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Enhancement of corn stover conversion to carboxylates by extrusion and biotic triggers in solid state fermentation

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Abstract

Solid-state fermentation is a potential technology for developing lignocellulosic biomass-based biorefineries. This work dealt with solid-state fermentation for carboxylates production from corn stover, as building blocks for a lignocellulosic feedstock based biorefinery. The effect of extrusion pretreatment, together with the action of a microbial consortia and hydrolytic enzymes as biotic triggers, was investigated on corn stover conversion, microbial metabolic pathways and populations.

The extrusion caused changes in the physical and morphological characteristics, without altering the biochemical composition of the corn stover. Extrusion also led to remarkable differences in the composition of the indigenous microbial population of the substrate. Consequently, it affected the structure of community developed after fermentation and the substrate conversion yield, which increased by 118% (from 23 ± 4 gCOD/kgVSi obtained with raw substrate to 51 ± 1 gCOD/kgVSi with extruded corn stover) with regards to self-fermentation experiments.

The use of activated sludge as inoculum further increased the total substrate conversion into carboxylates, up to 60 ± 2 gCOD/kgVSi, and shaped the microbial communities (mainly composed of bacteria from the *Clostridia* and *Bacteroidia* classes) with subsequent homogenization of the fermentation pathways. The addition of hydrolytic enzymes into the reactors, further increased the corn stover conversion, leading to a maximum yield of 142 ± 1 gCOD/kgVSi. Thus, extrusion pretreatment combined with the use of an inoculum and enzyme addition increased by 506% corn stover conversion into carboxylates. Beside biomass pretreatment, the results of this study indicated that biotic factor greatly impacted solid-state fermentation by shaping the microbial communities and related metabolic pathways.

Keywords: **Solid-State Fermentation; mixed culture; pretreatment; lignocellulosic biomass; carboxylates; environmental biorefinery**

Introduction

Lignocellulosic biomass is the most abundantly available organic raw material on Earth and it is considered to be one of the most promising resource for biofuels and biomolecules production at industrial scale (Adekunle and Okolie 2015). While lignocellulosic biomass cultures dedicated to energy production have been widely criticized for the competition with food dedicated cultures, agricultural residues, such as corn stover, do not compete with food production. In addition, the constantly increasing quantity of crop residues issued from the agricultural activities constitutes an opportunity for developing new valuable processes to produce bioenergy or biomolecules. Corn is currently the world largest crop, with production of about 885 million MT of grain (FAO 2013). Two tons of lignocellulosic residues are produced for each ton of grain, making corn stover (CS) one of the most abundant lignocellulosic resources issued from agricultural residues. Currently, this resource is poorly valorized since the stoves are incorporated into soil (buried in the fields or not) and/or used for animal farming as litter.

An interesting alternative is to use CS as raw material in biorefineries to maximize its exploitation and produce value added products and energy by fermentation and anaerobic digestion (AD). The remaining nutrients could here be returned into the soils in form of digestate as bio fertilizer. Biorefinery pathways to produce higher value products from heterogeneous low value feedstock could be based on the conversion by anaerobic fermentation of the organic matter into short chain organic acids (mainly acetate, propionate, butyrate) and alcohols, but also lactate: these compounds are all referred to carboxylates (Agler et al. 2011). Compared to bio-H_2 and bio-CH_4 that can be produced as well by anaerobic microbial consortia, carboxylates are not in competition with low price energy sources, such as natural gas (~0.5 $US·m^{-3}), as they can be further converted into value-added products, such as polyhydroxyalkanoate bioplastics. Carboxylates may also be used as intermediate building blocks for the production of commodity chemicals and biofuels (e.g via chain elongation processes) notably in integrated bioconversion steps (Agler et al. 2011).

However, due to their large content in lignocellulose, fermentative degradation of agricultural residues remains a technological challenge. Indeed, pretreatment is considered to be the most important and expensive step for obtaining an efficient conversion of biomass to value-added product (Mussatto and Dragone 2016). Several physical, chemical and biological pretreatment methods are described which promote the accessibility of polysaccharides in a lignocellulose complex for enzymatic and microbial hydrolysis (Mussatto and Dragone 2016). Mechanical pretreatment of lignocellulosic biomass is an essential prerequisite to improve particle distribution and densification, enzymatic accessibility and bioconversion efficiency (Bhutto et al. 2017).

Compared to other pretreatment methods, which are mainly performed in batch mode, extrusion has the advantage to be a continuous process, with a short residence time and high solid content (50-80%), which makes it easy to be scaled-up and with great adaptability to process modification (Muthukumarappan and Karunanithy 2016). Moreover, the total energy requirement of extrusion process has been estimated as 0,22-0,69 kWh/ t TS (Lamsal et al. 2010), which is quite low compared to the energy required for other pre-treatments. Indeed, Menardo et al. (2012) reported that the energy required for straw bale breaker and shredder was 15 kWh/ t TS while the energy required for thermo-alcaline pre-treatment has been estimated around 42 kWh/ t TS (Monlau et al. 2015).

Extrusion process consists in making chipped material passing between twin-screws and the compression of the screw causes a deconstruction of the organic material. Advantages of this process over others is that the compression causes a size reduction and a defibrillation of the material. Since the process is carried out at moderate temperature, the formation of degradation and oxidation products of lignin and polysaccharides is prevented (Vrije et al. 2002). Originally used in the plastic industry and later adapted to the food industry, extrusion process is a new promising physical pretreatment method for biomass conversion to sugars platform for biorefinery purposes (Zheng and Rehmann 2014; Muthukumarappan and Karunanithy 2016). In the context of environmental biorefinery, extrusion pre-treatment has been evaluated to increase the biogas yield in anaerobic digestion (Vrije et al. 2002; Panepinto and Genon 2016) and to increase sugar recovery by enzymatic hydrolysis (Karunanithy and Muthukumarappan 2011).

After having pretreated the lignocellulosic biomass by extrusion, solid-state (high-solids) fermentation (SSF) processes present several operational and technological advantages compared to the conventional “liquid” bioconversions: (i) a reduction of water requirements, (ii) a technical simplicity of extraction of metabolic by-products because of their higher concentrations in the digestate, (iii) a smaller reactor volume, (iv) less energy requirements to heat the reactor and (v) easier handling of the digestate residues (Kothari et al. 2014).

Usually, the digestate or leachate of SS-Anaerobic Digestion is used to inoculate the fresh feedstock but some studies reported an increase in the production of VFAs from lignocellulosic feedstock when rumen fluid was used as inoculum in Li et al. (2011).

However, a simple mechanical pretreatment alone is insufficient for attaining economically feasible biomass bio-conversion yields (Vidal et al. 2011). Thus, a combination of mechanical pretreatment with enzymatic or chemical treatment is required to significantly improve the conversion of organic material by reduction in particle size and enhancement of the biological hydrolysis and fermentation processes (Vrije et al. 2002).

To the best of our knowledge, there is a lack of investigation concerning the use of extrusion pre-treatment to enhance carboxylates production from lignocellulosic biomass by mixed-culture solid-state (SS-)fermentation.

The present work aims to evaluate CS deconstruction and bioconversion by combining a pretreatment by extrusion with the action of a microbial consortium and enzymes to produce carboxylates in SSF process. The effects of the extrusion pretreatment on CS conversion, microbial metabolic pathways and populations, in mesophilic SS-fermentation alone (self-fermentation) and in combination with the use of biotic triggers such inoculation and enzymatic addition were investigated.

Material and methods

Substrate

The substrate corresponded to a raw CS. Samples were chopped to a particle size of approximately 10-20cm using a vegetable milling machine. The chopped samples were then shredded using knife milling (Retsch SM 100, Germany) with a screen size of 4mm. The grinded biomass was then impregnated with water using a spray system to reach 400g/L of total solid then treated using twin screw extruder (Fig. 1). Impregnated CS was extruded at 200 rpm and 37°C in an automatic weight feeder extrusion (Thermo scientific Eurolab 16). The substrate was dried and kept at room temperature in a dry and dark room.

