Dark fermentative hydrogen production from neutralized acid hydrolysates of conifer pulp

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Abstract

Concentrated acid hydrolysis of cellulosic material results in high dissolution yields. In this study, the neutralization step of concentrated acid hydrolysate of conifer pulp was optimized. Dry conifer pulp hydrolysis with 55 % H_2SO_4 at 45ºC for two hours resulted in total sugar yields of 22.3-26.2 g/L. The neutralization step was optimized for solid $Ca(OH)_2$, liquid $Ca(OH)_2$ or solid CaO, mixing time and water supplementation. The highest hydrogen yield of 1.75 mol H_2/mol glucose was obtained with liquid $Ca(OH)_2$, while the use of solid $Ca(OH)_2$ or CaO inhibited hydrogen fermentation. Liquid $Ca(OH)_2$ removed sulfate to below 30 mg SO_4^{2-}/L. Further optimization of the neutralization conditions resulted in the yield of 2.26 mol H_2/mol glucose.

Key words: Biohydrogen, dark fermentation, acid hydrolysis, neutralization, conifer pulp

1. Introduction

Hydrogen has obtained increasing attention as a source of renewable energy due to its carbon-neutrality, clean breakdown, and efficient use in chemical fuel cells [1]. Dark fermentation results in high hydrogen production rates (for reviews, see [2,3]). Feasibility at large-scale requires hydrogen production from renewable and low-cost materials, such as wastewaters or agricultural wastes [4]. Hydrolysis of source materials is often required to increase sugar content for hydrogen fermentation.

Lignocellulosic materials can be hydrolyzed with mechanical, thermal, chemical and enzymatic treatments (for reviews, see [5,6]). Chemical hydrolysis has been accomplished with alkalis, e.g. calcium or potassium hydroxide, or acids, e.g. sulfuric or phosphoric acid [7-11]. Diluted acid hydrolysis requires elevated temperature and pressure [12,13], while concentrated acid hydrolysis proceeds at otherwise

37 milder conditions [10,14]. However, acid recovery and/or neutralization is required after acid hydrolysis
38 increasing costs and energy requirements. Hydrolysis may result in the production of inhibitory
39 compounds, such as furan derivatives (furfural and hydroxymethylfurfural (HMF)), phenolic compounds
40 or weak acids (acetate), that have to be removed prior to hydrogen fermentation [15,16]. Lime, $Ca(OH)_2$,
41 is often used for neutralization and removal of inhibitors resulting in the production of gypsum [13,17].
42 Detoxification of the hydrolysate has also been done with activated carbon or anion exchange resin
43 [10,14,18].
44
45 The advantages of concentrated acid hydrolysis include high sugar yields (90 %), amenability to diverse
46 feedstocks, fast hydrolysis (10 – 12 h), and low sugar losses [19]. However, the process is costly due to
47 equipment and acid recycling requirements. In addition, sulfate has to be removed after sulphuric acid
48 hydrolysis, since SO_2^{4-} in dark fermentation media may support sulfate reducing bacteria that can utilize
49 hydrogen and thus, decrease hydrogen yields [20]. Use of overliming for the removal of sulfate and other
50 inhibitory compounds requires an additional step and gypsum removal may lead to a potential waste
51 disposal problem [15]. Recycling of gypsum, e.g., as agricultural soil conditioner may improve the
52 process economy [19]. Inhibitory effects of lignocellulosic acid hydrolysates on ethanol fermentation
53 have been extensively studied (e.g., [21,22]). Potential inhibitors in synthetic hydrogen production media
54 and their inhibitory effects on dark fermentation have been reported [23,24]. However, to the authors´
55 knowledge inhibitory effects of neutralized acid hydrolysates on dark fermentative hydrogen production
56 has not been reported.
57
58 In this study, conifer pulp obtained from a paper factory was hydrolyzed with concentrated sulphuric acid
59 and the neutralized hydrolysate was used for dark fermentative hydrogen production. Preliminary
60 experiments indicated inhibitory effects of hydrolysates on hydrogen fermentation. Thus, the
61 neutralization step was optimized with different chemicals and under different experimental conditions.

Furthermore, the characteristics of neutralized hydrolysates and their effects on dark fermentative hydrogen production were determined.

2. Materials and methods

2.1 Acid hydrolysis followed by neutralization

Dry conifer pulp was obtained from a pulp and paper factory (UPM-Kymmene Oyj, Jämsänkoski, Finland) and cut into smaller pieces (1 cm x 1 cm). Acid hydrolysis was done with 55 % H_2SO_4 at 45°C for two hours, if not otherwise mentioned. After hydrolysis the hydrolysate was neutralized with CaO or $Ca(OH)_2$, which stopped the hydrolysis. Solid CaO was obtained from First Edition Enterprise Co., LTD (Hsinchu, Taiwan) and had a 70 % purity. Solid $Ca(OH)_2$ was from Union Chemical Works LTD (Taichung, Taiwan). Liquid $Ca(OH)_2$ (17.67 % w/v) was obtained from Diamond Nano-Biochem Co., LTD (Taichung, Taiwan) and contained nano-particles with a large surface area. After neutralization the solids were separated by filtration (1 µm, Advantec) and the pH of the liquid hydrolysate was adjusted to 7.0 ± 0.2.

