Two-Stage Alkaline-Enzymatic Pretreatments To Enhance Biohydrogen Production from Sunflower Stalks

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Due to their rich composition in carbohydrates, lignocellulosic residues represent an interesting source of biomass to produce biohydrogen by dark fermentation. Nevertheless, pretreatments should be applied to enhance the solubilization of holocelluloses and increase their further conversion into biohydrogen. The aim of this study was to investigate the effect of thermo-alkaline pretreatment alone and combined with enzymatic hydrolysis to enhance biohydrogen production from sunflower stalks. Low increase of hydrogen potentials from 2.3 ± 0.9 mL $H_2.g^{-1}$ VS to 4.4 ± 2.6 mL $H_2.g^{-1}$ and 20.6 ± 5.6 mL $H_2.g^{-1}$ VS were observed with raw sunflower stalks and after thermo-alkaline pretreatment at 55°C, 24h, 4% NaOH and 170°C, 1 h, 4% NaOH, respectively. Enzymatic pretreatment alone showed an enhancement of the biohydrogen yields to 30.4 ml H_2 g^{-1} initial VS whereas it led to 49 mL H_2 g^{-1} initial VS and 59.5 mL H_2 g^{-1} initial VS when combined with alkaline pretreatment at 55°C and 170°C, respectively. Interestingly, a diauxic effect was observed with sequential consumption of sugars by the mixed cultures during dark fermentation. Glucose was first consumed and, once glucose was completely exhausted, xylose was utilized by the microorganisms, mainly related to *Clostridium* species.

Graphical abstract

Introduction:

Development of renewable energy such as hydrogen has recently taken a considerable place in the scientific community due to oil crisis and the wish of mitigating global warming by reducing greenhouse gas emission. Biohydrogen production by dark fermentation of lignocellulosic substrates appears to be one of the most promising ways, especially when using agricultural residues since they are cheap, abundant and provide new incomes to farmers through a profitable reuse of the residues.[1,2] Among them, sunflower stalks are present in large quantities and represent renewable and low-cost raw materials for the production of biofuels such as biohydrogen.[3] In particular, holocelluloses (hemicelluloses and cellulose) which are polymers of pentose and hexose simple sugars can be efficiently converted into biohydrogen by dark fermentation.[4] Fermentative biohydrogen can be produced by either pure or mixed bacterial cultures. Even though pure cultures exhibit higher hydrogen yields, the high costs for maintaining these pure cultures restrict the development of industrial applications. Mixed cultures such as anaerobic sludge are easier to be used since they do not require sterile conditions, present a high metabolic flexibility and can therefore transform a wide range of feedstock.[5] However, direct conversion of lignocellulosic substrates into hydrogen is recalcitrant due to the intrinsic structure of the organic materials, and so far, only very low hydrogen yields of 1, 3 and 9 ml H_2 g^{-1}VS have been reported for wheat straws, cornstalks and corn straws, respectively.[1] The main bottleneck of using lignocellulosic biomass by dark fermentation is to convert holocelluloses into readily fermentable products as previously reported.[2,6] Recent studies[1,6] suggested that hydrogen yields correlate strongly with the content in easily accessible sugars of the organic substrates. Hydrolysis of cellulosic biomass is thus required since most of the hydrogen-producing bacteria find soluble substrates much easier to degrade.[7]

Among hydrolysis technologies that convert holocelluloses into soluble sugars, two conventional methods using mineral acids or enzymes are commonly used. Enzymatic hydrolysis is considered as an efficient, energy saving and environment-friendly process. Recently, hydrogen yields from poplar leaves were increased from 15.04 to 44.92 mL H_2 g^{-1} dry materials after pretreatment with 2% Vicozyme L (mixture of arabinase, cellulase, β-glucanase, hemicellulase and xylanase).[8] However, the accessibility of holocelluloses to the enzymes for further conversion into fermentable sugars is limited by the lignin content and the cellulose crystallinity.[8,9,10] Consequently, additional pretreatments need to be applied prior to the enzymatic attack to reduce this strong physical barrier and enhance holocellulose hydrolysis.[11] Alkaline pretreatments are known to remove efficiently lignin and lignin-hemicellulose complex, and thus can increase enzymatic hydrolysis of holocelluloses.[9,10] Combining alkaline pretreatment with enzymatic hydrolysis appears to be a promising approach to enhance hydrogen production from lignocellulosic residues. So far, alkaline pretreatment followed by enzymatic hydrolysis has been widely investigated for bioethanol production [3,12] and, to our knowledge, only one study investigated alkaline pretreatment combined with enzymatic hydrolysis for biohydrogen production of lignocellulosic residues and using anaerobic mixed cultures.[13] In this study, alkaline pretreatment (4 % NaOH (w/v) at 100°C for 2h) was combined with enzymatic hydrolysis (cellulases from *Trichoderma Reesei* at 20 U g^{-1}) to enhance biohydrogen production from bagasse.[13] A hydrogen yield of 300 mL H_2 g^{-1} VS (Volatile Solids) was observed compared to 31 mL H_2 g^{-1} VS with only enzymatic hydrolysis. Nevertheless, the use of hemicellulases was not considered in their study although fermentative biohydrogen can be produced from both hemicelluloses and cellulose.

The objectives of our study were: (1) to investigate the effect of thermo-alkaline pretreatment (55°C, 24h, 4% NaOH or 170°C, 1h, 4%

NaOH) or two-stage thermo-alkaline and enzymatic pretreatments on biohydrogen production, (2) to monitor the dynamics of the bacterial communities supporting the fermentation process of the sugars released from enzymatic hydrolysis.

Experimental

Feedstocks:

"Serin" sunflower stalks were dried at 37°C for 48 h and milled to a particle size of 2-3 mm using a SM-100 cutting mill. Total Solids (TS) and Volatile Solids (VS) were analysed according to the APHA standard methods.[14] All measurements were carried out in duplicates.

