pulp fermentation to hydrogen belonged mainly to phyla *Bacteroidetes*, *Firmicutes* and *Proteobacteria*.
3) Hydrogen was produced from chemically hydrolyzed pulps resulting in the highest sugar yields of 33 – 37 % and 70 – 84 % after 90 and 180 min treatment with wet and dry pulps,

respectively.

4) The highest hydrogen yield from dry conifer pulp hydrolysate was 63 mL H_2/g TS corresponding to energy yield of 0.6 kJ/g TS.

5) Direct fermentation produced higher hydrogen yields than that of chemically hydrolyzed pulp. The direct pulp fermentation to hydrogen and methane required 28 days. Less than 10 days was required for hydrogen fermentation of chemically hydrolyzed pulp.

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Figure captions:

Figure 1. Hydrogen, methane and carbon dioxide yields from dry (A) and wet (B) conifer and birch pulps during simultaneous cellulose hydrolysis and hydrogen production at different pH values (6,7,8,9) in the 1st enrichment step. Standard deviations are shown in the figures.

Figure 2. Production of soluble metabolites from dry (A) and wet (B) conifer and birch pulps at different pH values (6,7,8,9) during simultaneous bacterial hydrolysis and hydrogen production.

Figure 3. Bacterial community profiles determined with PCR-DGGE of partial 16S rRNA genes of cultures producing hydrogen from dry conifer and birch substrates at different pH values. See Table 1 for the labeled bands. Inoc. = inoculum.

Figure 4. Production of total sugars during acid hydrolysis of dry (A) and wet (B) conifer and birch pulps. Standard deviations are shown in the figures. The standard deviations from dry conifer hydrolysis are so small that they cannot be detected in the figure.

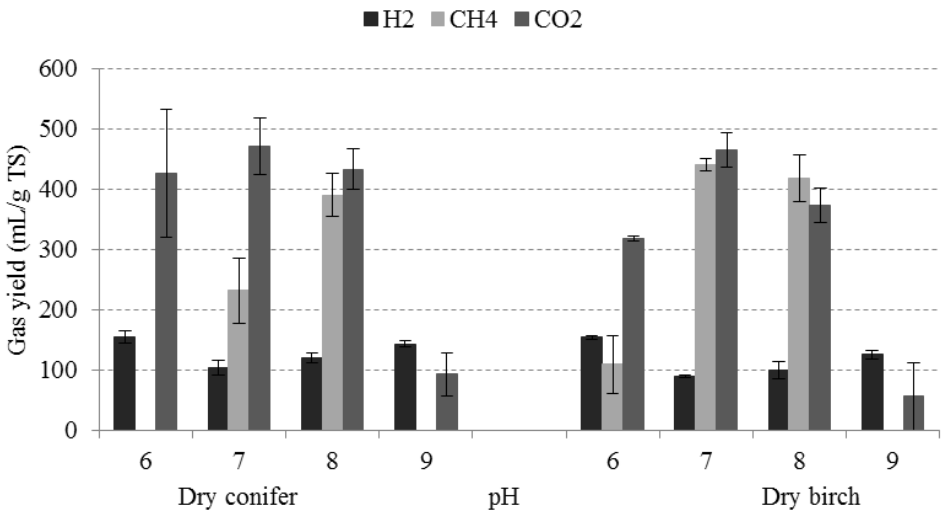
Table captions:

Table 1. Affiliations of DGGE fragments determined by their 16S rRNA sequence from cultures fermenting dry conifer and birch pulps at different pH values.

Table 2. Hydrogen production from dry conifer pulp hydrolysate at different pH values including cumulative hydrogen production (mL H_2), hydrogen yield per utilized sugars (mol H_2/mol sugar), utilization of total sugars (DE) and the amount of total (g/L) and individual (%) soluble metabolites. Standard deviations are shown in the table.

Table 3. Hydrogen production results from dry conifer pulp hydrolysate at different temperatures including cumulative hydrogen production (mL H_2), hydrogen yield per utilized sugars (mol H_2/mol sugar), utilization of total sugars (DE) and the amount of total and individual soluble metabolites. Standard deviations are shown in the table.