Cellulose swelling with 0.5 % NaOH was tested before acid hydrolysis for 1 or 2 hours to reveal the effect on final sugar yield after acid hydrolysis. After NaOH swelling the solids were separated by filtration and further used for acid hydrolysis. Neutralization step was optimized by determining the optimal mixing time and water supplementation. The experimental design was as presented in Figure 1. Samples were taken from the hydrolysates for the analysis of total sugar and sulfate concentration and for the composition of sugars, furfural compounds, volatile fatty acids (VFAs) and alcohols.

2.2 Hydrogen production from hydrolysate

Hydrogen was produced from hydrolysates with a heat-treated (80ºC, 1 h) culture enriched for hydrogen production. Endo-medium was used in the experiments, one liter containing 5.24 g NH_4HCO_3, 6.72 g $NaHCO_3$, 0.125 g K_2HPO_4, 0.1 g $CaCl_2$*2 H_2O, 1 mL mineral stock-solution (0.1 g/l $MgCl_2$*6 H_2O, 0.015 g/L $MnSO_4$*6 H_2O, 0.005 g/L $CuSO_4$*5 H_2O, 0.000125 g/L $CoCl_2$*5 H_2O), and 1 mL stock-solution containing 0.025 g/L $FeSO_4$*7 H_2O. Hydrogen production was studied at 37ºC in 200 mL batch bottles having a working volume of 50 mL and with sugar concentration of 4.5 g total sugars/L. Initial pH was adjusted to 7.0 ± 0.2 and the medium was purged with argon gas for 5 min before inoculation (10 % v/v). Batch incubations were done in triplicate with mechanical mixing (150 rpm). The effects of calcium on the hydrogen producing culture were studied with Ca^{2+}-concentrations from 0 to 300 mg/L. The experimental conditions were the same as in the hydrolysate studies with the exception of substrate that was 20 g/L glucose.

2.3 Chemical analyses

Total sugars from the hydrolysates were analyzed with a modified phenol-sulphuric acid method [25], where the volumes of sample, 5 % phenol and sulphuric acid were 1 mL, 0.5 mL and 2.5 mL, respectively. Sulfate concentrations were analyzed with modified APHA method [26]. Analysis was done with 5 mL diluted sample by adding 1 mL buffer solution (1 liter contained 30 g $MgCl_2$*6 H_2O, 5 g CH_3COONa*3 H_2O, 1 g KNO_3, 0.111 g Na_2SO_4, and 22 mL 99 % CH_3COOH) and 0.02 g $BaCl_2$*2 H_2O. The sample was mixed well and after 5 min the spectrophotometer reading was recorded at 420 nm. A standard curve was used to calculate the concentration of sulfate (mg/L).

111 The contents of H_2 and CO_2 in the gas phase were analyzed with a gas chromatograph (Shimadzu GC-
112 14B) equipped with a RT-Msieve 5A column and a thermal conductivity detector (TCD). The
113 temperatures of injector, oven and detector were 160, 170 and 160ºC, respectively. Argon was used as
114 carrier gas with a pressure of 200 kPa. The concentrations of sugars, furfural, HMF, VFAs and alcohols
115 were analyzed with liquid chromatograph (Shimadzu LC-10AT) equipped with Coregel 87H3 column
116 (35ºC) and refraction index detector (RID). The mobile phase was 0.005 N H_2SO_4 with a flow rate of 0.6
117 mL/min. Ions in the hydrolysates were analyzed with Inductively Coupled Plasma (ICP, Optima) Optical
118 Emission Spectrometry at 160 - 900 nm. The samples were prepared according to [27].
119
120 **3. Results and discussion**
121
122 ***3.1 Hydrogen fermentation after neutralization with different chemicals***
123
124 Conifer pulp was hydrolyzed with 55 % sulphuric acid at 45ºC for two hours. The reaction was stopped
125 and the hydrolysate was neutralized by using three different chemicals: solid or liquid $Ca(OH)_2$ or solid
126 CaO (70 % purity). The concentrations of sulfate, total sugars, furfural compounds, and ions in the
127 hydrolysates were as presented in Table 1. After neutralization, the hydrogen production potentials from
128 the hydrolysates were determined.
129
130 The sugar yields after neutralization with different chemicals were between 22 and 26 g/L and furfural
131 compounds were not detected in the hydrolysates. Hydrogen production potentials from the three
132 neutralized hydrolysates were as shown in Figure 2. The highest hydrogen yield of 230 ± 40 mL H_2/g
133 total sugars (1.75 ± 0.28 mol H_2/mol glucose) was obtained with liquid $Ca(OH)_2$ hydrolysate. Sugar
134 removal was 48 ± 6 % with butyrate as the main soluble metabolite. The superiority of liquid $Ca(OH)_2$ as
135 neutralizing agent was likely due to the presence of nano-particles that increased the available surface