Thermo-alkaline and enzymatic pretreatments

Thermo-alkaline pretreatments were performed on sunflower stalks at an initial solid organic load of 35 g_{TS} L^{-1}. At 55°C, pretreatment was performed in 500 mL flasks with 4% NaOH ($g/100g_{TS}$) for 24 hours, in a "Edmund Butler" heating shaker series SM-30-control with an agitation speed of 150 rpm. Pretreatment was performed at 170°C in a 1 L Zipperclave autoclave series 02-0378-1 (Autoclave France) with 4% NaOH for 1 hour under an agitation speed of 150 rpm. After pretreatment, the liquors were separated from the solid fraction by filtration through a 0.25 mm sieve. Biological Hydrogen Potentials (BHP) batch tests were carried out on total, liquid and solid fractions of the residual solids after alkaline pretreatment (Fig. 1). The remaining liquors were kept at 4°C for further chemical analysis and the solid fractions were washed several times with distilled water to remove water soluble compounds, and then dried at 40°C for 24 h. This washing step allowed removing phenolic compounds which are known to inhibit or deactivate enzymes.[15] Solid fractions were kept at 4°C for further chemical analysis and enzymatic hydrolysis. Concerning enzymatic hydrolysis, untreated and dried alkaline-pretreated solid fractions of sunflower stalks were autoclaved during 20 min at 121°C. This sterilization step was required to avoid consumption of the sugars released during enzymatic hydrolysis by indigenous microorganisms present naturally in lignocellulosic substrates.[16] Enzymatic hydrolysis of the untreated and alkaline-pretreated solid fractions was performed at a solid organic load of 50 g remaining TS L^{-1} in flasks incubated at 35°C in an "Edmund Butler" heating shaker series SM-30-control under 150 rpm agitation. The pH was buffered at 5 using 50 mM of 2-(N-morpholino) ethanesulfonic acid (MES) buffer. Cellulase from *Trichoderma reesei* (Sigma-Aldrich), β-glucosidase from *Aspergillus niger* (Sigma-Aldrich) and xylanase from *Thermomyces lanuginosus* (Sigma-Aldrich) were added at a concentration of 50 U g^{-1} TS, 25 U g^{-1} TS and 50 U g^{-1} TS, respectively. After enzymatic hydrolysis, BHP tests were performed on the total fraction (residual solids + liquor rich is xylose and glucose).

Biochemical analysis:

All samples were milled using a cutting miller Ika Werke MF 10 basic, and a 1 mm mesh. Total Kjeldahl Nitrogen (TKN) was titrated using Buchi 370-K after mineralization of the samples. Proteins were determined by multiplying TKN by 6.25. The carbohydrate composition of the untreated and solid fractions of pretreated sunflower stalks were measured using strong acid hydrolysis adapted from Effland (1977).[17] Samples (200 mg) were hydrolyzed with 12 M H_2SO_4 for 2h at room temperature. They were then diluted to reach an

acid concentration of 1.5 M and kept at 100°C for 3 h. The insoluble residues were separated from the supernatant by filtration with a GFA-WHATMAN glass fiber filter. The insoluble residues were washed with 100 mL of deionised water and then placed in a crucible. The crucible and the glass fiber filters were dried at 100°C during 24 h and then placed at 550°C during 2 h to determine the Klason lignin content. The supernatant was filtrated with nylon filters (0.2 µm) and analyzed for quantification of sugars by High-Pressure Liquid Chromatography (HPLC).

HPLC analysis was used to quantify monosaccharides (glucose, xylose, arabinose, glucuronic and galacturonic acids). The analysis was performed with a combined Water/Dionex system, using a Biorad HPX-87H column at 50°C and a refractive index detector. The eluent corresponded to a 0.005 M H_2SO_4 solution continuously pumped under a flow-rate of 0.3mL.min^{-1}. The system was calibrated with glucose, xylose, arabinose, glucuronic and galacturonic acids standards (Sigma–Aldrich).

Cellulose and hemicellulose contents were estimated on the basis of monomeric sugar contents. Cellulose is a polymer of glucose while hemicelluloses consist of branched chains of monomeric sugars composed mainly of pentoses such as xylose, arabinose. Consequently, the cellulose and hemicellulose contents were estimated as follows:

Cellulose (% TS) = Glucose (%TS) / 1.11 (1)

Hemicelluloses (% TS) = [Xylose (%TS) + Arabinose (%TS)] / 1.13 (2)**,**

where 1.11 is the ratio of the molecular weights of glucose to glucan (180/162) and 1.13 is the ratio of the molecular weights of xylose and arabinose to xylan (150/132).

Metabolites and byproducts contents

Volatile Fatty Acids (VFA) composition, *i.e*. acetic (C2), propionic (C3), butyric and iso-butyric (C4 andiC4), valeric and iso-valeric (C5 and iC5) and caproic (C6) acids were determined in the liquid phase using a gas chromatograph (GC-3900, Varian) equipped with a flame ionization detector. The concentrations of non-VFA metabolic byproducts such as lactate and ethanol and other byproducts (furfural, 5-hydroxylmethylfurfural) were measured by HPLC analysis and refractometric detection (Waters R410). The components were separated in an Aminex HPX-87H column (Biorad) maintained at 35°C. The eluent corresponded to 0.005 M H_2SO_4 and flow-rate was 0.4 mL.min^{-1}. Total phenols in the liquid fraction of alkaline pretreated samples were determined using a microtube test (Spectroquant, Merck) followed by a 4-aminoantipyrine colorimetric measurement after a two-hundred dilution.

