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Total solid content drives hydrogen production through microbial selection during thermophilic fermentation

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Abstract

In this study, the effect of total solid content (TS) on thermophilic hydrogen production from wheat straw was investigated. Six TS contents ranging from wet to dry conditions (10% to 34% TS) were tested in batch tests. A decrease of H_2 yields was observed and three statistical groups were distinguished according to the TS content: wet conditions (10% and 14% TS) with 15.3±1.6 $NmlH_2\ g_{TS}^{-1}$, intermediate conditions (19% TS) with 6.4±1.0 $NmlH_2\ g_{TS}^{-1}$ and dry conditions (25% to 34% TS) with 3.4±0.8 $NmlH_2\ g_{TS}^{-1}$. Such a decrease in biohydrogen yields was related to a metabolic shift with an accumulation of lactic acid under dry conditions. Concomitantly, a microbial population shift was observed with a dominance of species related to the class *Clostridia* under wet conditions, and a co-dominance of members of *Bacilli*, *Clostridia* classes and *Bacteroidetes* phylum under dry conditions.

KeyWords

Biohydrogen; Dark Fermentation; *hyd*A genes; Lactic acid bacteria ; Lignocellulosic residues; Solid-state processes

1 Introduction

Dark fermentation of lignocellulosic residue is a topic of wide interest in which a renewable energy *i.e.* biohydrogen (H_2) and high value biomolecules (volatile fatty acids, organic acids or alcohols) are produced simultaneously (Guo et al., 2010). Recent studies reported improvement of dark fermentation process performances through the optimization of several operating conditions (Valdez-Vazquez and Poggi-Varaldo, 2009; Valdez-Vazquez et al., 2006). Widely studied in anaerobic digestion of lignocellulosic residues, digester size and specific energy consumption for heating or effluent drying can be reduced by increasing the Total Solid content (TS) (Vandevivere et al., 2002). Nowadays, two technologies of high solid processes are used at industrial scale: wet (<15% TS) and dry (>20% TS) processes (Vandevivere et al., 2002). The reduction of water content in bioprocesses can lead to a decrease of specific energy consumption and reactor size (Vandevivere et al., 2002). These advantages applied to dark fermentation might favor the economical balance and develop biohydrogen production at industrial scale. To date, only few studies have been devoted to the effect of TS content on dark fermentation (Valdez-Vazquez and Poggi-Varaldo, 2009; Motte et al., 2013). Overall, a decrease of H_2 yields was reported under dry conditions. However, these studies were mainly performed under mesophilic conditions, while thermophilic conditions is more favourable to hydrogen production from lignocellulosic substrates by increasing substrate hydrolysis and selecting more specifically thermo-resistant H_2-producing bacteria (Ivanova et al., 2009; Kaparaju et al., 2009; Quéméneur et al., 2012).

Indeed, dark fermentation involves many microorganisms able to produce hydrogen and organic acids (volatile fatty acids and lactic acid) as well as alcohols (Guo et al., 2010; Motte et al., 2013), that might be impacted by process parameters such as temperature or

water content. Although several microbial populations are involved in the anaerobic process of fermentation, several microbial signatures obtained by 16S rDNA fingerprinting were found to be highly representative of the specific microbial diversity and activity under dry acidogenic conditions (Abbassi-Guendouz et al., 2013). However, 16S genes are global and non-specific regarding to metabolic pathways involved specifically in dark fermentation. Recently, molecular methods using the *hyd*A functional gene encoding for [Fe-Fe]-hydrogenase, an essential enzyme involved in hydrogen production, were reported to characterize more accurately the overall functional diversity (Huand et al., 2010; Quémeneur et al., 2010; Quémeneur et al., 2011).

The aim of this study is to investigate the effect of TS content on microbial metabolic pathways in thermophilic dark fermentation of wheat straw and characterize the associated microbial populations, by testing six TS contents ranging from wet to dry conditions (10% to 34% TS).