Substrate characterization

Physical state

Images of particles observed in bulk were acquired using a macrovision system including a CCD color video camera (JVC KY F55B, Alliance Vision, Montélimar) equipped with a 50 mm Nikon lens (AF Nikkor, 1/1.8) (Devaux et al. 2008). Two neon tubes (2 X9 W) were used for illumination. A specific lighting box was built in order to illuminate samples with diffuse light. Samples were put into a petri dish and inserted in the box using a drawer that closed the box. The height of the camera and the lens focus were manually adjusted to observe a 3.5 cm x 2.6 cm area. The size of pixel was 8.21 µm.

To observe the disintegration of particles on hydration, a torus reactor equipped for visualization of particles was used (Devaux et al. 2006). 300 mg of powder were suspended in water and introduced into the reactor. The volume was completed to 200 ml with water. The particles were maintained under stirring with a propeller and continuously moved

within the reactor. Images (10 per time) constituted of 768 x 576 pixel, each pixel being of size 17.3 µm x 17.3 µm were acquired every ten min per hour. The grey levels were coded on 256 values starting from 0 (black) up to 255 (white).

Quantitative information concerning particle size was extracted by image analysis using grey level granulometry through line opening as structural element (Devaux et al. 2006). The method can be considered as a sieving of the objects in images which provides granulometric curves that can be interpreted as usual granulometric curves.

Composition

Extractive-free samples were ground 3 min in nitrogen liquid (SPEX 6700 freezer Mill).

Infrared spectroscopy

FT-IR spectra were recorded from KBr pellets made of 2 mg extractive-free samples mixed with 100 mg of KBr. The spectra were collected in transmission mode between 4000 and 400 cm^{-1} at 4 cm^{-1} interval (Brucker vector 22 spectrometer). The infrared spectra resulted from the co-addition of 200 interferograms. Eight spectra per sample were collected. The infrared spectra in the 2000-700 cm^{-1} region were baseline corrected and unit vector normalized using the OPUS software (version 6.5).

Cellulose crystallinity

The crystallinity percentage of cellulose was determined by ^{13}C solid state NMR as previously described (Timpano et al. 2015).

Hydration properties

Water soluble sugars were determined as follows. Samples (360mg) were suspended in distilled water (30 ml) and gently stirred overnight at 4°C using a rotating shaker. Each suspension was centrifuged at à 20°C-3000g for 20 min. The supernatant was recovered, filtered on G2 sintered glass and analyzed for neutral sugars by the automated orcinol method (Tollier and Robin, 1979). Swelling, ‘volume occupied by a known weight of particles under the conditions used’ and water retention capacity ‘the amount of water retained by by a known weight of particles under the conditions used’ was determined as described by Robertson et al. (Robertson et al. 2000). Swelling was expressed as volume/g original substrate weight and water retention capacity as the amount of water retained by the insoluble material (g/g dry weight).

Fermentation tests

Inoculum

The microbial inoculum corresponded to dewatered Activated Sludge (AS) collected from a municipal wastewater treatment plant (Narbonne, France). The choice of this inoculum was dictated by the necessity of operating the fermentation experiment at high solid load. The initial total (TS) and volatile (VS) solids of the AS were 17.3% and 13.9% respectively. Preliminary tests were carried out to determine the optimal storage condition. Storage at at -20°C was chosen among the use of fresh AS, heat pre-treated AS (90°C, 30'), storage at 4°C and storage at -80°C, since it ensured the highest conversion yield and reproducibility of results (data not shown). The inoculum was placed at 4°C one day before the start-up of the reactors, before inoculating the reactors, an inoculum solution (20 gVS/L) was prepared by suspending the inoculum into demineralized water and mixing for 30 min.

Batch tests

Fermentation experiments were carried out in 120 mL flask. Each reactor contained: 4 g of substrate; 1 mL of NH_4Cl solution 16 %(m/v); 0.2 mL oligo-element solution (1.5 $g.L^{-1}$ of $FeCl_2,H_2O$; 62 $mg.L^{-1}$ of H_3BO_3,H_2O; 117 $mg.L^{-1}$ of $MnSO_4,H_2O$; 26 mg.L-1 of $CoCl_2,6H_2O$; 120 mg.L-1 of $ZnCl_2$;28 mg.L-1 of $NiCl_2,6H_2O$; 38 $mg.L^{-1}$ of $CuCl_2,2H_2O$; 31.8 $mg.L^{-1}$ of $NaMoO_4,2H_2O$; and 4.4 mL of HCl 37%); phosphate buffer (0.05M, pK = 6.4); in case of inoculated reactors, inoculum at substrate/Inoculum ratio of 18 (g_{VS}/g_{VS}) and distilled water. Enzymatic addition, with the objective of increasing the accessibility of the cellulose, was performed by directly adding a mix of enzymes (cellulase and endo-1,4-xylanase from *Trichoderma longibrachiatum*, *SIGMA-ALDRICH*®), at 20 U/gTS each, into the fermentation reactors following the procedure proposed by Sambusiti et al. (Sambusiti et al. 2015). Demineralized water was added according to a mass balance on the TS contents (including CS, inoculum, buffer, and solution addition) to obtain a final TS concentration of 15%. To ensure the homogeneity, a medium solution containing all the above mentioned components was prepared and the pH was adjusted at 6.4 with KCl or NaOH solutions (1N) before to be added into the reactors. After the solution was added, the reactors were mixed by hands with spatula to obtain a homogenous composition. Then, the reactors were closed with rubber septa and plastic plugs and the headspace was flushed with N_2 for 5 minutes to ensure anaerobic conditions. The batch tests were then incubated at 37°C for 9 days. The experiments were stopped at 9 days since no significant increase of conversion yield were observed during longer operation (up to 15 days) (data not shown).

The effect of the extrusion pretreatment on CS fermentation was evaluated in three different conditions (Table 2): first, the effect of extrusion pretreatment was assessed by fermenting the substrate without the use of biotic triggers (self-fermented); second, the use of AS as inoculum was evaluated (inoculated); third, the increase of hydrolysis with enzymes was carried out (enzymes addition). Experiments were performed in duplicates on CS Ext-CS.

Analytical methods

Total solids (TS) and volatile solids (VS) content were measured according to the standard Methods (APHA 2005). Biogas production was periodically monitored during fermentation by measuring the pressure in headspace (100 mL) using a digital manometer (LEO 2, Keller), and sampling biogas with a 0.2 mL syringe for analysis. If pressure of one reactor was superior to 1.75 bars, after gas sampling, all reactors were degased to reach 1.25 bar. Biogas composition was determined using a gas chromatograph (Perkin Elmer Clarus 580), equipped with two capillary columns: a R-Q-bond column for CO_2 determination (30 m x 0.32 mm) and an Rt-Molsieve 5 Å (30 m x 0.32 mm) column for O_2, N_2, CH_4 and H_2. Argon (350 kPa at 34 mL min^{-1}) was used as carrier gas to transport gas sample from the injector (250°C) to the thermal conductivity detector (150°C).

To extract the liquid fractions of the reactors, a gravimetric dilution to 5% TS was performed. The liquid phase was then extracted after filtration with a microfiber filter GF/D (2.7 µm Whatman, Kent, UK). The pH was immediately measured after dilution. Sugars and metabolites produced during fermentation were analyzed by High Performance Liquid Chromatography (HPLC) equipped with an Aminex HPX 87H column (Biorad) at 35°C, with a 0.005 M H_2SO_4 water solution (0.4 mL min^{-1}) as eluent. The concentration of each metabolite (g/L) provided by the HPLC was expressed in gram of Chemical Oxygen Demand (gCOD) and was referred to the VS initial amount (kg) of substrate. Total substrate conversion was used to assess fermentation performances, it was defined as the sum of metabolites measured in the reactor at the end of fermentation: succinate, acetate, propionate, butyrate, valerate, isovalerate, caproate, ethanol and lactate.

Microbial community analysis

Genomic DNA was extracted and purified as reported elsewhere (Pierra et al. 2014). The V3 region of 16S rRNA genes was amplified using universal primers for bacteria (Moscoviz et al. 2016). The resulting PCR products were purified and sequenced, using the MiSeq v3 chemistry (Illumina) at the GenoToul platform (www.genotoul.fr).