Accessible surface area and porosity

Nitrogen adsorption–desorption was performed on a micromeritics ASAP 2010 volumetric apparatus at 77 K. Before adsorption measurements, samples (0.5–0.3 g) were outgassed at 373 K under vacuum during 72 h. Specific surface area and porosity of the samples were evaluated using the BET method.[18]

Biochemical Hydrogen Potential (BHP) tests

The H_2 production experiments were carried out in batch in 600 mL glass bottles. An anaerobically digested sludge was pretreated by heat shock (90°C, 15 min) and then used as inoculum. One milliliter of the inoculum (final concentration around 250 mg-COD L^{-1}) was added into a culture medium (final working volume of 200 mL) containing 100 mM of 2-(N-morpholino) ethanesulfonic acid (MES)

buffer, 5 g $VS.L^{-1}$ of untreated or pretreated sunflower stalks. The initial pH was adjusted to 5.5 using HCl 1 M. In a first series of experiments, BHP tests were performed on thermoalkaline pretreated samples and on the separated liquid and solid fractions to determine the part of hydrogen originating from both fractions. In a second series of experiments, BHP tests were performed only on the solid residues after alkaline pretreatment with or with no enzymatic hydrolysis. All BHP tests were performed in duplicates. Once prepared, the flasks were bubbled with nitrogen to obtain anaerobic conditions, and closed with air impermeable red butyl rubber septum-type stoppers. Bottles were then incubated at 35°C. Two milliliters of the mixed cultures were periodically collected and centrifuged (13,000 rpm, 10 min). The supernatants were stored at 4°C for further metabolites analysis.

DNA extraction, PCR amplification and CE-SSCP fingerprinting

Two milliliters of the cultures were collected after 40 h and 92 h of incubation from all batch experiments. Microbial cells were then centrifuged at 12,100 g for 15 min. Genomic DNA was extracted and purified using the Wizard Genomic DNA Purification kit (Promega). DNA purity and quantity in the extracts were measured by spectrophotometry (Infinite NanoQuant M200, Tecan). 16S rRNA genes were amplified for further fingerprinting analysis using Capillary Electrophoresis Single Strand Conformation Polymorphism (CE-SSCP) as described elsewhere.[19] Fingerprint normalization and comparison were performed using the StatFingerprint library [20] of the R software version 2.10.1. [21]

Identification of microbial communities by pyrosequencing

Comparison of fingerprints led to the selection of one genomic DNA representative for each experimental condition. Identification of the main dominant bacterial populations was performed by pyrosequencing of the V4-V5 regions of the 16S rRNA gene (Molecular Research Laboratory, TX, USA). An average of 5161 sequences per sample was obtained, and sequence data derived from the sequencing process was processed using a proprietary analysis pipeline (Molecular Research Laboratory, TX, USA). Sequences were first clustered at 3% of divergence and then taxonomically classified using BLASTn against a curated GreenGenes database.

Results and discussion:

Effect of thermo-alkaline and two-stage alkaline-enzymatic pretreatments on biochemical and structural features

To investigate the biochemical changes induced by the thermo-alkaline pretreatment, variations in proteins, uronic acids, hemicelluloses, cellulose and Klason lignin contents were estimated (Figure 1). Uronic acids which are constituents of hemicelluloses and pectins were removed at 64% after alkali treatment at 55°C, and were totally removed at 170°C, as a consequence of the cleavage of the lignin-hemicellulose linkages through uronic acids, so-called lignin–carbohydrate complexes (LCC). LCC remain the main obstacle for the utilization of lignocellulosic biomass.[4] Their cleavage makes holocelluloses more accessible for enzymatic hydrolysis and microbial fermentation. Thermo-alkaline pretreatment at 170°C was found efficient to solubilize proteins since 54% were removed. Significant hemicelluloses removals of 26% and 58% were observed after thermo-alkaline pretreatment at 55°C and 170°C, respectively. In contrast,

it was shown less than 8% of cellulose removal after thermo-alkaline pretreatment at both 55°C and 170°C. Finally, high lignin removals of 22% and 36% were observed after thermo-alkaline pretreatment at 55°C and 170°C, respectively. Such results are in accordance with previous studies reporting that alkaline pretreatment is efficient to delignify and remove partly lignin and hemicelluloses whereas cellulose is preserved.[9,10] As a result of such concomitant and similar removal of lignin and holocelluloses, no significant variation of the lignin content ranging from 32.2 to 33.5 g/100g remaining VS was observed between alkaline pretreated solids and untreated sunflower stalks. In contrast, a relative increase of 24% and 60.5% of the cellulose content compared to raw sunflower stalks was observed after alkaline pretreatment at 55°C and 170°C, respectively. This result shows that cellulose was less removed compared to the other compounds during thermo-alkaline pretreatments.

Table 1 shows the structural features such as accessible surface area (SA) and pore volume (Vp) of the untreated and alkaline-pretreated solid fractions of the sunflower stalks. Low increase of SA and Vp was observed after thermo-alkaline pretreatment at 55°C. SA increased from 1.55 m^2 g^{-1} TS (sunflower stalks) to 1.59 m^2 g^{-1} TS and Vp increased from 0.083 cm^3 g^{-1} TS (sunflower stalks) to 0.106 cm^3 g^{-1} TS. Thermo-alkaline pretreatment at 170°C was found more efficient in increasing SA and Vp with final values of 2.55 m^2 g^{-1} TS and 0.235 cm^3 g^{-1} TS, respectively. Similarly, Gharpuray et al. (1983) observed an increase of the accessible surface area from 0.64 to 1.7 m^2 g^{-1} TS by pretreating wheat straw at 100°C with 10% NaOH (w/w) during 30 min.[22]