area and thus, increased the reaction rate between sulphate and calcium-ion [28]. Liquid $Ca(OH)_2$ removed sulfate below 30 mg/L. Removal of sulfate before dark fermentation is required due to sulfate reducing bacteria that can utilize hydrogen as electron donor decreasing the H_2 yields [29] and simultaneously reducing sulfate to hydrogen sulfide that may be inhibitory to anaerobic bacteria [30].

The hydrogen yields with other two hydrolysates remained low, even though the sugar removal after solid $Ca(OH)_2$ treatment was 49 ± 25 %. The fermentation of solid $Ca(OH)_2$ hydrolysate was directed towards ethanol production (yield 13 ± 2 g/L) instead of hydrogen. The neutralization with $Ca(OH)_2$ resulted in 480 mg Ca^{2+}/L in the hydrolysate. The Ca^{2+}-ions may inhibit dark fermentative hydrogen production at concentrations above 300 mg Ca^{2+}/L [31]. However, inhibitory effects of Ca^{2+}-ions on ethanol fermentation have not been reported. The effects of Ca^{2+}-ions on hydrogen production were, therefore, determined in three parallel batch bottles with Ca^{2+}-concentrations of 0 - 300 mg/L (Figure 3). The highest hydrogen yield of 1.44±0.11 mol H_2/mol glucose was obtained at Ca^{2+}-concentrations of 200 mg/L, after which the hydrogen yields decreased. Hydrogen yield decreased by 50 % (0.74±0.13 mol H_2/mol glucose) at the highest Ca^{2+}-concentration of 300 mg/L. Thus, the high Ca^{2+}-concentration of 480 mg/L likely inhibited hydrogen production and directed fermentation towards ethanol.

Solid CaO treatment did not degrade most of the sugars. After solid CaO neutralization the concentrations of sulfate and magnesium were 70 and 135 mg/L, respectively, i.e. below inhibitory concentration to hydrogen fermentation. Wang et al. [32] reported that increase in Mg^{2+} concentration up to 1.7 g/L increased the hydrogen production, and according to Lin and Chen [20] inhibitory effects of sulfate on dark fermentative hydrogen production were observed at 500 mg SO_4^{2-}/L. The purity of the solid CaO was 70 % and thus, may have contained other impurities that were not detected in our analyses but likely affected the hydrogen fermentation. Based on these results, the hydrolysates in further experiments were neutralized with liquid $Ca(OH)_2$.

3.2 Optimization of the neutralization step

NaOH swelling

Dry conifer pulp was pretreated with 0.5 % NaOH for 0, 1 or 2 h followed by acid hydrolysis. Alkaline pretreatment with diluted NaOH causes solvation and saphonication that swells the biomass, increases the surface area of the substrate, and decreases the crystallinity of cellulose [5,6]. The characteristics of the hydrolysates after NaOH pretreatment and acid hydrolysis were as shown in Table 2. Pretreatment with 0.5 % NaOH did not affect the sugar yields and sugar- and VFA-compositions were also similar. Furthermore, adding an extra pretreatment step would increase process costs and energy requirements. Thus, further experiments were done without NaOH swelling.

Effect of mixing time

The neutralization of acid hydrolysate with liquid $Ca(OH)_2$ was tested with different mixing times from 20 min to 1 h. The hydrolysate:liquid $Ca(OH)_2$:water ratio was kept at 1:4.32:2. The mixing time did not affect the sugar yields or individual sugar concentrations considerably (Table 3) and thus, was considered unimportant in further experiments.

Effect of water addition

The effect of water volume on neutralization of acid hydrolysate with liquid $Ca(OH)_2$ was determined (Table 4). The volume of added water determines whether the hydrolysate has to be concentrated to obtain adequate sugar concentration. Concentration can be done, e.g., in a hot air oven [12]. However, concentration of hydrolysate increases energy requirements and treatment time.

The sulfate concentrations as well as the concentrations of inhibitory compounds remained negligible in all conditions (data not shown). Sugar concentration was the highest with the lowest water addition and decreased when the volume of added water increased. However, the recovery of sugars (as grams) increased with increasing water volume. The optimal water addition depends on whether the optimization is based on final sugar concentration or sugar recovery. The lowest sugar concentration needed for continuous hydrogen production should also be determined. In this study, the lowest water addition was used in further experiments to obtain high total sugar concentrations. However, the hydrolysate obtained even with the highest water addition (1.9 g/L sugars) may be suitable for hydrogen fermentation, since hydrogen fermentation has been reported with concentration as low as 1.5 g COD/L corresponding to 1.6 g/L sugars [12].