Sequences (32393 ± 4295 in average per sample) were retrieved after demultiplexing, cleaning, and affiliating sequences using mother (Schloss et al. 2009). They were grouped into operational taxonomic units (OTUs) at a 97% similarity and most abundant sequences were affiliated to their closest relatives in the NCBI database (Table 3). Sequences were submitted to GenBank, with accession No. MG578513 - MG579773. Shannon (H) and Simpson (1-D) diversity indices were calculated by using R package 'vegan'. Quantitative PCR (qPCR) of 16S rDNA was performed as described elsewhere (Moscoviz et al. 2016).

Calculation

Hydrogen and carbon dioxide daily yields (in NmL/g_{VS}) were calculated according to eq.1:

$$Y_{biogas_{(j+1)}} = \frac{(\%Biogas_{j+1} \times P_{j+1}) - (\%Biogas_j \times P_j) \times T_0 \times V_{HS}}{P_0 \times T_{reactor} \times m_{VS}} \quad \text{(eq.1)}$$

Where $\%Biogas_j$ is the percentage (mol/mol) of H_2 or CO_2 at day j, P_j is the pressure of reactor headspace (bar) at day j, $T_{reactor}$ is the Temperature of the reactor, i.e. 37°C, T_0 and P_0 the temperature and pressure at Normal conditions (T_0 = 273 K, P_0=1.000 bar), V_{HS} the volume of the headspace in mL and m_{vs} the mass of volatile solids in g_{VS}.

Results

Substrate characterization

Particle morphology

Raw biomass was composed of particles with different sizes and shapes. Elongated particles corresponded to fibre enriched fragments while large particles originated mainly from pith parenchyma. In extruded biomass, small fragments and larger particles with a rounded shape and coarse surface could be observed, suggesting that extrusion led to particle fragmentation and aggregation. Images of the raw and extruded particles in bulk acquired with the macro-imaging device are shown in Supplementary Materials. To quantify more precisely the particle size distribution and its evolution when hydrating the samples, particles were imaged by the torus reactor. Quantitative information was extracted from the images by applying image texture analysis. The curves resulted from an averaging of the individual curves calculated for the images recorded at different times of sample hydration. The position of the peak reveals the particle size for which the major grey level variations occurred. At t0, large distributions were observed for both the raw and extruded samples indicating a heterogeneity in particle size as previously viewed in images of particles in bulk

(Fig. 2). After one-hour hydration in water, no evolution of the particle size was observed in the raw samples. In contrast, for the extruded sample, the initial peak clearly shifted to lower values. Grey level mean sizes were assessed from the granulometric curves. For the raw substrate, the grey level mean size was 1178 ± 87 µm. For the extruded sample, the grey level mean size decreased from 683 ± 50 µm at t0 to 411 ± 48 after 1h under stirring in water, suggesting a fragmentation of the aggregates.

Cell wall composition and properties

To investigate the changes in composition induced by the extrusion pretreatment, the cell wall composition was analysed by Fourier-transformed infrared spectroscopy (FITR spectra are shown in Supplementary materials). The spectra from extruded and raw biomass revealed differences in the intensity of the bands at 1730, 1513 and 1250 cm^{-1} which could be assigned to ester linked acetyl groups and hydroxycinnamic acids. There is no major change in cellulose crystallinity. (Table 1).

The ability to swell in presence of water, to retain water and the amount of soluble sugars are factors that can influence the rate and extent of fermentation. Extrusion decreased the swelling and water retention capacity (Table 1). The amount of water soluble sugars was low (less than 3% of the dry matter) and in the same range for raw and extruded substrates.

Solid State Fermentation

The effect of the extrusion step on substrate conversion during SSF (15%TS) was here evaluated under the same operating conditions for Ext-CS and CS. The effect of the use of biotic triggers, such as inoculation and the addition of hydrolytic enzymes, to improve substrate conversion was also investigated. The three different conditions performed are summarized in Table 2. The total conversion of CS and Ext-CS into metabolites of the three conditions is presented in Fig. 3 while soluble COD (SCOD) composition (gCOD/KgVS_i) and pH are reported in Table 2.

Substrate conversion highly varied between the different tests, but the replicates were highly reproducible (Fig. 3). The total conversion into fermentation metabolic products of extruded substrate was 50 ± 2 gCOD/kgVS_i for self-fermented, 60 ± 02 gCOD/kgVS_i for inoculated and 142 ± 1 gCOD/kgVS_i for enzymatic/inoculated test. The total conversion significantly increased after extrusion independently of the tested conditions, by 118%, 59% and 56% when compared to self-fermented, inoculated and enzymatic augmented/inoculated unextruded tests, respectively. This also

led to lower pH values measured at the end of fermentation for Ext-CS compared to CS in all the tested conditions (Table 2).

Inoculation with AS led to an increase of the total conversion for both substrates. This was particularly noticeable for CS which had a substrate conversion yield of self-fermentation of 23.4 ± 3.7 gCOD/kgVS$_i$, while inoculated CS yielded 37.6 ± 0.5 gCOD/kgVS$_i$. As a consequence, inoculum addition led to an increase of metabolites production of more than 61%. Comparatively, Ext-CS conversion increased by only 17% (i.e. from 50.9 ± 0.5 to 59.8 ± 2.0 gCOD/kgVS$_i$).

A significant improvement of the total conversion was observed after addition of the enzymes. The total substrate conversion was four times higher than the one obtained by self-fermentation for CS and almost three times for Ext-CS, reaching yields of 90.8 ± 1.9 gCOD/KgVS$_i$ and 141.9 ± 0.9 gCOD/KgVS$_i$ with CS and Ext-CS, respectively. Consecutively, with the enzymatic addition the final pH values were the lowest for both substrates.

Experimental conditions highly affected the metabolites composition since at the end of fermentation different metabolic behaviors were observed. Lactate was the main product of CS self-fermentation. Acetate, propionate and other metabolites (mainly succinate, data not shown) represented only 5% of the COD. In contrast, lactate was produced in a lesser extent during self-fermentation of Ext-CS. Here, the main metabolite was butyrate, followed by acetate, ethanol and propionate (Table 2).

Interestingly, with no inoculum, fermentation was incomplete for both substrates, with soluble sugars detected at the end of the self-fermentation. The use of AS as inoculum led to a total consumption of soluble sugars during the course of fermentation of CS and Ext-CS, independently of the tested conditions and enzyme addition. After inoculation, metabolites distribution was mostly similar for the two inoculated substrates, while, compared to self-fermentation different metabolites distribution were observed, indicating different metabolic pathways. Acetate was the main product, followed by propionate and butyrate (Table 2). Ethanol represented a minor part of the metabolites produced and no lactate was detected. In inoculated tests, medium chain carboxylates, *i.e.* valerate and other metabolites (mainly iso-butyrate and iso-valerate) were observed after fermentation of both substrates. Caproate was also produced in fermentation of Ext-CS.

The enzymatic addition induced a change in the proportions of each metabolite when compared to the simple inoculation, but not in the overall spectrum of metabolites produced. With enzymatic addition, acetate, despite still representing the major end-product, decreased at the expense of butyrate and ethanol produced from the fermentation

of both substrates (Table 2). Propionate and valerate production did not significantly increase, while in this condition, caproate was produced from the fermentation of both substrates. Interestingly, metabolic distribution was very similar for the self-fermentation and the enzymatic augmented fermentation of Ext-CS with the exception of lactate (produced during self-fermentation) and medium chain carboxylates (produced only with enzymatic augmented fermentation).

To further investigate the fermentation dynamics, the produced biogas over the fermentation time was daily analyzed and quantified. CH_4 was never detected in reactors headspace, while CO_2 production followed a similar trend in all the tested conditions: CO_2 production rate increased during the first three days of fermentation and afterward remained almost constant. The volume of CO_2 produced was linearly correlated (R^2= 0.87) with the accumulated amount of metabolites (data not shown). Regarding H_2 gas production, different behaviors between self-fermented and inoculated experiments were observed (Fig. 4). Almost no H_2 was produced during self-fermentation of CS, while H_2 was continuously produced during self-fermentation of extruded CS, reaching a concentration of 34% in the headspace, that was equivalent to a cumulative volume of 15.4 ± 0.7 mL H_2 (4.4 mL H_2/gVS_i) after nine days of fermentation. In all the experiments inoculated with AS, independently of enzymes addition, H_2 production followed a similar trend: a sharp increase of H_2 concentration was observed in the headspace of the reactors during the first three days and then H_2 was completely consumed between days three and six. The maximum cumulative H_2 was observed in the test with enzymatic addition for both CS and Ext-CS, reaching at three days of fermentation respectively 12.2 ± 0.2 and 17.0 ± 0.1 mL.