2 Material and methods

2.1 Hydrogen production in dry batches reactors

Four replicates of batch tests were carried out in 600 ml flasks at six different TS contents: 10%, 14%, 19%, 25%, 39% and 34% TS. The initial inoculum corresponded to the soluble fraction of two mesophilic and thermophilic industrial dry anaerobic digesters treating the organic fraction of municipal solid waste (OFMSW) mixed with sludge outlets of two upflow anaerobic sludge blanket (UASB) reactors treating a sugar factory effluent (mesophilic) and urban wastewaters (thermophilic). After mixing, the inoculum was heat-treated at 90°C during 15 minutes to avoid methanogenic activity prior to batch inoculation. The initial inoculation rate was adjusted at 20 $g_{wheat\ straw}.g_{inoculum}^{-1}$ (in volatile solid basis),

corresponding to 21 g of raw wheat straw (milled at 1mm) and 64 mL of inoculum. Fermentation medium was composed of a MES buffer (2-(N-morpholino)-ethanesulfonic acid) at 100 mM to keep the pH around 5.5, and 1 mL of a trace element solution as described elsewhere (Quéméneur et al., 2012). Deionized water was finally added to reach the TS content conditions and the initial pH was adjusted at 5.5. After flushing the headspace of the reactors with nitrogen gas, the flasks were incubated at 55°C for 14 days.

Biogas production and composition were monitored online with a multiplexed R3000 micro-gas-chromatograph (SRA instruments) as described previously in Motte *et al.* (2013). At the end of the experiment, medium soluble phase was extracted by adding water up to 5% TS. Samples were then centrifuged and filtrated at 2.7 µm (Whatman GF/D glass microfiber filters). The pH was measured immediately after dilution. Volatile fatty acids from C2 (acetate) to C6 (caproate) were quantified with a gas chromatograph (VARIAN 580) as described elsewhere in Motte *et al.* (2013). Other metabolic end-products, such as lactic acid and ethanol, were quantified by HPLC (Bio-Rad®) (Motte *et al.*, 2013).

Statistical analysis of the experimental data were performed with R software (version 2.15.1) using the Rcmdr library version 1.8.4.

2.2 Microbial community analysis

500 µL of the liquid fraction were sampled for each reactor at the beginning and the end of the experiment and stored at -20°C for subsequent DNA analysis. Total DNA was extracted and purified according to Abbassi-Guendouz et al. (2013). Both 16S rDNA and *hyd*A genes were amplified using universal primer pairs, respectively, W49 and W104 for bacterial communities (Abbassi-Guendouz et al., 2013) and primers hydAClosF and *hyd*AClosR for hydrogen producing communities (Quéméneur et al., 2011). PCR reactions were performed with a Mastercycler ep gradient S thermal cycler (Eppendorf) and amplified DNA fragments

were checked by electrophoresis using a 2100 Bioanalyzer (Agilent), according to Quéméneur *et al.* (2011). After sample denaturation, DNA fingerprinting by Capillary Electrophoresis of Single-Strand Conformation Polymorphism (CE-SSCP) was performed as described in Quéméneur *et al*. (2011), using an ABI prism 3130 genetic analyser (Applied Biosystems). CE-SSCP raw data were then analysed using GeneScan software (Applied Biosystems) and the StatFingerprint R library (Quéméneur *et al.*, 2011).

One sample for the inoculum and three samples at the end of batch tests operated with a TS content of 10%, 19% and 29% TS were selected for microbial identification. Pyrosequencing of the V4-V5 regions of 16S rDNA genes was performed to identify the microbial populations (Molecular Research Laboratory, TX, USA). An average of 2800 sequences per sample was processed using a proprietary analytical pipeline (Molecular Research Laboratory, TX, USA). By clustering with 3% of divergence, taxonomical classification was performed using BLASTn against a curated GreenGenes database.

Finally, principal component analysis of the CE-SSCP fingerprinting profiles was performed for statistical analysis of the molecular data. The correlation with the most discriminant parameters was determined using the *vegan* library of the R software.

3 Results and discussion

3.1 Impact of TS content on hydrogen yields

At 10 and 14% TS, *i.e.* under wet conditions, the highest hydrogen yields were obtained after less than 9 days of experiment with 15.2±1.7 and 15.4±2.0 $NmlH_2\,g_{TS}^{-1}$, respectively. The hydrogen content was 45% (Supplementary material 1). These H2 yields are lower than the values already reported in the literature under similar thermophilic conditions, *i.e.* up to

45 $NmlH_2\ g_{TS}^{-1}$ (Ivanova et al., 2009). Nevertheless, H2 yields are higher than under mesophilic conditions, *i.e.* 5-10 $NmlH_2\ g_{TS}^{-1}$ (Quéméneur et al., 2012).