3.3 Hydrogen production from neutralized acid hydrolysate at optimized conditions

Neutralization of the acid hydrolysate was optimized (Figure 1) and the hydrogen production potential from the optimal hydrolysate was determined in triplicate (Figure 4.A). After optimization, the hydrogen yield increased from 230 ± 40 to 300 ± 10 mL H_2/g total sugar (from 1.75 ± 0.28 to 2.26 ± 0.08 mol H_2/mol glucose). The sugar degradation increased from 48 ± 6 % to 54 ± 9 %. Optimizing the neutralization step resulted in 29 and 11 % increase in hydrogen yield and sugar degradation, respectively. Butyrate was the main soluble metabolite (Figure 4.B) followed by propionate, acetate and small amounts of lactate. Theoretically, acetate and butyrate as the only soluble metabolites result in hydrogen yields of 4 and 2 mol H_2/mol glucose, respectively. After optimization, the acetate/butyrate – ratio increased together with increased hydrogen yields.

The highest hydrogen yields obtained from acid hydrolysates in this study and in other studies are presented in Table 5. The hydrogen yield of 2.24 mol H_2/mol sugar reported by Cao et al. [33] after diluted acid hydrolysis was 2.26 mol H_2/mol TS as also obtained in this study, while the other reported hydrogen yields remained lower. The highest hydrogen yield obtained in this study was over two times higher than the yields of 0.99 mol H_2/mol RS [10] and 0.49 mol H_2/mol RS [14] reported after concentrated acid hydrolysis. Chu et al. [10] and Li et al. [14] removed the sulfate-ions from the acid hydrolysates with anion exchange resin before dark fermentation. Thus, some inhibitory compounds may have remained in the hydrolysate as indicated in the study of Li et al. [14] where the cumulative hydrogen production decreased with increasing hydrolysate concentration. This shows that optimizing the neutralization process of acid hydrolysates plays an important role in dark fermentative hydrogen production from cellulosic substrates.

Direct cellulose fermentation to hydrogen has resulted in hydrogen yields of 0.76 [36], 1.4 [37], 2.09 [38] and 2.4 mol H_2/mol hexose [39]. Thus, the hydrogen fermentation of neutralized acid hydrolysate in this study resulted in higher or as high H_2 yields that have been reported from direct hydrogen fermentation of cellulosic materials. In addition, acid hydrolysis followed by hydrogen fermentation (less than 10 days) was reported to be a faster process than direct cellulose fermentation to hydrogen (28 days) [40].

4. Conclusions

Dry conifer pulp was hydrolyzed with concentrated sulphuric acid. The neutralization of the acid hydrolysate was optimized with liquid $Ca(OH)_2$ containing nano-particles and with hydrolysate:$Ca(OH)_2$:water ratio of 1:4.3:4. Sulfate was removed to concentrations below 30 mg SO_4^{2-}/L with liquid $Ca(OH)_2$. Optimizing the neutralization conditions increased the hydrogen yield from 1.75 to 2.26 mol H_2/mol glucose and the sugar degradation from 48 to 54 %. These results show that optimization

of neutralization step of acid hydrolysates of conifer pulp increases the efficiency of hydrogen fermentation.

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References

1. Hallenbeck, P.C. (2009) Int. J. Hydrogen Energy 34, 7379-7389.
2. Wang, J. and Wang, W. (2009) Int. J. Hydrogen Energy 34, 799-811.
3. Hallenbeck, P.C., Abo-Hashesh, M., Ghosh, D. (2012) Biores. Technol. 110, 1-9.
4. Logan, B.E. (2004) Env. Sci. Technol. 38, 160A-167A.
5. Hendriks, A. and Zeeman, G. (2009) Biores. Technol. 100, 10-18.
6. Agbor, V.B., Cicek, N., Sparling, R., Berlin, A., Levin, D.B. (2011) Biotechnol. Adv. 29, 675-685.
7. Kim, S. and Holtzapple, M.T. (2005) Biores. Technol. 96, 1994-2006.
8. Lee, M.J., Song, J.H., Hwang, S.J. (2009) Biores. Technol. 100, 1491-1493.
9. Kim, D.H., Kim, S.H., Kim, K.Y., Shin, H.S. (2010) Int. J. Hydrogen Energy 35, 1590-1594.
10. Chu, C.Y., Wu, S.Y., Tsai, C.Y., Lin, C.Y. (2011) Int. J. Hydrogen Energy 36, 8743-8750.
11. Lo, Y.C., Su, Y.C., Cheng, C.L., Chang, J.S. (2011) Int. J. Hydrogen Energy 36, 13955-13963.
12. Pattra, S., Sangyoka, S., Boonmee, M., Reungsang, A. (2008) Int. J. Hydrogen Energy 33, 5256-5265.
13. Chang, A.C.C., Tu, Y.H., Huang, M.H., Lay, C.H., Lin, C.Y. (2011) Int. J. Hydrogen Energy 36, 1480-1488.
14. Li, Y.C., Wu, S.Y., Chu, C.Y., Huang, H.C. (2011) Int. J. Hydrogen Energy 36, 14245-14251.
15. Palmqvist, E. and Hahn-Hägerdal, B. (2000) Biores. Technol. 74, 17-24.
16. Ren, N., Wang, A., Cao, G., Xu, J., Gao, L. (2009) Biotechnol. Adv. 27, 1051-1060.
17. Dwivedi, P., Alavalapati, J.R.R., Lal, P. (2009) Energy Sust. Dev. 13, 174-182.
18. Sainio, T., Turku, I., Heinonen, J. (2011) Biores. Technol. 102, 6048-6057.
19. Hamelinck, C.N., Hooijdonk, G., Faaij, A.P.C. (2005) Biomass Bioenergy 28, 384-410.
20. Lin, C. and Chen, H. (2006) Int. J. Hydrogen Energy 31, 953-960.
21. Lee, W.G., Lee, J.S., Shin, C.S., Park, S.C., Chang, H.N., Chang, Y.K. (1999) Applied Biochem. Biotechnol. 78, 547-559.
22. Cantarella, M., Cantarella, L., Gallifuoco, A., Spera, A., Alfani, F. (2004) Process Biochem. 39, 1533-1542.
23. Cao, G.L., Ren, N.Q., Wang, A.J., Guo, W.Q., Xu, J.F., Liu, B.F. (2010) Int. J. Hydrogen Energy 35, 13475-13480.