Microbial community analysis

The microbial communities were analyzed by Illumina-based 16S sequencing after nine days of fermentation of the CS and Ext-CS substrates, in all the tested conditions, as well as the ones from the initial inoculum (AS) and the indigenous populations of the substrates. Table 3 reports the relative abundances (%) of the key microbial groups (at the genus level) and the alpha diversity indices (Shannon and Simpson) calculated for each microbial community.

Taxonomic affiliation of metagenomic data showed that sequences were mainly affiliated to the phyla *Firmicutes* (54.5%), *Proteobacteria* (21.4%), *Bacteroidetes* (13.1%) and *Actinobacteria* (9.0%). Other phyla such as *Nitrospirae*, *Chlorobi*, *Acidobacteria*, *Acidobacteria* and *Chloroflexi* were present but at very low abundances (<2%). Sequences with more than 3% of similarity were grouped into single Operational Taxonomic Unit (OTU). By excluding AS inoculum and CS substrate, only sixteen OTUs explained the overall diversity (> 92%) found. Taxonomic affiliation is shown in Table 3.

The *Proteobacteria* phylum dominated in the AS (49%) and the indigenous community of CS substrate (63%), with bacteria representative of the three classes of *Gammaproteobacteria*, *Alphaproteobacteria* and *Betaproteobacteria*. Meanwhile, the phylum *Firmicutes*, with bacteria belonging to the classes of *Bacilli* and *Clostridia*, strongly dominated the indigenous microbial communities of Ext-CS substrate and the community found in all the reactors, independently of the fermentation condition. Thus, surprisingly, extrusion pretreatment had a large impact on the microbial community. Indeed, wide difference was found in the indigenous populations of the two substrates. Whereas the number of 16S gene copies found in Extr-CS was similar to the ones found in the inoculum and the inoculated samples (in the order of 1010 copies 16S/g VS), the number of 16S gene copies found in CS (5.68E+08 copies 16S/g VS) was two orders of magnitude lower. Despite the lower bacterial abundance, the microbial community of CS showed high level of diversity, showing Shannon index (4.34) and 1-Simpson index (0.98) comparable to AS values, which, as expected, showed the highest diversity values among all samples (i.e. 5.42 and 0.99 for Shannon and 1- Simpson indices respectively). Conversely, Ext-CS was characterized by a low value of Simpson diversity (0.58), indicating a clear dominance of only few species, since the Simpson Index gives more weight to the most abundant species. The microbial community of the Ext-CS substrate was mainly composed of bacteria from the *Bacillaceae* family. Only two OTUs were dominant, from *Bacillus* and *Lysinobacillus* genera respectively, representing 71 % and 18 % of the total bacterial community. Nucleotide sequence analyses of their 16S rRNA genes revealed respectively 99 and 100 % of sequence homology with *Bacillus licheniformis* and *Lysinibacillus halotolerans*. Both are known to be facultative anaerobic species, commonly found in the soil (Kong et al. 2014), which is consistent with the origin of the substrate. The presence of these two OTUs represented a sort of signature of the Ext-CS, since they were found, even in a minor proportion (<10%), in all the microbial communities developed during fermentation of Ext-CS while they were never found in samples of CS substrate.

After nine days of self-fermentation an inverse trend was observed where the self-fermented CS showed a lower diversity (and higher dominance) when compared to Ext-CS (Table 3). The microbial community of self-fermented CS was mainly composed of bacteria from the class of *Bacilli* (63%), with an OTU affiliated to *Enterococcus* genus representing 57% of sequences, and more particularly 100% of homology with *Enterococcus hirae*. This OTU was also detected in the community of CS substrate but at very low abundance (1.4%). Apparently the fermentation condition imposed in the reactor (O_2 depletion, 37°C, initial pH 6.4, 15%TS) favored its development. This is confirmed by the fact that sequences from this OTU represented 19% of the sequences found in the self-fermentative

community of Ext-CS substrate, while they represented only 0.3% of the sequences of the community of Ext-CS. The remaining part of the microbial community of self-fermented CS was mainly composed of bacteria belonging to the family of *Enterobacteriaceae*. Specifically, two OTUs represented 36% of the total number of sequences. Nucleotide sequence analyses of their 16S rRNA genes revealed resp. 99 and 100% of sequence homology respectively with *Cronobacter sakazakii* (24%) and *Enterobacter cloacae* (12%), which are known to be facultative anaerobic fermentative species. The OTU affiliated with *Enterobacter cloacae* seems to be characteristic of CS substrate, since after fermentation, independently of the conditions, it explained a non-negligible part of the sequences (6-14%).

Self-fermenting Ext-CS community, a part of the bacteria belonging to the class of *Bacilli* (35%), was composed of bacteria of the *Clostridia* (39%) and *Coriobacteria* (17%) classes. Among *Clostridia*, the *Ruminococcaceae* family was predominant and the genus *Caproiciproducens* was mostly represented (22%), being the sequences of the two most abundant OTUs homologues of *Caproiciproducens galactitolivorans* (showing respectively 98 and 94% of identity). As indicated by the name, this species is known for the ability to produce caproic acid (Jeon et al. 2010), but this metabolite was not detected during self-fermentation of Ext-CS.

Inoculation led to the development of fermentative communities showing similar diversity values for the two substrates but the observed diversity was impacted by the fermentation conditions: the enzyme addition led to more diverse microbial communities with respect to simple fermentation. Strong similitudes were found in the community structure of the fermentative communities developed after inoculation, independently of the conditions (with or without enzymes). Few differences were observed, mostly linked to the substrate. Indeed, the four communities (Inoculated and enzymatic augmented CS and Ext-CS) were all mainly composed of bacteria from the *Clostridia* and *Bacteroidia* classes (representing respectively 44 and 22 % of sequences in average). Bacteria belonging to the class of *Clostridia* were mostly affiliated to the family of *Ruminococcaceae*, a family that contains a number of cellulolytic and amylolytic species isolated from several types of anaerobic environments, including AD reactors (Nelson et al. 2011). Specifically, two OTUs were affiliated to the *Caproiciproducens* genus. As above mentioned, these species are capable of producing caproic acid (Jeon et al. 2010), which is consistent with macroscopic observations.

The class of *Bacteroidia* was mostly represented by a unique OTU belonging to the *Prevotella* genus from the family of *Prevotellaceae*. This OTU represented 1% of the sequences found in the microbial community of AS. Microorganisms from this family are saccharolytic bacteria, which have already been found in fermentative communities developed from AS from wastewater treatment plant (Xing et al. 2008). *Prevotella* species showed

potential to produce propionate via the randomizing (succinate) pathway, which consume H_2, through the fermentation of sugars and lactate (Purushe et al. 2010). Thus, the presence of these bacteria could explain the H_2 consumption during fermentation observed into the reactors which were inoculated.

Discussion

Effect of extrusion pretreatment on SSF

During extrusion process, biomass undergoes physical and chemical changes via shearing, mixing, heating. In this study, extrusion results in particle size decrease, changes in morphology, surface aspect, swelling and water retention capacity. No drastic changes were observed in the biochemical characteristics of biomass (apart from a slight decrease in ester-linked compounds) or on the crystallinity of the cellulose. Consistently, Chen et al. (Chen et al. 2014) observed that extrusion-pretreated rice straw showed significant particle size reduction, physical properties improvement and volume expansion, but no changes in the chemical composition as compared to the milling-pretreatment and unpretreated rice straw. The particle size decrease and thus increase in specific surface area are the well-known effects of extrusion. They result from the shear friction between particles during the extrusion process. These phenomena are further enhanced by low moisture content and large particle size of biomass (Karunanithy and Muthukumarappan 2011).