The increase of TS content (from 19% to 34% TS) led to a significant decrease of hydrogen production with a H2 yield lower than 8 $NmlH_2\ g_{TS}^{-1}$ and a decrease of the biohydrogen content in biogas down to 30%. A decrease of the reaction rate was also observed with around 13 days to reach maximal H2 yield (Supplementary material 1). At 25%, 29% and 34% TS, low hydrogen yields were observed with 3.4±0.4, 3.9±1.1 and 2.9±0.4 $NmlH_2\ g_{TS}^{-1}$, respectively. The decrease of hydrogen content in biogas was concomitant with an increase of carbon dioxide. This might be attributed either to direct hydrogen consumption in a local environment by homoacetogenesis, or the use of metabolic routes concurrent to hydrogen production such as solventogenesis and lactic acid, ethanol pathways. Interestingly, at 19% TS, an intermediate hydrogen yield, *i.e*. 6.4±1.0 $NmlH_2\ g_{TS}^{-1}$, was observed, indicating a progressive effect of TS content between wet (<15% TS) and dry conditions (>20% TS), which is highly consistent with literature data reported for methanogenesis (Abbassi-Guendouz et al., 2012).

3.2 Metabolic routes associated to hydrogen production

The Figure 1 presents the molar distribution of fermentative end-products after 14 days of fermentation according to the TS content. Hydrogen yields were expressed in mmol kg_{TS}^{-1} for assessing the contribution of each fermentative pathway. The overall substrate conversion corresponding to the total production of fermentative end-products (metabolites, hydrogen and carbon dioxide), was evaluated and three statistical groups were distinguished according to the TS content (***p-value=9e-14): (i) a group of “wet” conditions (*i.e*. 10 and 14% TS) where the production of fermentative products was the

highest with a total conversion efficiency of 1,935±123 $mmol_{products}$ kg_{TS}^{-1}; (ii) an "intermediate" group at 19% TS, having intermediate performances for substrate conversion of 1323±70 mmol kg_{TS}^{-1} and (iii) a final group gathering the "dry" conditions from 25% to 34% TS, where metabolites production was the lowest with only 931±90 mmol kg_{TS}^{-1}.

Furthermore, not only the total substrate conversion was impacted by the TS content but also the metabolic pattern. Under wet conditions, a high production of acetic and butyric acids, *i.e*. 204±19 and 205±22 mmol kg_{TS}^{-1} respectively, was observed concomitant to a high hydrogen yield, *i.e*. 685±71 mmol kg_{TS}^{-1}. Theoretically, acetic and butyric acid pathways lead to the production of two moles of hydrogen per mole of acetic or butyric acid (Guo et al., 2010). Here, the experimental molar ratio of $H_2/(C_2+C_4)$ was 1.7±0.2 at 10% and 14% TS , suggesting that slight hydrogen consumption likely occurred. In addition, the butyric/acetic acid molar ratio can be used as an indicator of a good achievement of the fermentation process in mixed culture (Guo et al., 2010). Here, this molar ratio was 0.8±0.1 at 10% TS and 1.3±0.3 at 14% TS, which is consistent with Quéméneur, *et al.* (2012) who reported values around 1.2, but lower than the usual ratio of 1.5 (Guo et al., 2010). Both ratios support the hypothesis that acetic acid was partly produced by homoacetogenesis. This is consistent with recent findings suggesting that butyric acid pathway is more representative of H_2 production due to homoacetogenesis (Guo et al., 2014). Other metabolites were ethanol, caproic and lactic acids, counting for only 4%±1 of the total fermentative products.