24. Quéméneur, M., Hamelin, J., Barakat, A., Steyer, J., Carrére, H., Trably, E. (2012) Int. J. Hydrogen Energy 37, 3750-3759.
25. Dubois, M., Gilles, K.A., Hamilton, J.K., Regers, P., Smith, F. (1956) Analyt. Chem. 28, 350-356.
26. APHA. (1998) Standard methods for the examination of water and waste water, 20th ed., American Public Health Association, Washington.
27. Method 3020A. (1992), in Test methods for evaluating solid waste, physical/chemical methods, 3rd ed., United States Environmental Protection Agency.
28. Levenspiel O. (1999) Chemical reaction engineering, 3rd ed., John Wiley & Sons, USA, pp. 170-171.
29. Mizuno, O., Li, Y.Y., Noike, T. (1997) Water Res. 32, 1626-1634.
30. Rittmann, B.E. and McCarty, P.L. (2001) Environmental biotechnology: Principles and applications, McGraw-Hill Book Companies Inc., New York, pp. 600-601.
31. Chang, F.Y., Lin, C.Y. (2006) Water Sci. Technol. 54, 105-112.
32. Wang, X.J., Ren, N.Q., Shen, X.W., Qian, G.W. (2007) Int. J. Hydrogen Energy 32, 748-754.
33. Cao, G., Ren, N., Wang, A., Lee, D.J., Guo, W., Liu, B., Feng, Y., Zhao, Q. (2009) Int. J. Hydrogen Energy 34, 7182-7188.
34. Sagnak, R., Kargi, F., Kapdan, I.K. (2011) Int. J. Hydrogen Energy 36, 12803-12809.
35. Ozmihci, S., Kargi, F., Cakir, A. (2011) Int. J. Hydrogen Energy 36, 2111-2117.
36. Liu, H., Zhang, T., Fang, H.H.P. (2003) Biotechnol. Letters 25, 365-369.
37. Nissilä, M.E., Tähti, H.P., Rintala, J.A., Puhakka, J.A. (2011) Biores. Technol. 102, 4501-4506.
38. Ren, N.Q., Xu, J.F., Gao, L.F., Xin, L., Qiu, .J, Su, D.X. (2010) Int. J. Hydrogen Energy 35, 2742-2746.
39. Ueno, Y., Kawai, T., Sato, S., Otsuka, S., Morimoto, M. (1995) J. Fermentation Bioeng. 79, 395-397.
40. Nissilä, M.E., Li, Y.C., Wu, S.Y., Lin, C.Y., Puhakka, J.A. (2012) Appl. Energy 2012;http://dx.doi.org/10.1016/j.apenergy.2012.06.015.

Figure captions:

Figure 1. The effect of cellulose swelling (step 1) on acid hydrolysis (step 2) was determined and neutralization of dry conifer pulp hydrolysate was optimized in three steps (3, 4 and 5). The optimal results of each step are underlined. After neutralization, liquids were separated by filtration (step 6) and used for hydrogen fermentation (step 7). [a] Mixing time did not have a considerable effect.

Figure 2. Hydrogen yields and soluble metabolite production from hydrolysates neutralized with three different chemicals. Standard deviations are shown in the figure.

Figure 3. Effect of Ca^{2+}-concentration on hydrogen production.