Some authors reported an increase in water soluble fractions in extruded plant biomass (Ralet et al. 1990; Ning et al. 1991; Ralet et al. 1993; Myat and Ryu 2014). The water solubility has been shown to be proportional to specific mechanical energy up to a certain value over which solubility did not further increase (Ralet et al. 1990; Ralet et al. 1993). In our study, the severity of extrusion was probably too soft to induce solubilization of the cell wall components. However, only few data are available on the impact of extrusion on water hydration properties. Our results are in agreement with those reported by Ning et al. (Ning et al. 1991) and Ralet et al (Ralet et al. 1993). The lower values obtained for hydration properties could be explained by the fact that extrusion caused particle disruption and aggregation thus reducing interstitial spaces between particles that can participate in retaining water. The impact of extrusion on cellulose crystallinity is more controversial. Zhang et al. (Zhang et al. 2012) reported no drastic change in cellulose crystallinity of CS submitted to twin-screw extrusion. The authors noticed that extrusion could result in the appearance of cellulose II structure under certain conditions. Similarly, no significant difference in crystallinity index between raw and extruded CS fiber was reported by Myat et al. (Myat and Ryu 2014). Zheng et al. (Zheng et al.

2015) noticed only a slight increase of the cellulose crystallinity index in extruded CS with no significant effect of screw configurations while Yoo et al. (Yoo et al. 2011) found an increase of cellulose crystallinity by 82 % with no change in sugar composition in extruded compared to untreated soybean hull. Variations in the biomass moisture content and extrusion operation condition may account for theses discrepancies.

The increase in total conversion observed for of Extr-CS compared to CS was probably due to a higher specific surface of the substrate (Vidal et al. 2011) that enabled a better access to enzymatic hydrolysis (Chen et al. 2014). When testing hydrolysis efficiencies of enzymes on extruded or 1 mm ground *Miscanthus* in addition with NaOH, Vrije et al. (Vrije et al. 2002) improved glucan hydrolysis by 34% and total biomass conversion by 15%. Similarly, Motte et al. (2015b) found an increase of 22% conversion of wheat straw into ethanol when substrate was pretreated with ball-milling (particle size of 0.3600 ± 0.004mm) instead of traditional milling (particle size of 1.03 ± 0.01 mm). Results presented in the present study showed the effectiveness of extrusion pretreatment for SS- fermentative conversion of CS. In addition, the increase in total conversion was always linked with a decrease in final pH. Probably, a higher substrate hydrolysis occurred with Extr-CS, followed by higher acidogenesis, consequently decreasing the pH due to organic acid accumulation (Zoetemeyer et al. 1982). Extrusion decreased the swelling and water retention capacity, meaning that more water was available for microorganisms during fermentation of Ext-CS compared to CS. García-Bernet et al. (García-Bernet et al. 2011) showed that the critical water content (corresponding to the amount of water under which there is no free water in the medium, only capillary and bound water) is proportional to the TS content; at 15% content of TS it can have a considerable effect on the effective diffusion volume available. Moreover, Bollon et al. (2013) reported that the transfer limitation has a threshold limit at 15% of TS. Therefore, the observed difference of water retention capacity of untreated CS and Ext-CS could have had a great impact on fermentation performances at 15% TS. The higher swelling of ground CS indicates that mass transfer may be lower compared to extruded CS, resulting in a more heterogeneous medium for ground CS. Low water content during the hydrolysis of CS implies a larger viscosity, low mass transfer of intermediates and end products, having consequent effect on limited enzymes diffusivity and fermentation performances (Pimenova and Hanley 2004; Felby et al. 2008; Hodge et al. 2009).

Effect of biotic triggers on SSF

Beside biomass pretreatment, the results of this study indicated that biotic factor greatly impacted SSF. According to previous researches (Marone et al. 2012; Marone et al. 2014), it was shown that the naturally-occurring microbes

hosted by the substrate can perform the biodegradation via so-called self-fermentation. However, the impact of inoculation with AS was very high, not only because it increased the total amounts of recovered products, but especially because it seemed to drive the fermentation, by stabilizing the community and consequently the spectra of produced metabolites.

During self-fermentation of CS, lactate accounted for almost half of the conversion products, which was consistent with the fact that microbial community was dominated by lactic acid bacteria (*Enterococcus* sp.). Since lactic fermentation was the major metabolic pathway carried out by endogenous bacteria, accumulation of lactate might have inhibited the fermentation process (Cabrol et al. 2017), which could explain a lower total conversion and the presence of remaining sugars at the end of fermentation. Moreover, the strong dominance of lactic acid bacteria could also explain the absence of H_2 production, since they are able to secrete bacteriocins which inhibit H_2 production (Noike et al. 2002).

Ext-CS showed a different behavior during self-fermentation, probably explained by the different microbial community found in this substrate. Indeed, the high amount of bacteria (4.52E+10) found on the Ext substrate, together with high dominance of few air tolerant strains, suggested that extrusion pretreatment favored the proliferation of these bacteria. This different initial community, together with the highest accessibility due to the higher specific surface of the substrate, led to the development of a more efficient fermentative community during self-fermentation of Ext-CS, which produced different spectra of metabolites. A possible explanation could be that the small particle size of Ext-CS allowed a faster hydrolysis rate, making soluble sugars rapidly available. Thus, *Clostridia*, which have fast growth rate and are known to be efficient fermenting bacteria, gained the competition over other microorganisms (Cabrol et al. 2017).

Inoculum addition shaped substantially the metabolite composition of SS- Fermentation. When adding inoculum, a different metabolic pathway was observed: no lactate was produced, no sugars remained after 9 days of fermentation and the two major metabolites were acetate and butyrate. As metabolites composition was significantly different from self-fermentation, it was concluded that bacteria from the inoculum won the competition over endogenous bacteria and imposed their metabolism as main metabolic pathway. That was confirmed by the analysis of microbial community structures after fermentation. The profiles of the different microbial communities developed during the inoculated fermentation were highly similar, independently of the conditions (with or without enzymes). Few differences were observed, mostly linked to the substrate. Although the strict conditions of fermentation imposed a strong selective

pressure leading to the establishment of a specific microbial community developed from the inoculum, the indigenous community brought by the substrate played a role in shaping the final structure of fermentative community. Indeed, a sort of substrate signature was identified only by the presence of specific strains or groups of bacteria, which were characteristic for each substrate.

Interestingly, some amounts of medium chain carboxylates were produced only after inoculation even though the bacteria responsible of chain elongation pathways (i.e. *Caproiciproducens* spp.) were found also in the self-fermentative community in Ext-CS. The inoculation led likely to a faster hydrolysis confirmed by the observation of a faster H_2 production rate, followed by a faster fermentation. This difference in hydrolysis kinetics affected the types of metabolites produced, making the substrates (i.e. ethanol, propionate, butyrate) (Angenent et al. 2016) for chain elongation sooner available.

In addition, differences in H_2 metabolism were observed between self-fermenting Ext-CS and inoculated reactors, which might be explained by the absence of hydrogen consuming bacteria in the self-fermenting Ext-CS. Indeed, H_2 consuming bacteria of *Prevotella* genus (Purushe et al. 2010) were found only in the fermenting communities developed after inoculation with AS.

Finally, the best result of 141.9 ± 0.9 gCOD/KgVS_i, which corresponded to a yield of 21 g COD/L of total metabolites, was obtained after enzymes addition, which allowed the simultaneous saccharification and fermentation into the reactors. This result is comparable with CS conversion yields obtained by applying different kinds of pre-treatment and bio-conversion processes. Darvekar et al. (Darvekar and Holtzapple 2016) obtained similar results (around 20 g/L acetic acid equivalent) from CS fermentation at 10% of TS, but adopting a time consuming procedure: after applying submerged lime pre-treatment, conducted at 50 °C for 28 days with lime loading of 0.15 g Ca(OH)2/g dry biomass, followed or not by shock (a mechanical process that uses a shockwave to alter the biomass structure) the fermentation lasted 35 days. A slightly higher conversion yield, equal to 25 g/L of total metabolites, was obtained after 12 days of anaerobic digestion at 22% of TS of grinded CS pre-treated with 5.0% NaOH (Zhu et al. 2010). Beside the different pre-treatment adopted, this better result it may be due to the use of a previously activated inoculum, i.e. the effluent of liquid anaerobic digestion. In our study, the microbial inoculum corresponded to dewatered AS, the dewatering procedures (the use of flocculants coupled with centrifugation) may have somehow made less active the microorganisms, probably the usage of more active microbiome, would gave different results.