In addition, enzymatic hydrolysis of the raw sunflower stalks and the residual solids obtained after alkaline pretreatment was further investigated by estimating the solubilization of cellulose and hemicelluloses to glucose and xylose which are the two main monomeric sugars found in holocelluloses (Figure 1). A low enzymatic hydrolysis of hemicelluloses (12%) and cellulose (35%) was observed on raw sunflower stalks. After thermo-alkaline pretreatments, the enzymatic hydrolysis yields increased for both cellulose and hemicelluloses. Indeed, after alkaline pretreatment at 55°C and enzymatic hydrolysis, an increase of 45% cellulose and 56% hemicelluloses solubilization was observed. After alkaline pretreatment at 170°C and enzymatic hydrolysis, a higher increase of 51% and 82% was observed for cellulose and hemicelluloses, respectively. Similar results were previously reported by combining alkaline-enzymatic pretreatment on spruce and where enzymatic hydrolysis of both cellulose and hemicelluloses was substantially enhanced.[23] Several factors may explain the increase of enzymatic hydrolysis after thermo-alkaline pretreatment. First, the increase of accessible surface area and pore volume, as previously observed after thermo-alkaline pretreatment, can enhance the efficiency of enzymatic hydrolysis. Consistently, good correlations between pore volume, accessible surface area and enzymatic digestibility of lignocellulosic residues were previously reported.[24] Gharpuray et al. (1983) showed that the specific surface area can impact biomass digestibility: an increase of the accessible surface area resulted in higher hydrolysis yields.[22] However, the low increase of both accessible surface area and pore volumes between raw sunflower stalks and sunflower stalks alkaline-pretreated at 55°C suggested that probably other factors were favorable for enzymatic hydrolysis. Among them, removal of pectins (polymers of uronic acids) after thermo-alkaline pretreatment might explain the increase of enzymatic hydrolysis efficiency. As described elsewhere, removal of pectins in hemp was shown to increase enzymatic hydrolysis by 26%. [25] Even though thermo-alkaline pretreatment did not change the lignin content in the solid residues, a physical redistribution of the lignin probably occurred with a cleavage of the LCC, that play a key role for enzyme accessibility and further biomass digestibility.

Biohydrogen performances

Effect of thermo-alkaline and two-stage alkaline-enzymatic pretreatments on biohydrogen yields

Gas product analysis of Biological Hydrogen Potential (BHP) tests showed that only H_2 and CO_2 were produced during dark fermentation, with no detectable CH_4 in the biogas. This suggests that heat shock treatment (90°C, 15 min) of the inoculum was efficient to suppress methanogens from anaerobic sludge. A low hydrogen yield of 2.3 ± 0.9 mL $H_2.g^{-1}$ VS was observed for the raw sunflower stalks (Table 1). A small increase in hydrogen yields to 4.4 ± 2.6 mL $H_2.g^{-1}$ VS and 20.6 ± 5.6 mL $H_2.g^{-1}$ VS were observed after thermo-alkaline pretreatments at 55°C and 170°C, respectively. Similar results were obtained by pretreating grass with 0.5% NaOH (w/v) boiled for 30 min with an increase of the hydrogen yield from 4.4 mL $H_2.g^{-1}$ dry grass for the untreated sample to 19.3 mL $H_2.g^{-1}$dry grass after treatment.[11] Alkaline pretreatment (0.4% NaOH, 24 h, room temperature) of sweet sorghum stalks led also to an increase of hydrogen potentials (127 mL $H_2.g^{-1}$ VS compared to 52 mL $H_2.g^{-1}$ VS for untreated).[26] The enhancement of hydrogen yields coincided with an increase in soluble sugars after alkali pretreatment compared to raw sweet sorghum stalks with a sugar concentration of 2.23 and 0.86 $g.L^{-1}$, respectively.[26]

Biohydrogen potentials of separated liquid and solid fractions obtained after thermo-alkaline pretreatment at 55°C and 170°C were further investigated to determine from which fraction the biohydrogen was produced (Table 1). At 55°C, biohydrogen production from the liquid and solid fractions was similar with 2.6 and 2 mL $H_2.g^{-1}$ VS, respectively. In contrast, at 170°C, biohydrogen yield of the liquid fraction was twice higher than solid fraction with 11.4 and 5.1 mL $H_2.g^{-1}$ VS, respectively. To analyze the performances of biohydrogen production on these different fractions, biohydrogen yields were expressed in terms of initial sugar contents (glucose, xylose and arabinose) according to the amount of holocelluloses present in the different fractions (total, solid and liquid) as shown in Table 1. Concerning the liquid fractions, hydrogen yields of 0.344 and 0.865 mol $H_2.mol^{-1}$ hexose were obtained after 55°C and 170°C pretreatments, respectively. Nevertheless, the hydrogen yields from the liquid phase remained lower than the values commonly reported in the literature with simple sugars and mixed cultures, *i.e.* 2 mol $H_2.mol^{-1}$ glucose and 1.92–2.25 mol $H_2.mol^{-1}$ xylose. [19, 27] Two hypotheses can be formulated to explain these results. The first one is that soluble sugars released in the liquid phase are present in the form of oligomeric and monomeric sugars. Recently, Quéméneur et al. (2011) showed the impact of the low carbohydrate structure where di- and tri-saccharides affected significantly hydrogen production, and longer chains of oligomers lowered the hydrogen yields.[28] The second hypothesis is the generation of byproducts during thermo-alkaline pretreatments such as furfural, 5-HMF and phenol compounds. Even though no byproducts of sugar degradation (furfural and 5-HMF) were detected in the liquid phase after thermo-alkaline pretreatments, phenols coming from lignin degradation accumulated in the liquid phase at a low concentration of 25.7 mg L^{-1} and 42.7 mg L^{-1} at 55°C and 170°C, respectively (data not shown), and phenolic compounds were previously shown to negatively impact biohydrogen production from xylose using mixed cultures [15,19,29].

Concerning the solid fractions, low hydrogen yields of 0.043 and 0.081 mol $H_2.mol^{-1}$ hexose were obtained, respectively, with raw sunflower stalks and after alkaline pretreatment at 55°C. The increase of the hydrogen yields to 0.380 mol $H_2.mol^{-1}$ hexose after thermo-

alkaline pretreatment at 170°C on the solid fraction was likely due to the increase of the accessible surface area as shown in Table 1. However, these hydrogen yields remained low likely because of the nature of the sugars present in the solid fractions which are in their polymeric and not monomeric form. Since low hydrogen potentials were obtained whatever the solids, enzymatic hydrolysis was performed on raw sunflower stalks as well as on the solid fractions after alkaline pretreatment of the straws at 55°C and 170°C to enhance the solubilization of holocelluloses into monomeric sugars.