Under dry conditions from 25% to 34% TS, fermentative end-products linked to hydrogen-producing pathways, *i.e.* hydrogen, acetic and butyric acids, counted for only 26 ±3% of total metabolites. Hydrogen, acetic and butyric acids production was reduced to 152±28 mmol kg_{TS}^{-1}, 41±4 mmol kg_{TS}^{-1} and 51±11 mmol kg_{TS}^{-1}, respectively. Interestingly, the molar ratio $H_2/(C_2+C_4)$ was 1.6±0.2 mol mol^{-1} and the butyric/acetic acid molar ratios was

around 1.3±0.3, suggesting that hydrogen consumption by homoacetogenesis occurred in similar proportion than under wet conditions. The main difference with wet conditions concerned the higher production of lactic acid (333±23 mmol kg_{TS}^{-1}, ***p-value=5e-15), which corresponded to approximately 36% of the fermentative products. All process parameter that can impact lactic acid production such as temperature, pH, carbon source and concentration, as reported previously by Hofvendahl and Hahn-Hägerdal (2000) were kept constant in the present experiment. Even though pH was maintained constant around 5.1±0.1 by using a proper amount of MES buffer, high solids environment induces invariably local heterogeneity where pH, but also substrate and products concentrations might affect locally the microbial activity (Abbassi-Guendouz et al., 2012). Therefore, such accumulation of lactic acid in dry conditions could likely be attributed to the effect of high TS content on the microbial consortium through heterogeneous environmental conditions and, probably to either unfavourable or stressing local conditions to hydrogen-producing bacteria.

Interestingly, the batch tests operated at 19% TS presented an intermediate metabolic pattern. Although the production of lactic acid was statistically similar to the values found for the “dry”- group (302±49 mmol kg_{TS}^{-1}, ns p-value=0.155), the hydrogen yield was significantly different (286±45 mmol kg_{TS}^{-1}, ***p-value=3e-4) and between the ones observed in dry and wet conditions. Compared to the dry conditions, higher accumulation (+ 33%) of metabolites related to hydrogen-producing pathways, *i.e.* hydrogen, acetic and butyric acids, was observed at 19% TS with, consequently, an increase of substrate conversion from 931±90 mmol kg_{TS}^{-1} for dry conditions to 1323±70 mmol kg_{TS}^{-1} at 19% TS. In conclusion, 19% TS corresponded to intermediate conditions where the specific H2-producing pathways were more efficient than in the “dry” group with an higher

accumulation of lactic acid and lower substrate conversion than under wet conditions (1935±123 mmol kg_{TS}^{-1}).

3.3 Shift of microbial populations from wet to dry conditions

Principal component analysis of the genetic variability assessed from the microbial fingerprints (CE-SSCP 16S profile) at final time and for each TS content was assessed (Figure 2a). After 14 days of fermentation, the three statistical groups that were previously defined by their specific metabolic pattern ("wet"-"intermediate"-"dry") can be statistically distinguished along the first axis representing 55.6% of the genetic variability (68.4% of total variability for first and second axes) (Figure 2a). The intermediate point, *i.e*. 19% TS, showed a larger dispersion of the profiles that were distributed amongst the wet and dry profiles, confirming an intermediate composition of the microbial community. The CE-SSCP profiles corresponding to the wet conditions (*i.e.* 10% and 14% TS) were the most distant profiles compared to the initial inoculum. This result suggests that microbial growth was more efficient than in dry batch tests (Supplementary material 2). Moreover, the genetic diversity of the microbial community under wet conditions was strongly correlated to the total substrate conversion (***p-value<0.001) and the hydrogen yield (***p-value<0.001), whereas the microbial structure in the dry group correlated with lactic acid production (***p-value<0.001) (Figure 2a). Therefore, the increase of TS content did not probably impact the microbial metabolism, but rather favoured the growth of lactic acid bacteria (LAB) likely because LAB are more resistant to acidic environment (Hofvendahl and Hahn-Hägerdal, 2000).

Furthermore, principal component analysis of the final CE-SSCP *hyd*A profiles, *i.e.* representing more specifically hydrogen-producing bacterial population was performed (Figure 2b). Two groups were clearly distinguished, mainly based on the first axis of variance

representing 81.5% of the total genetic variability: the wet group (10% and 14% TS) and, interestingly, a group from 19% to 34% TS. The analysis of *hyd*A genes revealed therefore that the production of hydrogen at 19% TS was carried out by similar populations than under dry conditions. It was concluded that the increase of TS content between wet and dry conditions affected first hydrogen-producing bacteria and at higher TS content was more favourable to lactic acid bacteria.