However, accordingly with the present study, the combination of mechanical treatment with chemical or enzymatic treatment was shown to be the key step to increase the bioconversion of lignocellulosic biomass (Motte et al. 2015a). These authors used SSF as both a pretreatment process and a metabolite production process. After 64 days of fermentation at 23.5% TS with an initial pH of 6.8 of 10 mm ground wheat straw, a substrate conversion into metabolites of 170.7 ± 6.5 gCOD/kgVS was observed, which is a little bit higher than the one obtained in this study (141.9 ± 0.9 gCOD/kgVS) with a different substrate and in a much shorter fermentation (9 days). It could be supposed that, in the present study, extrusion greatly decreased the reaction time. Similarly, Chen et al. (Chen et al. 2014) reported that extrusion of rice straw accelerated the hemicellulose and cellulose hydrolysis and led to higher cellulose and hemicellulose degradation efficiencies, thus contributing to the enhancement of substrate conversion (i.e. into methane in that case). Therefore, the increased production of metabolites, such as ethanol and butyrate (with a concomitant decreased in produced acetate), observed in this study after enzymatic addition could be due to a rapid decrease in pH. Due to the rapid production of acids and the low homogeneity due to the solid state environment, the pH could locally reach very low values, causing a shift in bacteria metabolism to the production of metabolites with no charge (ethanol) or higher carbon content per unit (butyrate).

In conclusion, a significant effect of an extrusion pretreatment combined with the action of microbial consortia and hydrolytic enzymes was shown in favour to the production of carboxylates from lignocellulosic biomass by SSF. The extrusion, while it did not alter the biochemical composition of CS, caused changes in the physical and morphological characteristics. These changes, such as the increase in surface area and the decrease of water retention capacity, enabled a better action of microbial consortia and a better access to enzymatic hydrolysis, leading to an increase up to 506% of CS conversion into carboxylates. Biotic factors greatly impacted SSF: the use of AS as inoculum, beside increased the total amounts of recovered products, drove the fermentation pathways by stabilizing the community and consequently the spectra of produced metabolites. Nevertheless, it was possible to identify a sort of substrate signature in the communities characterized at the end of fermentation process, by the presence of specific strains or groups of bacteria which were characteristic for each substrate. Further studies are required to clarify the effective impact of the microbial community present on the substrate itself on fermentation at high organic load. Beyond, such extrusion pretreatment could be potentially used for enhancing carboxylates production from solid-state fermentation of CS biomass; however, the choice of an appropriate inoculum and effective hydrolytic enzymes should be taken into account and optimized.

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Compliance with Ethical Standards

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FIGURE CAPTIONS

Fig.1 Pretreatment of corn stover using extrusion.

Fig. 2 Granulometry of raw and extruded samples. a) example of images of particles acquired at t0 and after 1h under stirring in water; b) average granulometric curves at different residence time in the reactor assessed using grey level granulometry .

Fig. 3 Substrate conversion (gCOD/kgVS$_i$) after 9 days of SS-fermentation of raw (CS) and extruded (Ext-CS) corn stover in the different conditions tested: self-fermented, inoculated and enzymatic bio-augmented. Only metabolic products of fermentation are presented, unfermented sugars are not taken into account. Average data and standard deviations of replicates are reported.

Fig. 4 Time course profiling of cumulative hydrogen (H_2) production during 9 days of SS-fermentation of ground (CS, plain lines) and extruded (Ext CS, dashed lines) corn stover in the differet conditions tested: self-fermented (diamonds), inoculated (triangles) and enzymatic bio-augmented (circles).

Table1

Table 1: Characteristics of raw and extruded corn stover (CS).

	Raw CS	Extruded CS
Amount of water extractible sugars ($mg.g^{-1}$ TS)	22.0 ±1.7	25.5 ± 0.3
Swelling ($ml.g^{-1}$TS)	17.5 ± 0.5	9.8 ± 0.2
Water retention capacity (g $H_2O.g^{-1}$TS)	15.5 ± 0.1	6.8 ± 0.0
Cellulose crystallinity (%)	48.0 ± 3.7	51.2± 3.4
Total Solid (TS%)	95.4 ± 0.1	91.2 ± 0.3
Volatile Solid (VS%)	91.2 ± 0.3	87.4 ± 0.5

Values are means ± SD

Table3

Table 3. Fermentative microbial communities developped after 9 days of SSF of raw (CS) and extruded (Ext CS) corn stover in the differet conditions tested, as well as the ones from inoculum (AS) and substrates: self-fermented, inoculated and enzymatic bio-augmented

CLASS	**AS**	**Substrate**		**Self-fermented**		**Inoculated**		**Enzymatic augmented**	
Family, Genus		**CS**	**Ext-CS**	**CS**	**Ext-CS**	**CS**	**Ext-CS**	**CS**	**Ext-CS**
BACILLI	1.3%	8.7%	**93.2%**	**62.6%**	**35.2%[a]**	**13.4%**	**10.2%**	**13.8%**	**19.0%**
Bacillaceae, Bacillus	0.2%	4.1%	**71.4%**	0.1%	8.9%	0.0%	2.8%	0.0%	3.8%
Enterococcaceae, Enterococcus	0.3%	1.4%	0.3%	**57.2%**	**18.9%[a]**	**10.8%**	4.8%	9.4%	**10.4%**
Bacillaceae, Lysinibacillus	0.1%	**0.0%**	**18.5%[0]**	0.0%	6.1%	0.0%	2.1%	0.0%	2.2%
Lactobacillaceae, Lactobacillus	0.3%	0.0%	0.0%	5.1%	0.4%	1.0%	0.0%	1.0%	1.6%
other Bacilli	0.4%	3.2%	3.1%	0.1%	0.9%	1.6%	0.4%	3.3%	1.1%
GAMMAPROTEOBACTERIA	**12.5%**	**17.1%**	6.7%	**35.9%**	0.4%	**10.7%**	3.4%	**20.3%**	1.6%
Enterobacteriaceae, Cronobacter	0.0%	4.0%	5.2%	**24.1%**	0.0%	0.0%	0.0%	**0.1%**	0.0%
Enterobacteriaceae, Enterobacter	0.3%	3.5%	1.5%	**11.5%**	0.4%	6.4%	1.7%	**14.1%**	1.4%
Enterobacteriaceae, Escherichia-Shigella	0.1%	0.0%	0.0%	0.0%	0.0%	3.8%	1.5%	5.6%	0.1%
others Gammaproteobacteria	**12.2%**	9.5%	0.0%	0.2%	0.0%	0.5%	0.2%	0.5%	0.1%
CLOSTRIDIA	3.2%	0.4%	0.1%	0.2%	**39.1%**	**49.9%**	**55.1%**	**37.9%**	**33.8%**
Ruminococcaceae, Caproiciproducens	0.8%	0.0%	0.0%	0.1%	**22.0%**	**20.7%**	**51.3%**	**31.2%**	**18.5%**
Ruminococcaceae, Ruminiclostridium	0.0%	0.0%	0.0%	0.0%	0.0%	**25.7%**	0.0%	0.0%	0.0%
Clostridiaceae, Clostridium	0.3%	0.0%	0.0%	0.1%	0.0%	0.0%	0.0%	0.0%	**10.9%**
Lachnospiraceae	0.0%	0.0%	0.0%	0.0%	7.4%	0.2%	0.1%	0.0%	0.0%
other Clostridia	2.1%	0.4%	0.1%	0.0%	**9.8%**	3.2%	3.7%	6.7%	4.5%
BACTEROIDIA	1.7%	0.0%	0.0%	0.1%	0.0%	**20.0%**	**25.4%**	**19.4%**	**22.1%**
Prevotellaceae, Prevotella	1.2%	0.0%	0.0%	0.1%	0.0%	8.0%	**19.6%**	**17.8%**	**22.1%**
other Bacteroidia	0.6%	0.0%	0.0%	0.0%	0.0%	**12.0%**	5.8%	1.6%	0.0%
CORIOBACTERIA	0.9%	0.0%	0.0%	0.1%	**17.0%**	0.5%	0.4%	0.7%	**15.3%**
Coriobacteriaceae, Collinsella	0.7%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.2%	**12.9%**
Coriobacteriaceae, Atopobium	0.1%	0.0%	0.0%	0.0%	**16.3%**	0.4%	0.1%	0.2%	0.4%
others Coriobacteriia	0.1%	0.0%	0.0%	0.0%	0.6%	0.1%	0.3%	0.3%	2.0%
ALPHAPROTEOBACTERIA	**20.5%**	**38.7%**	0.0%	0.9%	0.6%	1.3%	0.9%	1.2%	0.5%
ACTINOBACTERIA	13.4%	13.8%	0.0%	0.1%	7.6%	1.6%	1.7%	1.7%	5.7%
BETAPROTEOBACTERIA	12.8%	6.6%	0.0%	0.1%	0.0%	0.9%	0.4%	0.5%	0.2%
ACIDIMICROBIIA	11.1%	0.4%	0.0%	0.0%	0.0%	0.7%	0.9%	1.0%	0.6%
SPHINGOBACTERIIA	6.2%	6.3%	0.0%	0.1%	0.0%	1.1%	0.4%	0.1%	0.1%
other bacteria classes	16.4%	8.0%	0.0%	0.0%	0.0%	0.0%	1.3%	3.4%	1.0%
N° copies 16S/g VS	1.74E+10	5.68E+08	4.52E+10	2.14E+09	1.12E+10	7.69E+10	8.27E+10	4.51E+10	6.45E+10
Shannon Diversity (H)	5.42	4.34	1.36	1.22	2.34	2.96	2.51	3.23	3.02
Simpson Diversity (1-D)	0.99	0.98	0.58	0.60	0.88	0.89	0.79	0.89	0.92