Figure 4. Hydrogen yield and sugar degradation (A) and soluble metabolites (B) obtained from optimally neutralized hydrolysate.

Table 1. The effect of different neutralization conditions on sugar yields, sulfate removal and concentrations of ions in the hydrolysate.

Chemical used for neutralization	**Total sugars (g/L)**	**SO_4^{2-} (mg/L)**	**Ions[a] (mg/L)**				
			Ca^{2+}	**Fe^{2+}**	**Mg^{2+}**	**K^{+}**	**Na^{2+}**
Solid $Ca(OH)_2$	22.3	9.8	477	1.8	67.8	61.5	38.5
Liquid $Ca(OH)_2$	26.2	8.0	242.1	7.3	112.1	344.0	9.0
Solid CaO	24.7	70.3	13.3	51.7	135	19.7	74.1

[a] Other ions analyzed included Co^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+}, Si^{4+}, Ba^{2+} and Ag^{+}. Their concentrations in the samples were under 1 mg/L.

322 Table 2. The effect of NaOH swelling on acid hydrolysate.

0.5 % NaOH (h)	Sugars (g/L)	Sugars[a] (g)	SO_4^{2-} (mg/L)	Glucose (g/L)	Xylose (g/L)	HLa (g/L)	HFo (g/L)	HPr (g/L)	HBu (g/L)
0	2.82	0.51	0	0.64	2.18	0.73	1.21	nd	nd
1	2.89	0.58	0	0.67	2.22	0.57	0.76	0.89	0.36
2	2.90	0.46	0	0.79	2.11	0.73	0.79	nd	0.31

323 [a] Calculated based on the volume of the hydrolysate, which differed slightly between the experiments.
324 HLa: lactic acid, HFo: formic acid, HPr: propionic acid, HBu: butyric acid, nd: not detected.

325 Table 3. The effect of mixing time on neutralization of acid hydrolysate with liquid $Ca(OH)_2$.

Mixing (min)	Sugars (g/L)	Sugars[a] (g)	SO_4^{2-} (mg/L)	Sucrose (g/L)	Glucose (g/L)	Xylose (g/L)	HLa (g/L)
20	6.30	3.15	12.0	0.76	3.02	2.52	nd
40	6.30	2.84	21.5	0.83	2.98	2.49	0.52
60	6.01	2.88	29.5	0.76	2.95	2.30	nd

326 [a] Calculated based on the volume of the hydrolysate, which differed slightly between the experiments.
327 HLa: lactic acid, nd = not detected

328 Table 4. The effect of water addition on neutralization of acid hydrolysate with liquid $Ca(OH)_2$.

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Water addition[a]	Sugars (g/L)	Sugars[b] (g)	SO_4^{2-} (mg/L)	Sucrose (g/L)	Glucose (g/L)	Xylose (g/L)	HLa (g/L)	HFo (g/L)
4	4.83	0.58	4.7	0.54	1.82	2.47	0.61	0.46
6	3.93	0.95	9.6	0.27	1.35	2.31	0.53	0.39
8	3.20	0.98	8.6	nd	0.63	2.02	0.55	0.42
10	2.75	1.25	3.7	0.4	0.68	2.03	0.58	0.44
20	2.30	2.09	3.8	nd	0.45	1.85	0.62	0.43
30	1.93	2.76	4.0	nd	0.21	1.72	nd	nd

330 [a] The relationship between hydrolysate:liquid $Ca(OH)_2$:water was 1:4.32:x.
331 [b] Calculated based on the volume of the hydrolysate, which differed between the experiments.
332 HLa: lactic acid, HFo, formic acid, nd: not detected

333 Table 5. Dark fermentative hydrogen production after sulphuric acid hydrolysis of different substrates. A
334 review of literature.

Substrate	Hydrolysis	H_2 production	Culture	H_2 yield	Reference
Rice straw	150ºC, 1 h, 3 wt%[a] H_2SO_4	40ºC, pH 6.5, 3 g RS/L	Sewage treatment plant	5.28 mmol H_2/g RS 1.05 mol H_2/mol RS[d]	[13]
Sugarcane bagasse	121ºC, 1.5 kg/cm^3, 1 h, 0.5 % H_2SO_4	37ºC, pH 5.5, 20 g COD/L	Pure culture[b]	1.73 mol H_2/mol TS	[12]
Corn stover	121ºC, 117 min, 1.69 % H_2SO_4	60ºC, pH 7.0	Pure culture[c]	2.24 mol H_2/mol sugar	[33]
Waste ground wheat	90ºC, 15 min, H_2SO_4 (pH 3)	37ºC, pH 6.8, 10 g TS/L	Anaerobic sludge	1.46 mol H_2/ mol TS	[34]
Waste ground wheat	121ºC, 15 min, H_2SO_4 (pH 2.5)	55ºC, pH 7.0, 20 g TS/L	Anaerobic sludge	8.31 mmol H_2/g TS[d] 1.50 mol H_2/mol TS[d]	[35]
Cotton cellulose	40ºC, 1.5 h, 55 % H_2SO_4	37ºC, pH 8.2, 15 g RS/L	Seed sludge	0.99 mol H_2/mol RS	[10]
Musroom farm waste	40ºC, 20 min, 55 % H_2SO_4	37ºC, pH 7.0, 20 g COD/L	Anaerobic sludge	0.49 mol H_2/mol RS[d]	[14]
Dry conifer pulp	45ºC, 2 h, 55 % H_2SO_4	37ºC, pH 7.0, 4.5 g TS/L	Enrichment culture	2.26 mol H_2/mol TS 12.5 mmol H_2/g TS	This study