The microbial populations for each TS were identified by pyrosequencing (Table 1). The inoculum was composed of a large variety of microorganisms with more than 120 species assessed by richness estimation (Chao, 1984). The *Clostridia* class, *i.e*. main class reported for hydrogen production (Guo et al., 2010), represented about 31% of the total abundance in the inoculum. Under wet conditions, a strong enrichment of *Clostridia* class was observed with a microbial abundance increasing from 22% in the initial inoculum to 88% after fermentation, with three species substantially enriched: *Ruminococcus bromii* (53%), *Ethanoligenes cellulosi* (15%) and *Sarcina ventriculi* (12%). This result is consistent with the highest H_2 performances observed under wet conditions. Moreover, such composition is rather common in dark fermentation processes, since *Clostridia* class is known to be highly dominant in most of the hydrogen-producing ecosystems (Quéméneur et al., 2011). In the dry group, a larger variability of microbial species was observed. The *Clostridia* class represented only 28.4% of the community at the end with the emergence of two specific species: *Thermoanaerobacterium saccharolyticum* (11%), which is known to grown under thermophilic conditions and to produce hydrogen (Desai et al., 2004), and *Coprothermobacter proteolyticus* (12%) known to produce hydrogen and to grow under dry conditions also (Kersters et al., 1994). These two hydrogen-producing microorganisms were likely more adapted to dry environment where volatile fatty acids accumulate locally. In

addition, the emergence of other classes, such as *Bacilli* (19.4%) and *Bacteroidetes* (12.3%), was observed under dry conditions. This is consistent with the accumulation of lactic acid since most of the LAB are belonging to the *Bacilli* class, within the family of the *Lactobacillaceae*. The growth of members of the *Bacteroidetes* genus under dry conditions is consistent with their natural environments, such as soil, sediment and intestine (Thomas et al., 2011), where water content is fluctuating and rather low. Interestingly, *Bacillus coagulans*, found in dry conditions, was previously studied in solid-state fermentation for its strong resistance to local environmental conditions (large range of pH and temperature) as well as its ability to produce lactic acid in this environment (Payot et al., 1999). It was therefore concluded that S*porolactobacillus racemilacticus* and *Bacillus coagulans* were probably the bacteria that were involved in the production of lactic acid under dry conditions. At 19%, an intermediate composition of microbial populations was observed. The *Clostridia* class represented 58.2% of the total community with the emergence of *Ruminococcus bromii* (43.1%) as under wet conditions and *Coprothermobacter proteolyticus* (7.1%) as under dry conditions. Both *Bacilli* and *Bacteroidetes* were maintained at low abundance (3.7% and 2.0%, respectively), confirming that the emergence of these classes was only favoured under dry conditions. Considering that lactic acid accumulate partially at 19%TS, this result confirmed that H2-producing bacteria were first impacted in terms of population abundance and metabolism, and higher dryness favoured rather the selection of more resistant microorganisms to local environment such lactic acid bacteria.

4 Conclusion

This work showed that hydrogen production significantly decreased when increasing the solid content. While wet conditions favoured hydrogen-producing species, such as

Clostridia, thermophilic lactic acid bacteria that are more resistant and adapted to acidic environments were specifically selected under dry conditions. Interestingly, an intermediate behaviour was observed at 19% TS with transitional microbial community and metabolic pattern suggesting that H_2-producing bacteria were first impacted by their local environment in high solids. This work suggests that 19% TS is the limit of operation for producing hydrogen in thermophilic fermentation bioprocesses.

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Table captions

Table 1: Identification by pyrosequencing of the bacterial communities, in the inoculum and after 14 days of fermentation at 10%, 19% and 29% TS. Bacterial classes are represented in bold characters, while species are in normal character. Only the species with relative abundance higher than 1% are presented. Light grey represents the species with abundance higher than 5% and dark grey the species with abundance higher than 20%. Richness estimation was calculated according to the CHAO index (Chao, 1984).

