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AIM AND APPROACH USED

In order to drastically reduce the anthropogenic emissions of greenhouse gases, many new clean-energy-technologies are under development. Among them, hydrogen production by biological processes, and more especially by dark fermentation (DF), appears as one of the most sustainable solutions to produce green hydrogen. However, H_2 production can be significantly impacted by biotic factors as the inoculum origin or the presence of indigenous bacteria (bacteria naturally existing on the biomass) when dealing with complex organic residues (Dauptain et al., 2020). Indeed, exogenous or indigenous consortia contain both hydrogen-producing bacteria (HPB) and hydrogen-consuming bacteria (HCB). To increase the H_2 yield and specifically select HPB, the application of pretreatment technologies is often required. Moreover, the conversion yield can be increased after biomass pretreatment by breaking down the complex organic structure and releasing fermentable soluble sugars from cellulosic and hemicellulosic compounds (Parthiba Karthikeyan et al., 2018). The main objective of the present work was therefore to investigate, on two different biomasses (organic fraction of municipal solid waste (OFMSW) and sorghum), the impact of thermo-acid pretreatments on dark fermentation (DF) performances.

Biochemical Hydrogen Potential (BHP) tests were performed in quadruplicate in batch tests operated with sorghum and the OFMSW. DF was either performed with indigenous bacteria or by adding exogenous bacteria coming from a freeze-dried inoculum. Before use, for inoculated experiments, the stored inoculum was thermally pretreated at 90°C for 15 min to specifically select HPB (Parthiba Karthikeyan et al., 2018). OFMSW were freshly prepared each week in the laboratory and information regarding both biomasses are detailed elsewhere (Dauptain et al., 2020). BHP tests, metabolic by-product measurements (hydrogen, volatile fatty acid, formic acid, ethanol, lactic acid and succinic acid) and microbial community analyses were carried out as suggested elsewhere (Dauptain et al., 2020) and in quadruplicates. Regarding the microbial communities, the replicate with the closest H_2 yield to the quadruplicate mean was selected as representative of the test for further microbial composition analysis. Thermo-acid pretreatments were directly carried out in the BHP flask at a total solid (TS) rate of 10%. Pretreatment conditions are given in Table 1.

SCIENTIFIC INNOVATION AND RELEVANCE

The impact of pretreatment methods on lignocellulosic biomass has already been extensively investigated, especially regarding the organic matter solubilization or their content in lignin, cellulose and hemicellulose (Kumar et al., 2015). In order to increase the biomass conversion into valuable products as hydrogen, biomass pretreatments have been largely investigated on various biomasses but with low possibilities of inter-comparison due to the microbial variability (Rafieenia et al., 2018). To limit the impact of the microbial origin and to be able to compare the different pretreatment conditions, a freeze-dried stored inoculum can be used (Dauptain et al., 2021). Moreover, studies on biomass pretreatment scarcely investigated the pretreatment impact on final microbial communities (biotic factor) after DF, which may explain some variations in the total metabolite amount, the metabolic profiles or in H_2 yields. In addition, only a very limited number of research papers investigated the pretreatment impact on indigenous bacteria (biotic factor) despite their high influence (Dauptain et al., 2020). Regarding OFMSW pretreatments, some attempts have already been performed in the literature by Bru et al. (2012), who investigated the impact of CO_2 pretreatment and showed an increase from 56.7 mLH_2/gVS to 68.6 mLH_2/gVS for a flow rate of 30 $mLCO_2/min$. Cesaro et al. (2020) also investigated the impact of formic acid pretreatments on OFMSW. The authors reported an increase of hydrogen production from 13.3 mLH_2/gVS to 31.6 mLH_2/gVS after a pretreatment at 80°C for 70 min with 5% of formic acid. Dauptain et al. (2020) also investigated the impact of a thermal pretreatment performed at 90°C-15min on sorghum, with and without inoculum, and reported no increase in H_2 yield or total metabolite production.

RESULTS OR PRELIMINARY RESULTS AND CONCLUSION

Before performing pretreated biomass BHP tests, the soluble carbohydrate concentration was measured by the anthrone method (Koehler, 1952). As shown in Table 1, thermo-acid pretreatments significantly impacted the biomass characteristics with an increase in sugar solubilization (abiotic factor). As the hydrogen yield is correlated with the soluble carbohydrate content, an increase in H_2 yield or total metabolite amount was expected.

The increase in H_2 yield was observed only once for OFMSW pretreated with 0.35 mol/L HCl at 90°C for 30 min (condition "1P"). Surprisingly, for this condition, the total metabolite amount was lower compared to the non-pretreated one ("1C"), which is not consistent with the higher content in soluble sugars. In all other cases and notably in uninoculated experiments, the pretreatment did not impact H_2 yield nor total metabolite production. It can be inferred that the pretreatment negatively impacted the indigenous bacteria.

Table 1 – Hydrogen yield, lag phase and total metabolite amounts of pretreated and unpretreated biomasses (with and without inoculum). Letters show Tukey's test results for a same experiment number (1,2 or 3). Experiments with an asterisk (*) were not inoculated. Acid pretreated samples were afterwards thermally pretreated at 90°C for 30 min. A hashtag (#) indicates that the samples were not considered in Tukey's test. P= Pretreated, C= Control, NYD= Not Yet Determined.