After enzymatic pretreatment of raw sunflower stalks, a maximal cumulative hydrogen production of 30.4 (± 3.8) mL $H_2.g^{-1}$ VS was reached which was 13-fold higher than untreated sunflower stalks. Coupling alkaline-pretreatment with enzymatic hydrolysis led to a significant increase of the hydrogen yields compared to enzymatic hydrolysis alone with 59.9 (± 2.6) mL $H_2.g^{-1}$ VS and 80.9 (± 3.0) mL $H_2.g^{-1}$ VS at 55°C and 170°C, respectively. Such increase of hydrogen production was concomitant to the increase of enzymatic hydrolysis through the release of soluble monomer sugars (ie glucose and xylose). Hydrogen yields of 0.562, 0.959 and 1.158 mol $H_2.mol^{-1}$ hexose were obtained after enzymatic hydrolysis of raw sunflower stalks and alkaline pretreated sunflower stalks at 55°C and 170°C, respectively. Although a substantial increase was observed, these results remained lower than the average values of 2 mol $H_2.mol^{-1}$ glucose and 1.92–2.25 mol $H_2.mol^{-1}$ xylose commonly reported in the literature when using mixed cultures.[19,27] These results suggest that a part of holocelluloses was still not degraded during the dark fermentation process. Interestingly, if hydrogen potentials were expressed by considering only the initial content in soluble sugars monomers (ie xylose and glucose) released after enzymatic pretreatment, hydrogen yields ranged from 1.97 to 2.14 mol $H_2.mol^{-1}$ soluble sugars, in strict accordance with data previously published. Thus, hydrogen was mainly produced from soluble sugar monomers released during enzymatic hydrolysis which is also in accordance with the literature.[6,26]

Furthermore, once the maximum cumulative hydrogen production was reached, microbial metabolites were also analyzed. The fermentation metabolites produced with or without enzymatic hydrolysis of sunflower stalks and for the three conditions (raw, solid residue from alkaline treatment at 55°C and 170°C) were only acetate and butyrate (data not shown). This suggests that H_2 was produced only by the well described metabolic fermentative H_2-producing pathways of acetate and butyrate. Butyrate as well as acetate production are indeed typical of a dark fermentation process when using substrates rich in carbohydrates.[30]

General remarks on biohydrogen potentials

To assess the advantage of combining thermo-alkaline pretreatment with enzymatic hydrolysis, results were expressed according to the initial VS, as shown in Figure 2. In the case of thermo-alkaline pretreated stalks, the hydrogen produced from the liquid fraction after thermo alkaline pretreatment and the hydrogen produced from the solids alkaline-pretreated at 55°C and 170°C and with or without enzymatic hydrolysis were considered separately. Moreover, a theoretical hydrogen potential (100 L $H_2.kg^{-1}$ initial VS) was calculated by considering the content of holocelluloses in sunflower stalks and a hydrogen production of 2 mol $H_2.mol^{-1}$ eq glucose as commonly observed with mixed cultures.[16]

As mentioned previously, a hydrogen yield of 30.4 mL $H_2.g^{-1}$ initial VS was obtained after enzymatic pretreatment of sunflower stalks,

which was 13-fold higher than raw sunflower stalks (2.3 ± 0.9 mL $H_2.g^{-1}$ initial VS). When considering combined thermo-alkaline pretreatment at 55°C and 170°C with enzymatic hydrolysis, hydrogen potentials were respectively 21 to 26-fold higher than with untreated stalks. In both cases, the main part of hydrogen was coming from the enzymatic-alkaline-pretreated solid fraction which corresponded respectively to 81% and 95% of total biohydrogen production. When considering the initial VS content, coupling thermo-alkaline pretreatment at 170°C with enzymatic hydrolysis was not substantially beneficial compared to 55°C since hydrogen yields increased only from 49 to 59.5 mL $H_2.g^{-1}$ initial VS. Overall, hydrogen yields obtained after enzymatic hydrolysis with or without coupling with thermo-alkaline pretreatments remained always lower than the highest hydrogen yields that can be expected if holocelluloses would have been fully converted to H_2 during the dark fermentation process. In addition, and to make the process economically viable, the effluents of the H_2 fermentation process should also be considered for further valorisation as either hydrogen by photo-fermentation or methane by anaerobic digestion.[4,31]

Bacterial community analysis

By investigating the metabolic routes of the dark fermentation process, a diauxic behavior of the mixed culture was observed on the consumption of soluble sugars (ie glucose and xylose) released after enzymatic hydrolysis. Glucose was first consumed and, once glucose was completely exhausted, xylose was utilized by the microorganisms (Fig. 3). Such diauxic effect has been well described with pure cultures, but to our knowledge, no study reported such effect with complex mixed cultures. Diauxic growth usually occurs when bacterial cells of an individual species are exposed to multiple carbon sources leading to a catabolic repressive effect that does not permit simultaneous consumption of all sugars, *i.e.* sugars are sequentially consumed resulting in two successive exponential growth phases that are separated by an intermediate lag phase. Glucose is a preferred substrate for many microorganisms, and is responsible of a common catabolic repression which is commonly called the "glucose effect".[32] In mixed culture, several bacterial populations with different substrate affinities can use indifferently all sugars, and various microbial populations can very probably use several carbon sources simultaneously.[33] Therefore, a diauxic behaviour could result from a diauxic growth but also from a shift of highly specific microbial populations. To support the hypothesis of a diauxic catabolic repression effect, a bacterial analysis of the H_2-producing bacteria present in mixed culture was performed at two experimental times: 40 h and 92 h, corresponding to the end of glucose and xylose consumption, respectively (red arrows in Figure 3). The number of sequences analyzed per sample ranged from 3980 to 6366 and the number of identified species varied between 23 and 34 according to the sample. The bacterial species with a relative abundance above 1% at 40 h and 92 h in the dark H_2 fermentation batch are summarized in Table 2.