335 [a] acid/biomass, [b] *Clostridium butyricum*, [c] *Thermoanaerobacterium thermosaccharolyticum* W16, [d] calculated from given date
336 RS: reduced sugars, TS: total sugars
337

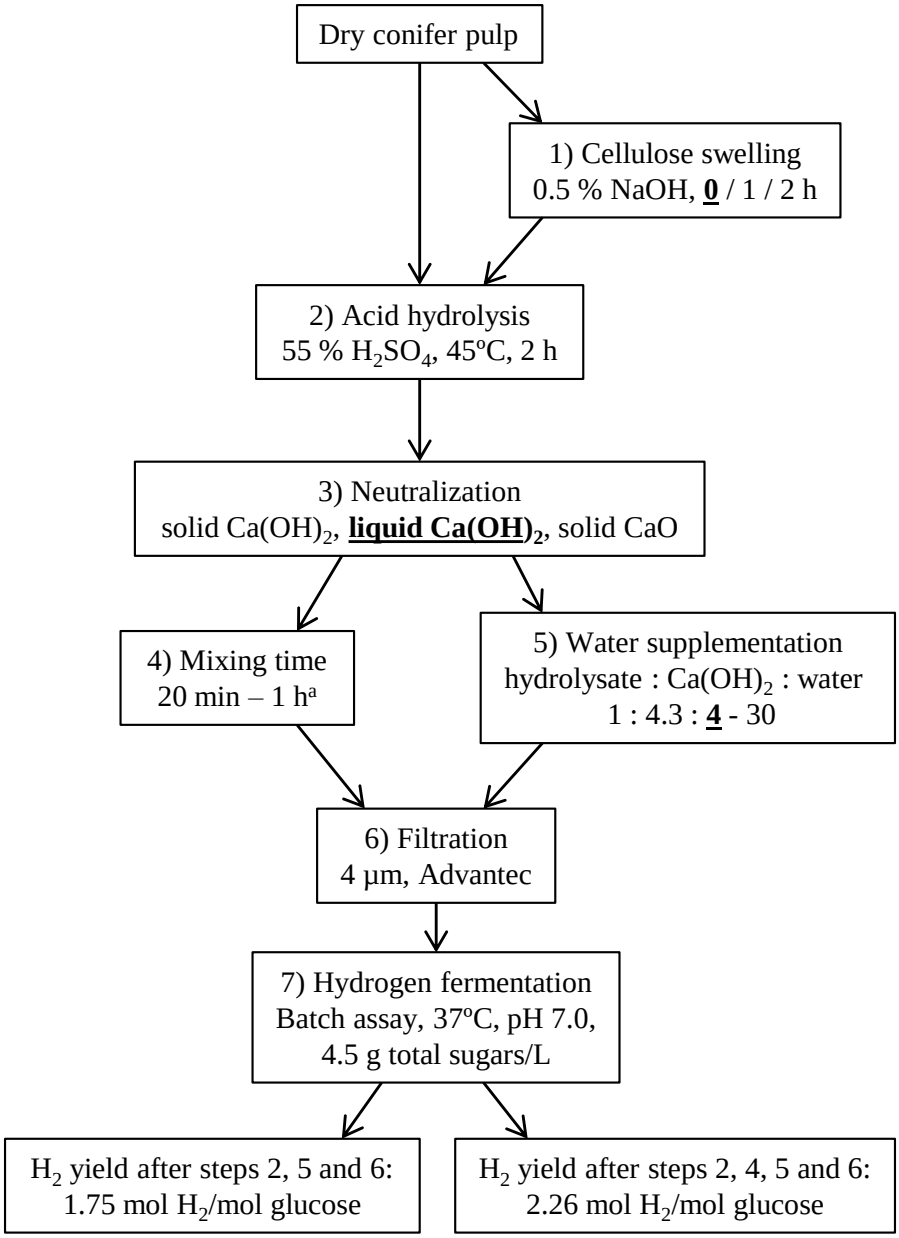
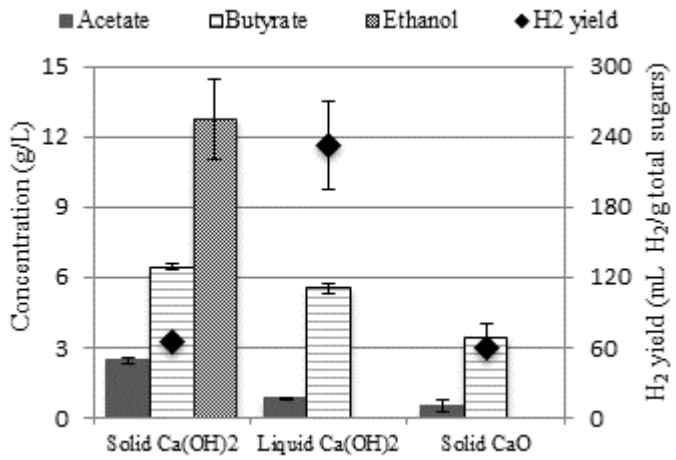