Figure captions

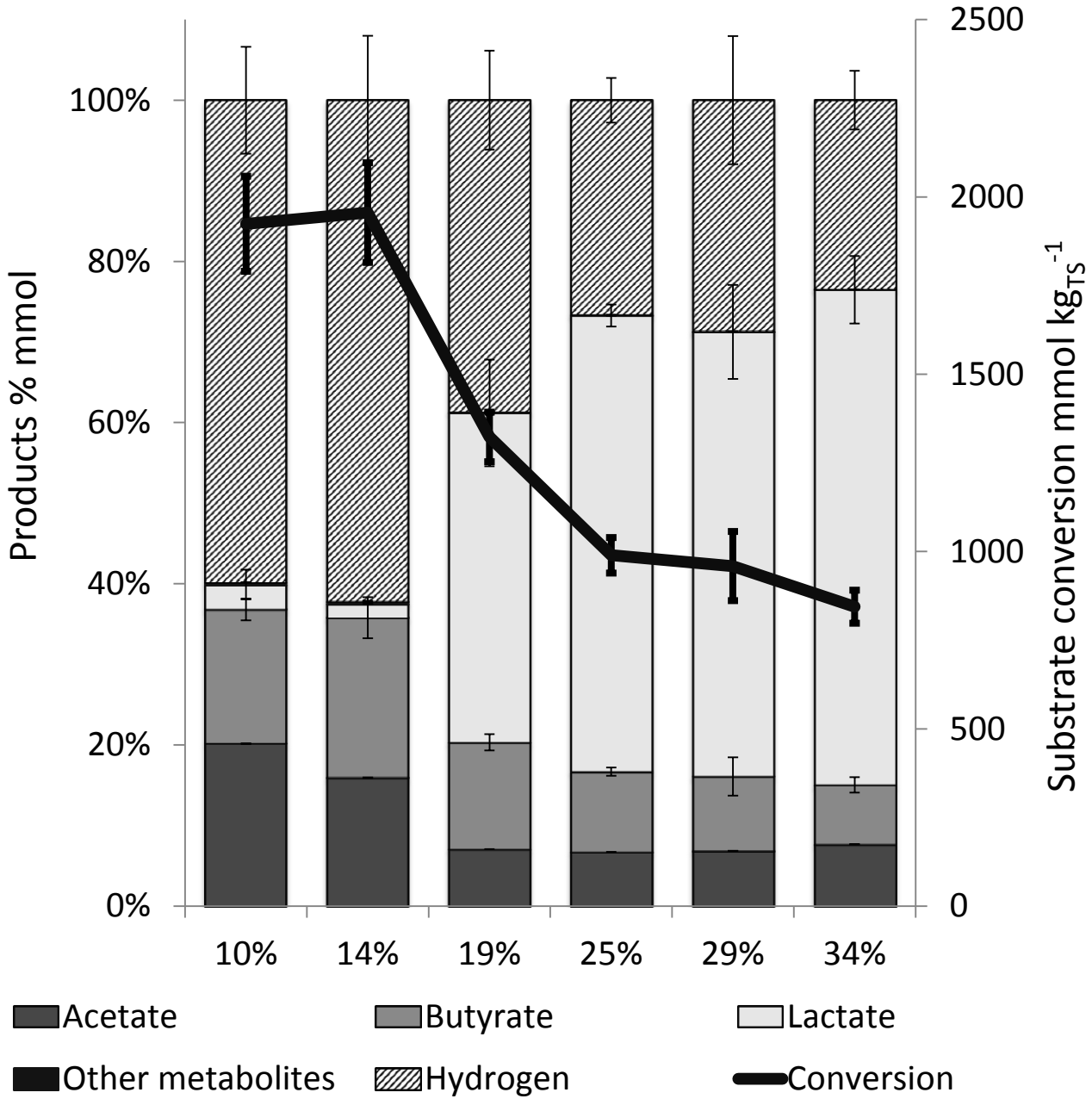
Figure 1: Molar distribution of fermentative end-products and substrate conversion according to TS content. The “other metabolites” group corresponded to propionic, valeric, caproic acids and ethanol.

Figure 2: Principal component analysis biplot of bacterial communities genetic variability assessed from CE-SSCP profiles. (A) Final 16s rDNA distribution classified by TS content. (B) Final *hyd*A genes distribution classified by TS content. Black arrows present the discriminant parameters with statistical significance.