Bacterial classes are represented in bold characters, families are in normal characters and genera are in italic. Only the species with relative abundance higher than 5% in at least one sample are presented. **Bold text** is used for the species with abundance higher than 10% and **bold emphasized text** for the species with abundance higher than 50%.

Table 2

Table 2. COD composition and pH after 9 days of SSF of raw and extruded corn stover.

CONDITION	**self-fermented**		**inoculated**		**enzymes addition**	
inoculum	no		yes		yes	
enzymes	no		no		yes	
Substrate	**CS**	**Ext CS**	**CS**	**Ext CS**	**CS**	**Ext CS**
Soluble Sugars	2.9 ± 0.1	1.8 ± 0.2	0	0	0	0
Lactate	10.3 ± 0.1	2.7 ± 0.1	0	0	0	0
Acetate	7.0 ± 0.9	14.4 ± 0.6	16.9 ± 0.8	28.2 ± 0.7	35.6 ± 0.0	47.2 ± 0.7
Propionate	2.0 ± 0.2	5.0 ± 0.2	8.2 ± 0.5	8.7 ± 0.2	10.2 ± 0.0	10.6 ± 0.1
Ethanol	0	10.4 ± 0.4	1.2 ± 1.7	2.6 ± 0.1	8.6 ± 0.2	31.6 ± 0.3
Butyrate	0	16.6 ± 0.4	6.9 ± 0.4	11.5 ± 0.6	27.4 ± 0.6	43.7 ± 0.2
Valerate	0	0	2.4 ± 0.2	3.3 ± 0.2	3.4 ± 0.2	3.3 ± 0.2
Caproate	0	0	0	2.7 ± 0.1	4.1 ± 0.4	4.9 ± 0.6
Other Metabolites*	1.2 ± 0.1	0	2.0 ± 0.7	2.8 ± 0.1	1.5 ± 0.8	0
Total SCOD	23.4 ± 3.7	50.9 ± 0.5	37.6 ± 0.5	59.8 ± 2.0	90.8 ± 1.9	141.9 ± 0.9
pH_f	5.4 ± 0.1	4.9 ± 0.0	5.4 ± 0.0	4.8 ± 0.0	5.0 ± 0.0	4.5 ± 0.2

Soluble COD (SCOD) composition (gCOD/kgVS_i) and pH after 9 days of SSF of raw (CS) and extruded (Ext CS) corn stover in the differet conditions tested: self-fermented, inoculated and enzymatic addition. Values are means ± Standard deviation. *sum of succinate + iso-butyrate + iso-valerate

Figure1

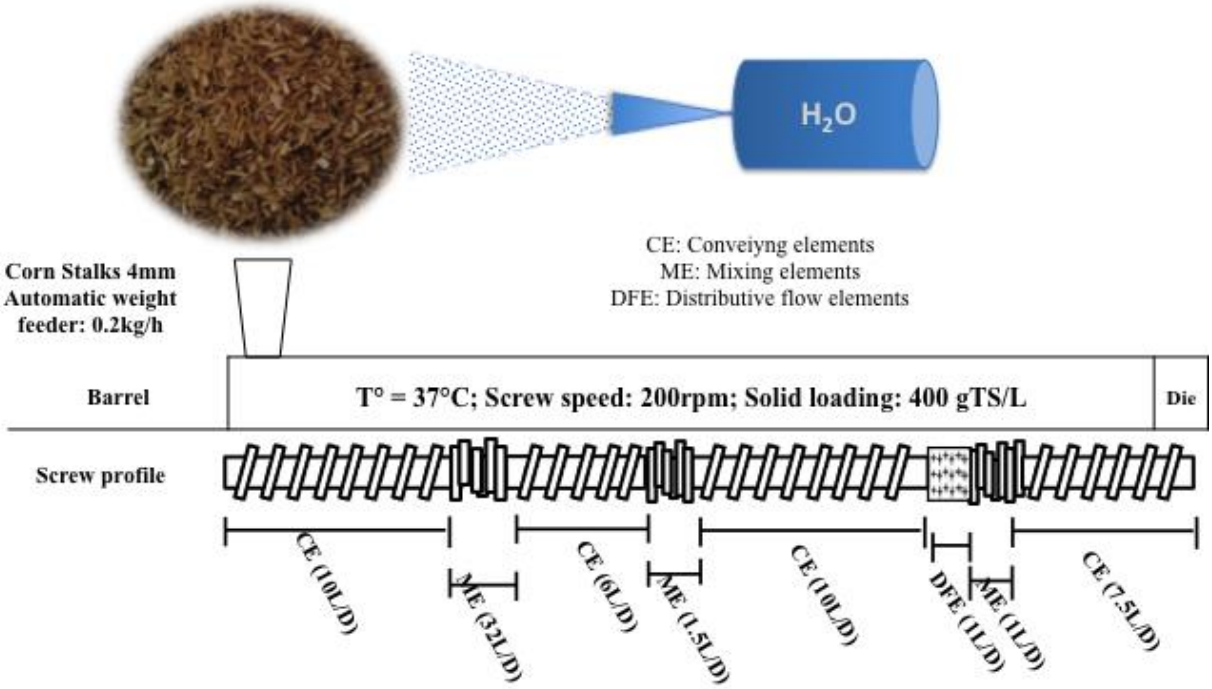

H2O
CE: Conveiyng elements
ME: Mixing elements
DFE: Distributive flow elements
Corn Stalks 4mm
Automatic weight
feeder: 0.2kg/h
Barrel
T° = 37°C; Screw speed: 200rpm; Solid loading: 400 gTS/L
Die
Screw profile
CE (10L/D)
ME (32L/D)
CE (6L/D)
ME (1.5L/D)
CE (10L/D)
DFE (1L/D)
ME (1L/D)
CE (7.5L/D)