Dry conifer pulp
1) Cellulose swelling
0.5 % NaOH, 0 / 1 / 2 h
2) Acid hydrolysis
55 % H2SO4, 45ºC, 2 h
3) Neutralization
solid Ca(OH)2, liquid Ca(OH)2, solid CaO
4) Mixing time
20 min – 1 ha
5) Water supplementation
hydrolysate : Ca(OH)2 : water
1 : 4.3 : 4 - 30
6) Filtration
4 µm, Advantec
7) Hydrogen fermentation
Batch assay, 37ºC, pH 7.0,
4.5 g total sugars/L
H2 yield after steps 2, 5 and 6:
1.75 mol H2/mol glucose
H2 yield after steps 2, 4, 5 and 6:
2.26 mol H2/mol glucose

Figure 1.



341

342 Figure 2.

343

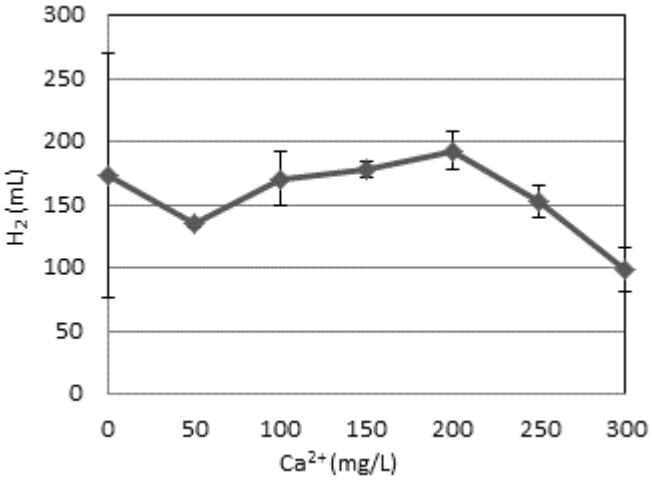
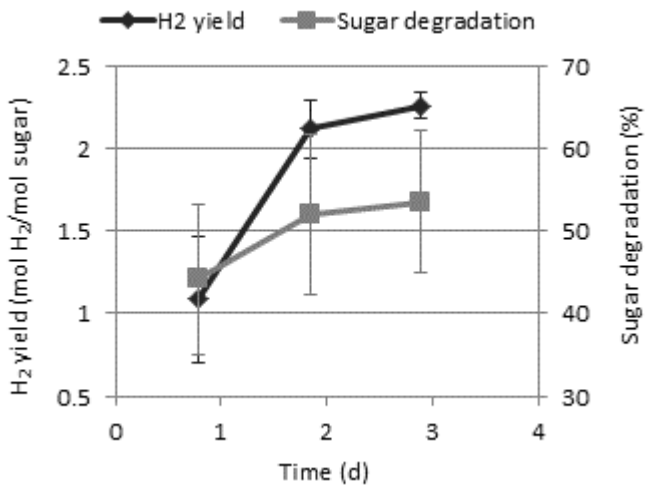
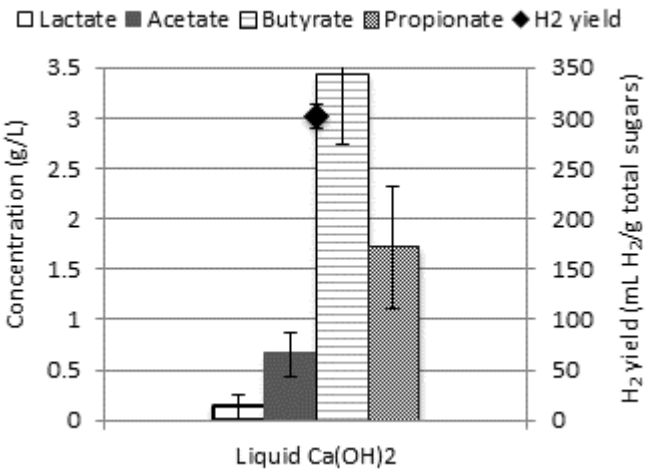


Figure 3.

A



B



347 Figure 4.

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