For all samples, the bacterial community was dominated by members of the *Clostridium* genus with an average of 92% of the total abundance. The composition of the bacterial communities in this study was similar to the ones reported in previous studies carried out in dark fermentation systems.[19,34] For instance, in granular sludge of H_2-producing bioreactor using sucrose as substrate, 69.1% of the clones were affiliated to four *Clostridium* species.[32] Similarly, a proportion of *Clostridium* species of 85.8% was observed during dark

fermentation of xylose and after heat shock pretreatment (90°C, 10 min) of the anaerobic mixed culture inoculum.[19]

During the glucose phase consumption, a specific enrichment of *Clostridium butyricum* amongst the other *Clostridium* species was observed with proportions varying from 80.3 % to 92.9%. In contrast, during xylose consumption, even though *Clostridium butyricum* remained the main dominant species, other *Clostridium* species clearly outcompeted. More particularly, and except for *Clostridium bifermentans*, the relative abundance of *Clostridium butyricum* decreased between 40 h and 92 h, while the abundance of minority species such as *Clostridium roseum, Clostridium aciditolerans, Clostridium saccharoperbutylacetonicum* and *Clostridium tunisiense* increased after 40 h. Consequently, the diauxic effect observed on glucose/xylose consumption was not only related to a diauxic growth of *Clostridium butyricum* but also to the development of highly competitive *Clostridium* species during the change of sugar degradation pathway. This result suggests that the diauxic effect could be common to the whole *Clostridium* genus. Diauxic effects were previously reported on ethanol production from glucose/xylose mixture using pure cultures, ie. *Saccharomyces cerevisiae* and *E. coli* [35, 36]. So far, and to our knowledge, such diauxic effect has not been so clearly reported with fermentative mixed cultures. In general, diauxic effect can be avoided by the use of mixed cultures instead of pure cultures since the presence of various bacterial populations enhances the possibilities to convert concomitantly a large range of substrates due to microbial diversity, metabolic flexibility, and the possibility of specific individual ecological niches.[35] In this study, the heat shock pretreatment initially applied on anaerobic sludge led not only to the complete suppression of *Archaea* methanogens, but also to a strong selection of *Clostridium* species with a low final number of bacteria species ranging from 23 to 34 only. This observation is in accordance with Baghchehsaraee et al. (2008) who showed that heat pretreatment (80°C or 95°C) of inocula led to a substantial decrease of bacterial diversity.[37] Comparatively, much higher numbers of bacteria ranging from 114 to 164 were reported on three granular and non-granular mesophilic cultures which were not heat treated.[38]

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TABLES and FIGURES CAPTIONS

Table 1 Accessible surface area (SA), Volume pores (Vp) Sugars contents in equivalent hexose (g eq hexose.g^{-1} VS) and biohydrogen potentials originating from total, liquid and solid fractions of raw sunflower stalks (SS) and alkaline-pretreated sunflower stalks at 55°C(SS 55°C) and 170°C (SS 170°C) with or without enzymatic hydrolysis

Table 2 Repartition of the bacterial population in % of order, genus and species during dark fermentation process at 40 h and 92 h after enzymatic hydrolysis of raw sunflower stalks (SS), alkaline pretreated sunflower stalks at 55°C (SS, 55°C) and at 170°C (SS, 170°C).

Figure 1 Scheme of pretreatment strategies used and biochemical composition of raw sunflower stalks (SS) expressed in g/100g initial VS and of the solid fractions after thermo-alkaline pretreatments at 55°C (SS 55°C) and at 170°C (SS 170°C) expressed in g/100g initial VS and in g/100g remaining VS. Values correspond to means of two replicates of independent values ± standard deviation.

Figure 2 Impact of pretreatments on the origin of the biohydrogen production (ml H_2 .g^{-1} initial VS) for raw sunflower stalks and solid residue of alkaline pretreated sunflower stalks at 55°C and 170°C with or without enzymatic hydrolysis. Values correspond to means of two replicates of independent values ± standard deviations (error bars).

Figure 3 Evolution of sugars monomers consumption (ie glucose and xylose) released from enzymatic hydrolysis during H_2 fermentation in mixed culture (a) from raw sunflower stalks (b) from solid residue of sunflower stalks pretreated at 55°C, 24 h, 4% NaOH (c) from solid residue of sunflower stalks pretreated at 170°C, 1 h, 4% NaOH. Values correspond to means of two replicates of independent values ± standard deviations (error bars).

Table 1 Accessible surface area (SA), Volume pores (Vp) Sugars contents in equivalent hexose (g eq hexose.g^{-1} VS) and biohydrogen potentials originating from total, liquid and solid fractions of raw sunflower stalks (SS) and alkaline-pretreated sunflower stalks at 55°C(SS 55°C) and 170°C (SS 170°C) with or without enzymatic hydrolysis

		Structural features		**Sugars content**	**Biohydrogen potentials**	
Conditions	Fractions	SA ($m^2.g^{-1}$ remaining TS)	Vp ($cm^3.g^{-1}$ remaining TS)	(g eq hexose. $100g^{-1}$ VS)	mL $H_2.g^{-1}$ VS	mol $H_2.mol^{-1}$ eq hexose
SS	Total	1.55	0.083	43.5	2.3 ± 0.9	0.043
SS (55°C)	Total	1.59	0.106	43.5	4.4 ± 2.6	0.081
	Liquid			6.1 (14%)[a]	2.6 ± 0.2 (57%)[b]	0.344
	Solid			37.4 (86%)[a]	2 ± 0 (43%)[b]	0.043
SS (170°C)	Total	2.55	0.235	43.5	20.6 ± 5.6	0.380
	Liquid			10.6 (24.3%)[a]	11.4 ± 0.3 (69%)[b]	0.865
	Solid			32.9 (75.7%)[a]	5.1 ± 0.4 (31%)[b]	0.125
SS + Enzymes	Total			43.5	30.4 ± 3.8	0.562
SS (55°C) + Enzymes	Total			50.2	59.9 ± 2.6[c]	0.959
SS (170°C) + Enzymes	Total			56.1	80.9 ± 3.0[c]	1.158

[a] Percentage of total glucose originating from the liquid and solid fractions. In the liquid fraction, sugar content in equivalent glucose was calculated according the removal of holocelluloses in the initial total fraction.