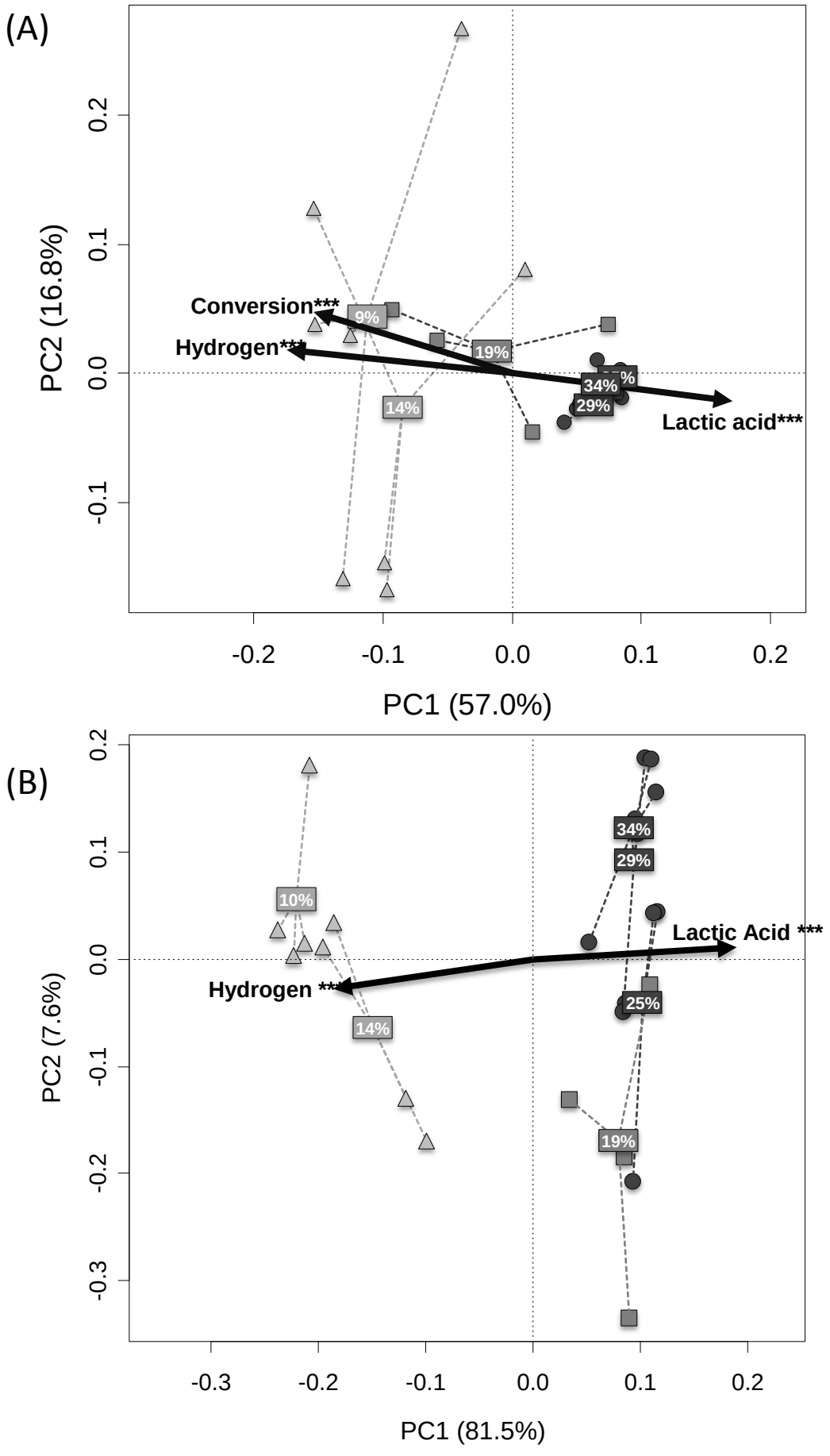
Table

CLASS *Genus & Species*	10% TS	19% TS	29% TS	Pretreated inoculum
CLOSTRIDIA	**88,8%**	**59,3%**	**29,7%**	**30,6%**
Ruminococcus bromii	53,0%	43,1%	1,4%	0,1%
Coprothermobacter proteolyticus	2,6%	7,1%	12,3%	19,5%
Ethanoligenens cellulosi	15,4%	3,4%	0,0%	0,0%
Sarcina ventriculi	11,9%	0,0%	0,0%	0,0%
Thermoanaerobacterium saccharolyticum	2,6%	3,2%	11,0%	0,0%
Halothermothrix orenii	0,1%	0,2%	0,3%	4,9%
Natranaerobius thermophilus	0,1%	0,4%	2,4%	0,9%
Thermaerobacter nagasakiensis	1,0%	0,0%	0,1%	0,1%
Other *Clostridium* sp. (17 species)	0,6%	0,5%	1,2%	1,3%
Other Clostridia (19 species)	1,4%	1,3%	1,1%	4,0%
BACILLI	**1,5%**	**5,1%**	**20,7%**	**5,2%**
Bacillus coagulans	0,4%	1,5%	9,9%	0,0%
Sporolactobacillus racemilacticus	0,4%	1,0%	7,7%	0,1%
Paenibacillus larvae	0,2%	1,1%	1,1%	2,5%
Aneurinibacillus thermoaerophilus	0,1%	0,1%	0,3%	0,9%
Other Bacilli (15 species)	0,4%	1,4%	1,7%	1,8%
BACTEROIDETES	**0,5%**	**2,1%**	**12,8%**	**4,5%**
Lewinella nigricans	0,3%	1,3%	7,3%	1,8%
Dysgonomonas mossii	0,0%	0,5%	2,9%	0,8%
Other Bacteroidetes (9 species)	0,2%	0,3%	2,6%	1,9%
ACTINOBACTERIA	**0,1%**	**0,3%**	**3,3%**	**3,3%**
Actinomyces lingnae	0,0%	0,0%	0,8%	1,1%
Georgenia muralis	0,0%	0,0%	0,5%	0,6%
Other Actinobacteria (18 species)	0,1%	0,2%	2,0%	1,6%