Figure2

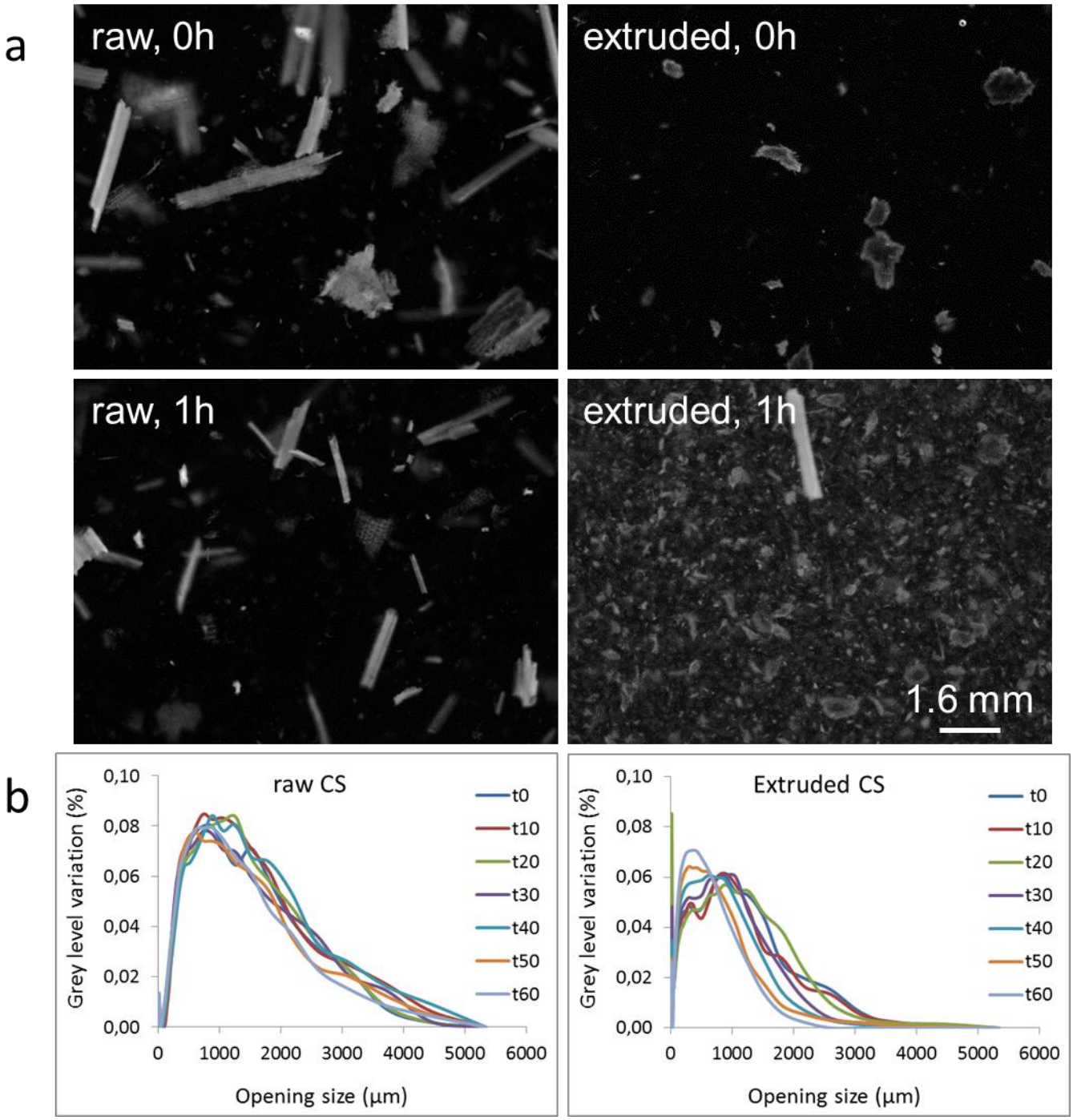

a
raw, 0h
extruded, 0h
raw, 1h
extruded, 1h
1.6 mm
b
raw CS
Extruded CS
Grey level variation (%)
Opening size (µm)
0,10
0,08
0,06
0,04
0,02
0,00
0
1000
2000
3000
4000
5000
6000
t0
t10
t20
t30
t40
t50
t60

Figure3

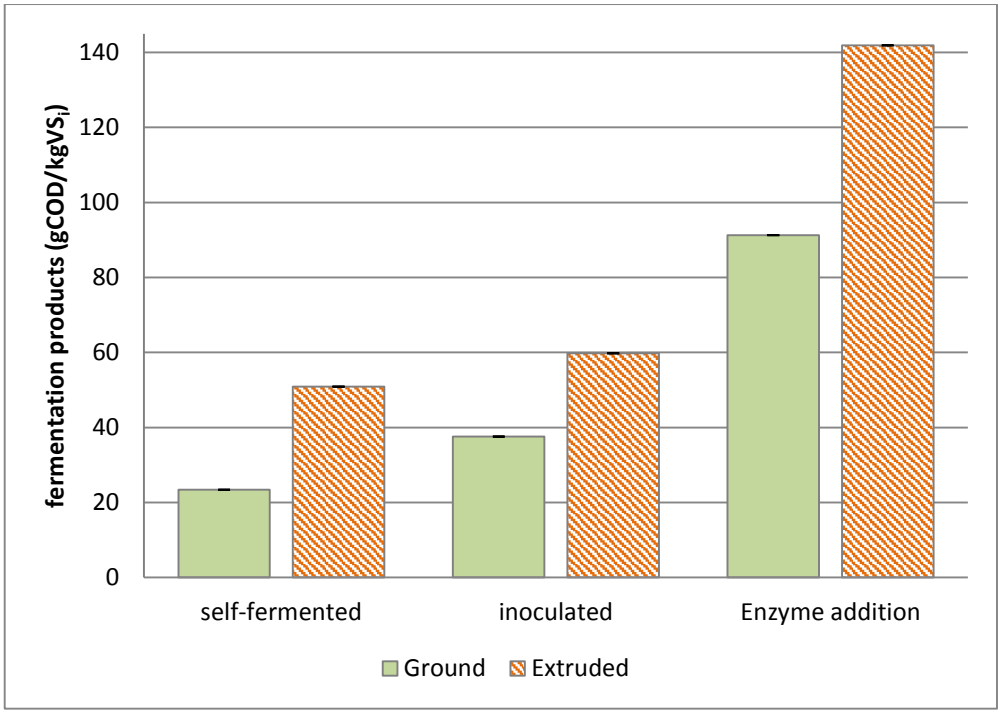
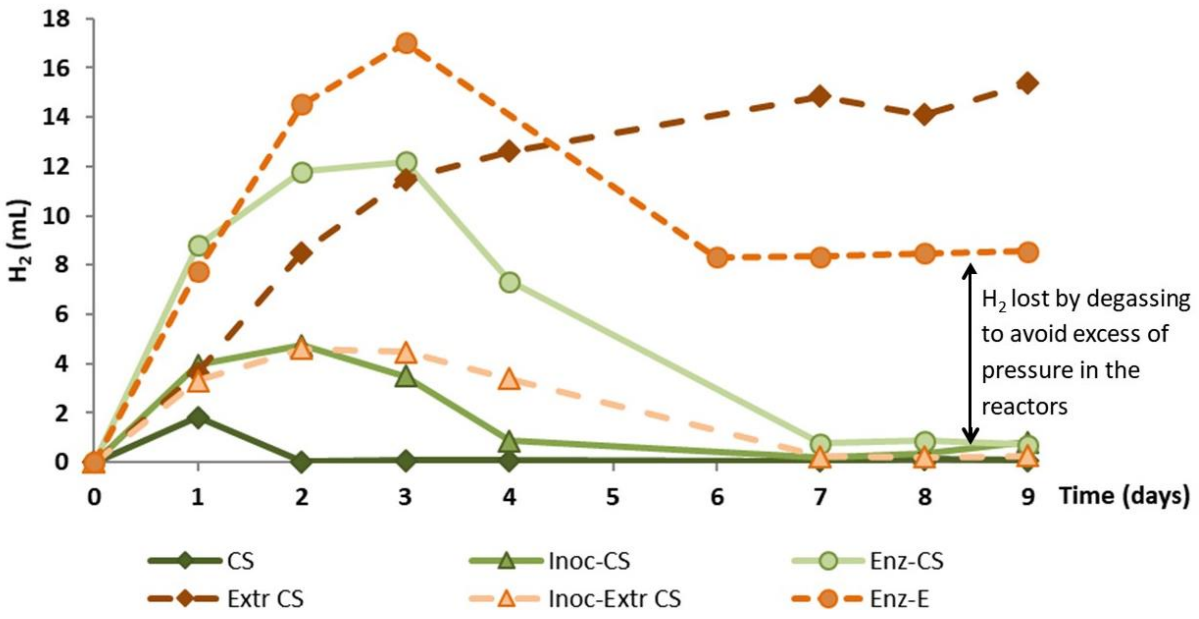

fermentation products (gCOD/kgVS$_i$)
140
120
100
80
60
40
20
0
self-fermented
inoculated
Enzyme addition
Ground
Extruded

Figure 4


18
16
14
12
10
8
6
4
2
0
H_2 (mL)
0
1
2
3
4
5
6
7
8
9
Time (days)
H_2 lost by degassing to avoid excess of pressure in the reactors
CS
Inoc-CS
Enz-CS
Extr CS
Inoc-Extr CS
Enz-E