A



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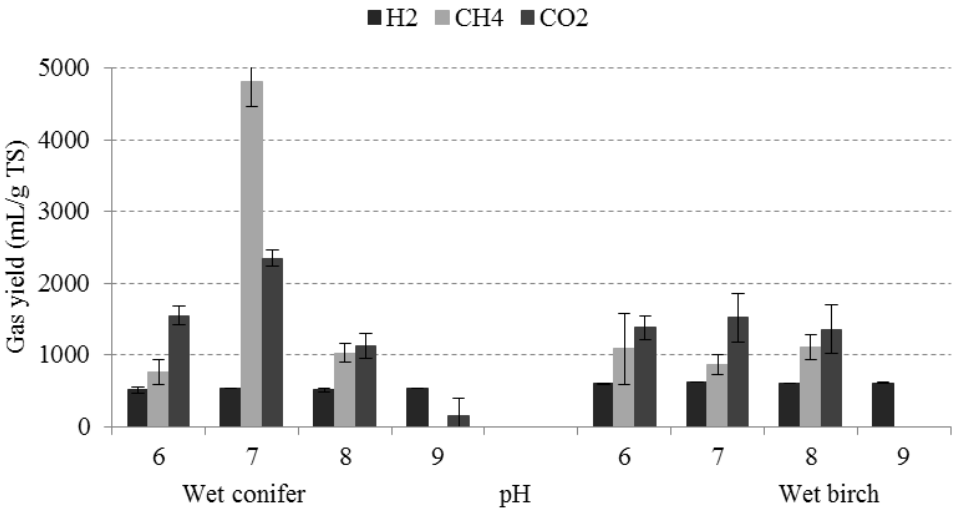

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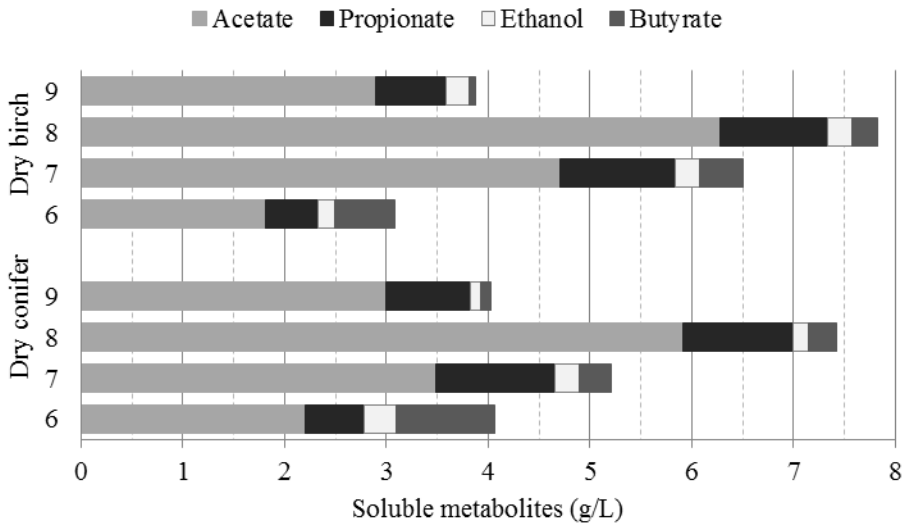
A



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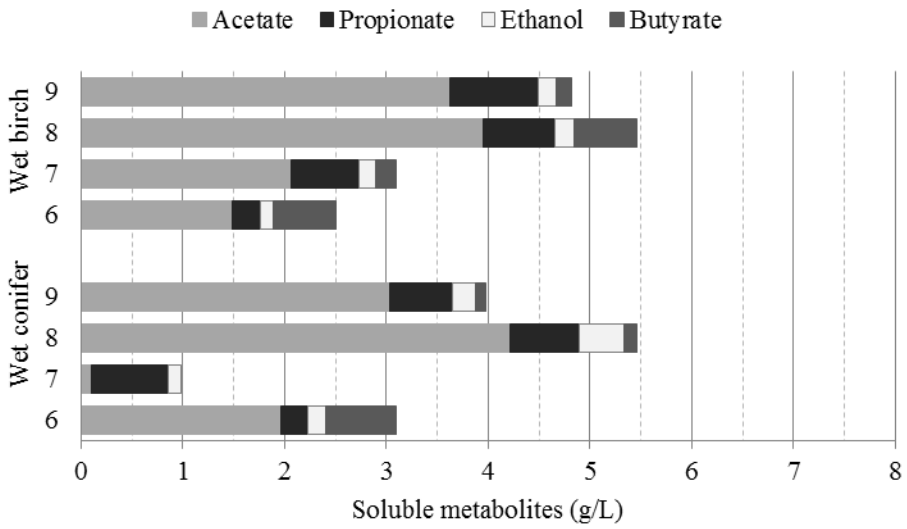