ARCHAEA	**0,2%**	**0,5%**	**2,1%**	**10,7%**
Methanobacterium beijingense	0,1%	0,3%	1,7%	7,3%
Methanothermobacter thermautotrophicus	0,1%	0,2%	0,3%	1,0%
Methanosaeta concilii	0,0%	0,0%	0,0%	0,9%
Other Archaea (5 species)	0,0%	0,1%	0,2%	1,5%
PROTEOBACTERIA	**0,2%**	**0,5%**	**2,2%**	**4,1%**
Aeromonas simiae	0,0%	0,0%	0,2%	1,9%
Pseudomonas mendocina	0,0%	0,2%	0,5%	0,3%
Other Proteobacteria (26 species)	0,2%	0,3%	1,5%	1,9%
SYNERGISTIA	**0,7%**	**2,7%**	**1,8%**	**9,5%**
Thermanaerovibrio acidaminovorans	0,5%	2,3%	1,2%	7,4%
Anaerobaculum mobile	0,2%	0,2%	0,6%	1,4%
Other Synergistia (2 species)	0,0%	0,2%	0,0%	0,7%
OTHER BACTERIA GROUPS	**8,0%**	**29,7%**	**27,5%**	**31,9%**
OP9 (candidate division)	6,8%	26,2%	22,2%	18,9%
Thermotoga / Petrotoga mobilis	0,9%	3,0%	4,5%	9,7%
Chloroflexi / Thermobaculum terrenum	0,0%	0,2%	0,1%	1,0%
Other Bacteria (15 species)	0,2%	0,3%	0,7%	2,3%
CHAO RICHNESS ESTIMATION	153±17	136±13	120±10	125±13

Figure


Products % mmol
100%
80%
60%
40%
20%
0%
2500
2000
1500
1000
500
0
Substrate conversion mmol kg$_{TS}^{-1}$
10%
14%
19%
25%
29%
34%
Acetate
Butyrate
Lactate
Other metabolites
Hydrogen
Conversion

Figure


(A)
PC2 (16.8%)
PC1 (57.0%)
Conversion***
Hydrogen***
Lactic acid***
9%
19%
34%
29%
14%
(B)
PC2 (7.6%)
PC1 (81.5%)
Lactic Acid ***
Hydrogen ***
10%
34%
29%
25%
14%
19%