[b] Percentage of biohydrogen potentials originating from the liquid and solid fractions

[c] Biohydrogen potentials are expressed in ml $H_2.g^{-1}$ remaining VS

Table 2 Repartition of the bacterial population in % of order, genus and species during dark fermentation process at 40 h and 92 h after enzymatic hydrolysis of raw sunflower stalks (SS), alkaline pretreated sunflower stalks at 55°C (SS, 55°C) and at 170°C (SS, 170°C).

	Conditions					
	SS 40 h	**SS 92 h**	**SS (55°C) 40 h**	**SS (55°C) 92 h**	**SS (170°C) 40 h**	**SS (170°C) 92 h**
Number of sequencing	6366	4715	4791	6172	3980	4940
Number of species	26	28	23	34	25	30
	% **ORDER** / *Genus species*[a]					
CLOSTRIDIALES	**91**	**92**	**96**	**92**	**85**	**95**
Clostridium butyricum	89.0	72.3	92.9	60.3	80.3	71.6
Clostridium perfringens	0.0	0.0	0.0	9.7	0.0	0.1
Clostridium roseum	0.4	6.2	0.7	6.7	0.7	13.5
Clostridium aciditolerans	0.9	1.5	0.5	6.5	1.5	1.5
Clostridium saccharobutylicum	0.0	0.0	0.0	2.7	0.0	0.1
Clostridium tertium	0.2	0.8	0.8	1.9	0.6	0.4
Clostridium lundensis	0.0	0.4	0.0	1.7	0.0	0.4
Clostridium peptidivorans	0.2	0.6	0.1	1.1	0.4	0.2
Clostridium saccharoperbutylacetonicum	0.3	3.3	0.3	1.1	0.5	2.6
Clostridium tunisiense	0.1	1.0	0.1	0.3	0.0	3.5
Clostridium bifermentans	0.0	0.0	1.0	0.1	0.9	0.3
Clostridium thiosulfatireducens	0.0	3.2	0.0	0.0	0.0	0.2
Clostridium botulinum	0.0	1.7	0.0	0.0	0.0	0.4
Clostridium paraputrificum	0.0	1.0	0.0	0.0	0.0	0.1
BACILLALES	**8**	**7**	**3**	**5**	**13**	**4**
Rummeliibacillus pycnus	0.0	0.0	0.6	3.0	0.0	0.0
Sporolactobacillus racemilacticus	0.0	0.0	0.0	1.1	0.0	1.0
Bacillus ginsengihumi	7.9	7.0	2.0	0.6	13.3	3.0
Others	**1**	**1**	**1**	**3**	**2**	**1**

[a] Among the species observed, only species with a relative abundance above 1% are represented in the table

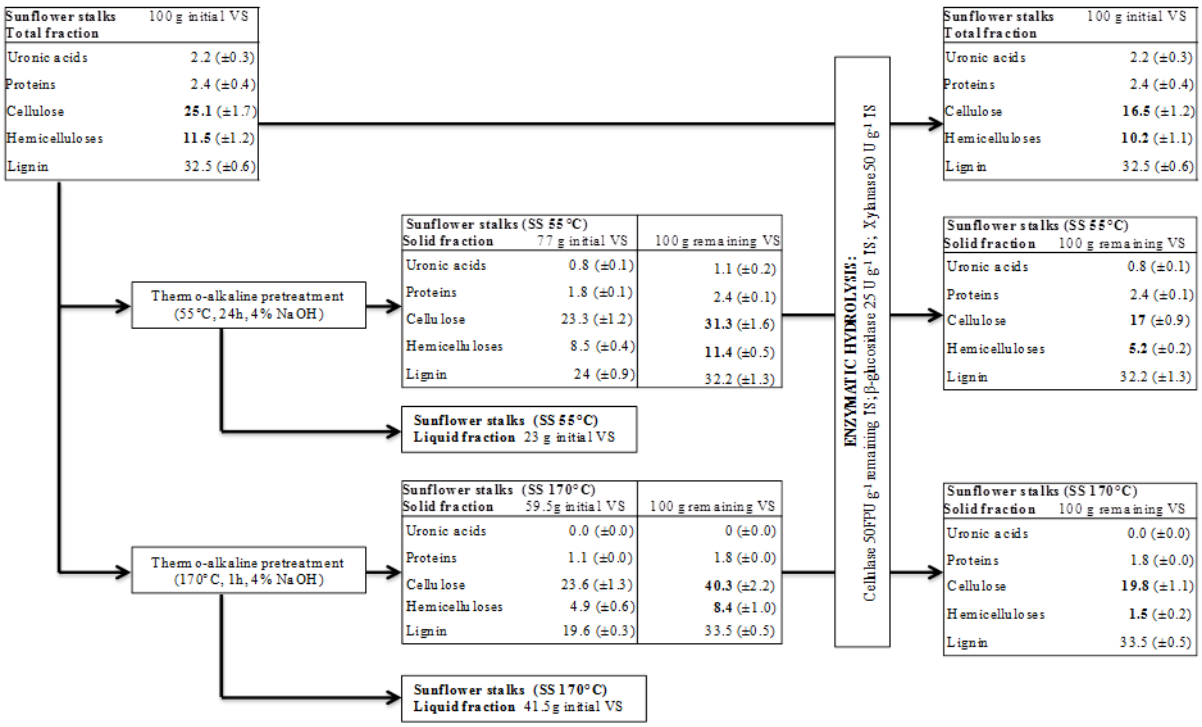
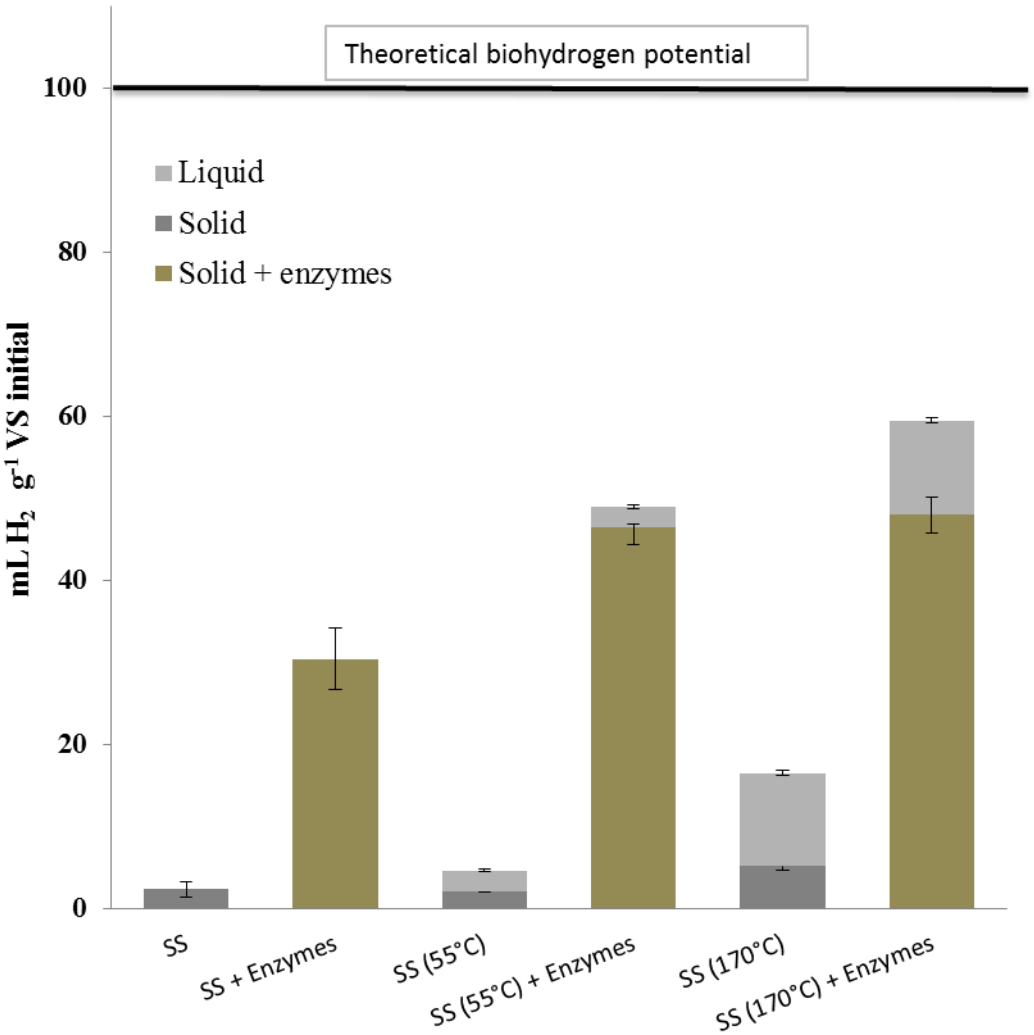