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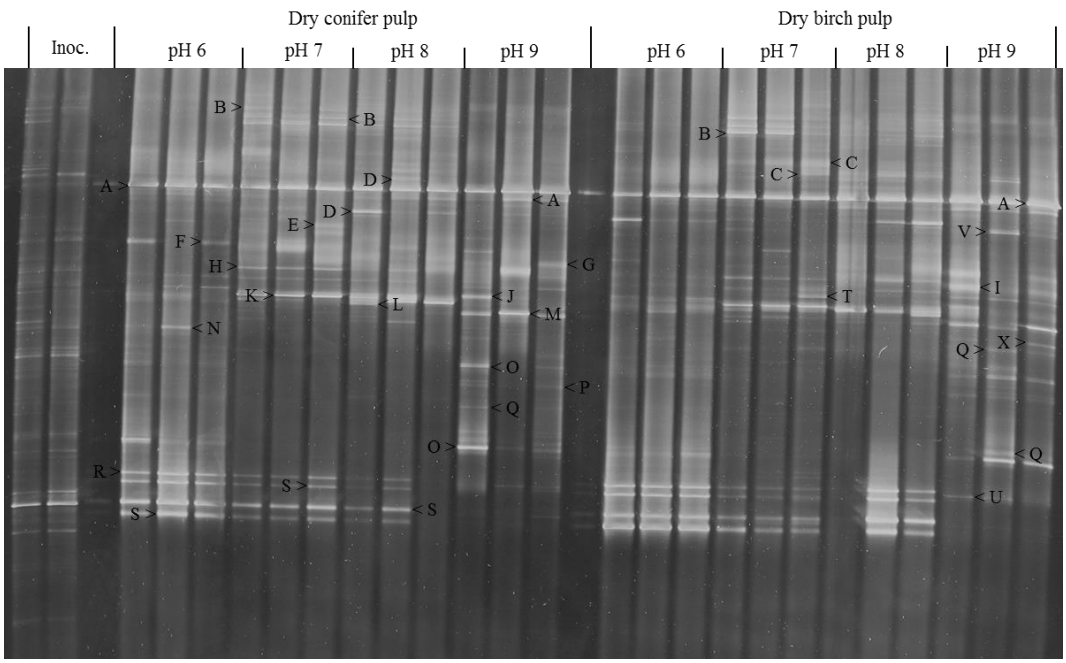

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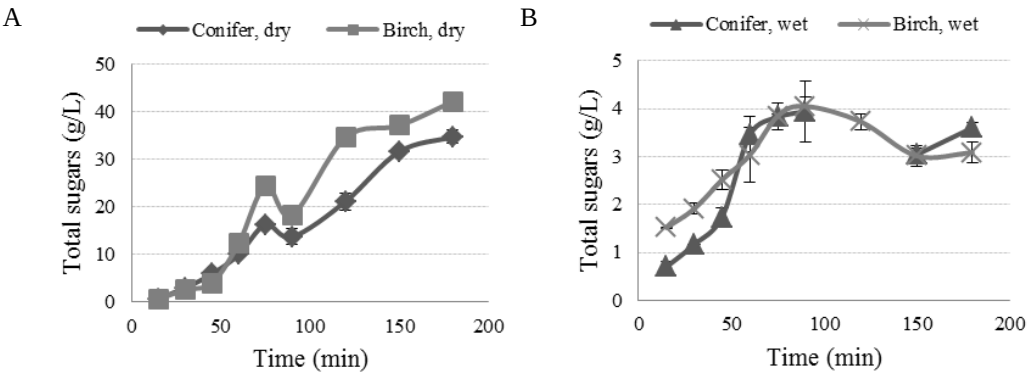

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Table 1. Affiliations of DGGE fragments determined by their 16S rRNA sequence from cultures fermenting dry conifer and birch pulps at different pH values.

Band[a]	**Phylum**[b]	**Affiliation (acc)**[c]	**Sim (%)**[d]	**SL (bp)**[e]
A		*Bacteroides* sp. (AY554420)	99.0-100	208-503
B	Bacteroidetes	*Ruminofilibacter xylanolyticum* (DQ141183)	97.5-99.4	451-506
C	Bacteroidetes	*Parabacteroides goldsteinii* (AB547650)	90.3-95.3	503-504
D		*Ruminobacillus xylanolyticum* (DQ178248)	96.0-96.3	502-507
E	Firmicutes	*Ruminococcus flavefaciens* (JN866826)	97.5	540
F	Firmicutes	Uncultured *Firmicutes* (AB646230)	100	451
G	Firmicutes	*Clostridium ultunense* (NR_026531)	95.4	473
H	Firmicutes	*Clostridium populeti* (NR_026103)	94.9	473
I	Proteobacteria	*Pseudomonas pertucinogena* (HM031486)	100	503
J	Proteobacteria	*Alcaligenes faecalis* (EF011115)	97.0	478
K	Proteobacteria	*Comamonas denitrificans* (DQ836252)	98.8	504
L		Uncultured bacterium (GQ134001)	100	484
M		Uncultured prokaryote (HQ154908)	100	488
N	Firmicutes	*Lysinibacillus fusiformis* (GU904698)	98.2	510
O	Proteobacteria	*Escherichia coli* (AY319394)	99.3-99.8	452-485
P	Proteobacteria	*Cronobacter sakazakii* (JQ312042)	97.9	189
Q	Proteobacteria	*Citrobacter sedlakii* (AB682286)	95.5-99.7	398-585
R	Spirochaetes	*Sphaerochaeta globus* (AF357916)	90.4	516
S	Spirochaetes	Uncultured *spirochete* (JF736651)	99.4-100	450-519
T	Firmicutes	*Clostridium straminisolvens* (NR_024829)	96.5	476
U	Actinobacteria	*Actinotalea fermentans* (AB639014)	96.0	426
V	Firmicutes	*Erysipelothrix inopinata* (NR_025594)	93.9	485
X	Firmicutes	Uncultured *Firmicutes* (GU958167)	95.9	412