Biomass	Experiment name	Acid conditions	H_2 yield (mL H_2/gVS$_{ini}$)	Lag phase (days)	Total metabolites (g COD/gVS$_{ini}$)	Soluble sugars (g/L)
OFMSW	1C*	Unpretreated	29 ± 1 ^{a}	0.48 ± 0.01	0.23 ± 0.00 ^{a}	1.5
	1P*	[HCl]=0.35 mol/L	23 ± 16 $^\#$	1.28 ± 0.81	0.20 ± 0.06 $^\#$	8.7
	1C	Unpretreated	39 ± 4 ^{b}	0.39 ± 0.01	0.27 ± 0.02 ^{b}	1.5
	1P	[HCl]=0.35 mol/L	45 ± 2 ^{c}	0.72 ± 0.03	0.24 ± 0.00 ^{a}	8.7
	2C*	Unpretreated	39 ± 4 ^{a}	0.46 ± 0.07	NYD	1.1
	2P*	[H_2SO_4]=0.72 mol/L	39 ± 7 ^{a}	1.63 ± 0.33	NYD	6.0
	2C	Unpretreated	44 ± 2 ^{a}	0.36 ± 0.06	NYD	1.1
	2P	[H_2SO_4]=0.72 mol/L	35 ± 5 ^{a}	1.32 ± 0.38	NYD	6.0
Sorghum	3C*	Unpretreated	48 ± 2 ^{b}	0.53 ± 0.10	0.31 ± 0.01 ^{a}	7.0
	3P*	[HCl]=0.42 mol/L	49 ± 4 ^{b}	1.74 ± 0.13	0.27 ± 0.03 ^{a}	9.2
	3C	Unpretreated	42 ± 2 ^{a}	0.41 ± 0.08	0.28 ± 0.01 ^{a}	7.0
	3P	[HCl]=0.42 mol/L	43 ± 9 $^\#$	1.15 ± 0.12	0.27 ± 0.03 ^{a}	9.2

In the case where H_2 yield increased ("1P"), the bacterial community analysis showed that the pretreatment induced a microbial shift from *Bacteroidales* order (20.2% for "1C") to the *Clostridiales* (54.5%) and *Enterobacteriales* orders (39.7%) for condition "1P" (Table 2). As *Bacteroidales* order is known to consume H_2 by homoacetogenesis (Yang and Wang, 2018), the increase in hydrogen production was clearly attributed to this microbial shift. For sorghum, the pretreatment led to a shift from the *Enterobacteriales* order (47.2% for condition "3C") to the *Clostridiales* order (94.5% for "3P (R1)" and 37.6% for "3C"). However, this shift was not correlated to better performances. Consistently, as reported by Dauptain et al. (2020), those orders seem to have similar H_2 yields. Interestingly, as shown in Table 2, thermo-acid pretreatments led to higher variability in H_2 production that can be explained by the final microbial community composition. For instance, for 2 replicates of "1P*" experiment, microbial compositions were very different, especially regarding *Clostridiales* and *Enterobacteriales* proportions. More information about metabolites and microbial communities will be presented in detail at the conference.

Table 2 – Microbial communities after dark fermentation of pretreated and unpretreated biomasses at the order level (with and without inoculum). Only microbial communities detected after DF (> 5%) are displayed. The microbial communities of one or two replicates among the quadruplicate were measured. Percentages have been rounded to one decimal place. Experiments with an asterisk (*) were not inoculated. P= Pretreated, C= Control, R= Replicate.

Biomass	Experiment name	H_2 yield of the replicate (mL H_2/gVS$_{ini}$)	Clostridiales	Enterobacteriales	Bacillales	Lactobacillales	Bacteroidales	Other
OFMSW	1C*	28.9	40.3%	29.4%	0.0%	18.2%	11.8%	0.2%
	1P* (R1)	25.3	19.0%	53.7%	13.9%	13.4%	0.0%	0.1%
	1P* (R2)	31.2	94.1%	0.3%	3.6%	0.0%	0.0%	1.9%
	1C	39.0	30.2%	23.5%	0.4%	25.4%	20.2%	0.4%
	1P	44.1	54.5%	39.7%	5.7%	0.1%	0.0%	0.0%
Sorghum	3C*	47.0	44.0%	53.3%	0.0%	2.6%	0.0%	0.1%
	3P*	48.9	64.7%	0.0%	23.0%	12.3%	0.0%	0.0%
	3C	42.1	37.6%	47.2%	0.5%	14.6%	0.0%	0.0%
	3P (R1)	38.9	87.3%	0.1%	9.9%	2.7%	0.0%	0.0%
	3P (R2)	51.6	94.5%	0.0%	5.5%	0.0%	0.0%	0.0%

In addition, thermo-acid pretreatments led to a significant increase in the lag phase (Table 1) and in T95 (time to produce 95% of the maximum hydrogen yield). This phenomenon is barely described in the literature. Indeed, most authors reported a similar or a decrease in the lag phase after an acid pretreatment as Cesaro et al. (2020). Indeed, a decrease in the lag phase from 20h to 2h after a formic acid pretreatment of OFMSW was reported. Decreases in H_2 production due to inhibition by furfural, 5-HMF (Monlau et al., 2014) or by the ionic strength (Paillet et al., 2020) were also reported, but only rare mentions on the impacts on the lag phase. However, in our study, no furfural nor 5-HMF was detected. Complementary experiments with immediate acid neutralization are in progress to verify whether the ionic strength was sufficient to increase the lag phase, to inhibit partially the hydrogen production or to negatively impact hydrolytic bacteria, which could have counterbalanced the increase in soluble sugars due to the pretreatment. Thermo-acid pretreatments are reported as an interesting method to increase DF performances of OFMSW and sorghum. However, some impacts on hydrolytic bacteria or on dark fermentative bacteria need to be elucidated first to determine whether thermo-acid pretreatments could be used to increase the overall biomass conversion yield of DF.

In conclusion, our study provides new insights on the abiotic and biotic mechanisms existing behind the application of pretreatment methods prior to environmental bioconversion.

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