Figure 1 Scheme of pretreatment strategies used and biochemical composition of raw sunflower stalks (SS) expressed in g/100g initial VS and of the solid fractions after thermo-alkaline pretreatments at 55°C (SS 55°C) and at 170°C (SS 170°C) expressed in g/100g initial VS and in g/100g remaining VS. Values correspond to means of two replicates of independent values ± standard deviation.



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464 **Figure 2** Impact of pretreatments on the origin of the biohydrogen production (ml H_2 .g^{-1} initial VS) for raw sunflower stalks and solid residue of alkaline
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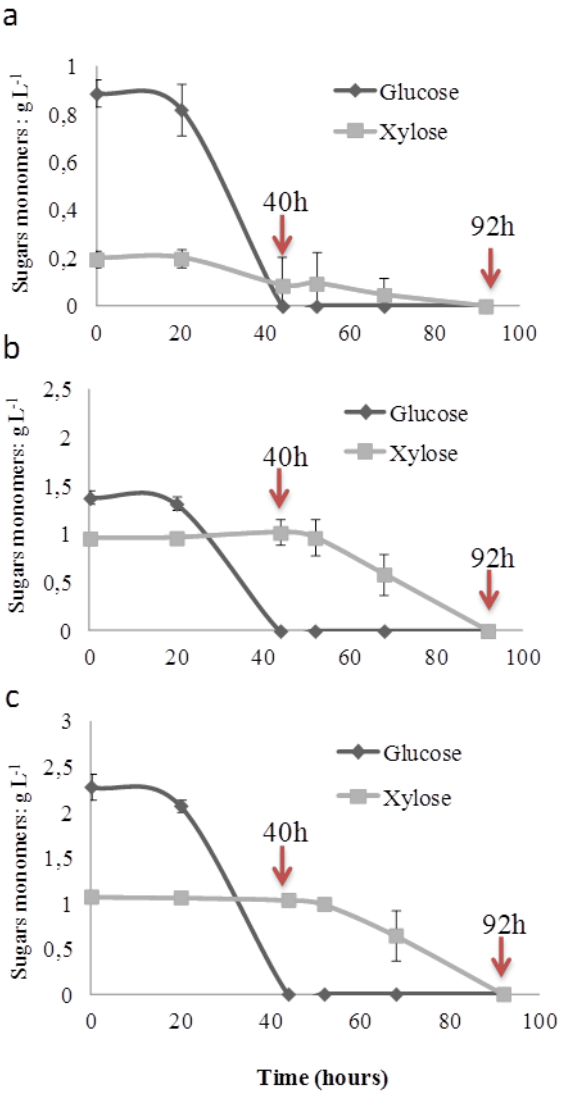

Figure 3 Evolution of consumption of monomeric sugars (ie glucose and xylose) released from enzymatic hydrolysis during H_2 fermentation in mixed cultures (a) from raw sunflower stalks (b) from solid residue of sunflower stalks pretreated at 55°C, 24 h, 4% NaOH (c) from solid residue of sunflower stalks pretreated at 170°C, 1 h, 4% NaOH. Values correspond to means of two replicates of independent values ± standard deviations (error bars).