[a] Band mark in Figure 5
[b] Phylum according to Ribosomal Database Project II
[c] Closest species in GenBank with accession number
[d] Similarity (%)
[e] Sequence length (base pairs)

Table 2. Hydrogen production from dry conifer pulp hydrolysate at different pH values including cumulative hydrogen production (mL H_2), hydrogen yield per utilized sugars (mol H_2/mol sugar), utilization of total sugars (DE) and the amount of total (g/L) and individual (%) soluble metabolites. Standard deviations are shown in the table.

pH	**mL H_2**	**mol H_2/ mol sugar**	**DE (%)**	**VFA (g/L)**	**HLa (%)**	**HFo (%)**	**HAc (%)**	**HPr (%)**	**EtOH (%)**	**HBu (%)**
5	4.5 ± 1.6	0.09 ± 0.03	73.0 ± 3.6	4.5 ± 2.0	90.4	ND	1.7	ND	2.4	5.5
6	45.6 ± 0.5	0.77 ± 0.01	86.8 ± 2.2	2.2 ± 0.3	39.3	6.0	23.1	1.9	21.3	10.3
7	30.9 ± 2.0	0.55 ±0.04	82.7 ± 1.0	4.6 ± 0.9	52.8	4.9	18.8	4.2	18.3	5.1
8	23.7 ± 2.5	0.42 ±0.04	83.0 ± 0.8	5.8 ± 1.5	63.3	3.8	16.4	3.2	15.2	1.3
9	15.6 ± 1.0	0.28 ±0.02	82.2 ± 0.4	6.1 ± 1.6	64.7	5.5	13.0	3.0	15.0	1.7

VFA: total amount of soluble metabolites; HLa: lactic acid; HFo: formic acid; HAc: acetic acid; HPr: propionic acid; EtOH: ethanol; HBu: butyric acid; ND: not detected.

Table 3. Hydrogen production results from dry conifer pulp hydrolysate at different temperatures including cumulative hydrogen production (mL H_2), hydrogen yield per utilized sugars (mol H_2/mol sugar), utilization of total sugars (DE) and the amount of total and individual soluble metabolites. Standard deviations are shown in the table.

T (ºC)	**mL H_2**	**mol H_2/ mol sugar**	**DE (%)**	**VFA (g/L)**	**HLa (%)**	**HFo (%)**	**HAc (%)**	**HPr (%)**	**EtOH (%)**	**HBu (%)**
25	1.9 ± 0.2	0.20 ± 0.03	69.7 ± 4.8	3.9 ± 2.1	95.5	ND	1.8	ND	2.7	ND
28	2.3[a]	0.24[a]	71.5	3.2	16.9	1.9	15.4	2.0	15.4	48.3
34	2.2 ± 0.6	0.21 ± 0.06	78.5 ± 2.0	5.5 ± 0.7	37.4	4.8	15.2	3.4	15.7	23.7
37	1.8 ± 1.1	0.18 ±0.11	73.5 ± 0.3	6.5 ± 1.4	56.9	1.8	16.6	3.1	13.2	8.4
40	1.2 ± 0.0	0.11 ± 0.00	75.1 ± 7.4	6.2 ± 1.3	55.6	4.8	13.3	3.0	14.2	9.1
44	0.4 ± 0.0	0.04 ± 0.00	71.5 ± 2.2	6.5 ± 1.5	60.7	5.9	12.0	2.8	13.9	4.7

[a] The other clture was omitted since it did not grow.

VFA: total aount of soluble metabolites; HLa: lactic acid; HFo: formic acid; HAc: acetic acid; HPr: propionic acid; EtOH: ethanol; HBu: butyric acid; ND: not detected.