Conditions for efficient alkaline storage of cover crops for biomethane production

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Abstract

An innovative process aiming to combine storage and alkali pretreatment of cover crops was investigated using lime as a low cost and environmental friendly reactant. Different lime loadings and Total Solid concentrations (TS) allowed to highlight the abiotic mechanisms of deacetylation during the early stages of the process. Long-term storage experiments of rye and sunflower cover crops at 100 g.kgTS^{-1} lime loading allowed to evaluate the fermentation kinetics and to compare performances in dry and wet conditions to classical silage storage. The dry condition allowed an efficient alkaline storage and up to a 15.7% Biochemical Methane Potential (BMP) increase, while the wet condition underwent a succession of fermentations with a high butyric acid accumulation and H_2 production, leading to a 13% BMP loss. Silage experiments allowed an efficient preservation of the BMP, with no significant variation over the 6-month storage duration.

Keywords

Intermediate crops; Anaerobic digestion; Silage fermentation; Pretreatment of lignocellulose; Biogas

1 Introduction

In order to face the increasing demand in renewable energies, extensive research and investments are currently made to develop new modes of production of clean and sustainable energies. Resulting from the degradation of a large panel of sewage, effluents, biowastes and agricultural residues, production of methane by anaerobic digestion (AD) processes provides a sustainable alternative to natural gas allowing significant reductions in greenhouse gases emissions when substituted to natural gas or other fossil fuels.

AD of waste-like substrates allows to produce a renewable fuel while valorizing a large variety of by-products. However, a larger amount of biomass is required to substitute the present natural gas consumption in France and worldwide. For this reason, energy crops were grown in various countries to provide a larger amount of biomass for anaerobic digestion. As energy crops have a high biomass yield per hectare and a high CH_4 yield, they allow to secure the intake supply of biogas plants. In Germany, about 78% of total energy production by AD referred to energy crops in 2016 (Daniel-Gromke et al., 2018), *i.e.* 25 TWh. However, despite these advantages, energy crops compete for arable land with food crops and the benefits of their use as a sustainable intake for AD are questioned. As an alternative to energy crops, cover crops (CC), also called intermediate crops, allow to produce biomass for AD without increasing the demand in arable land. CC are grown during the intercultural period of food or feed crop rotations. In addition, their use brings agro-ecological advantages, allowing to avoid erosion and nutrient leaching (Igos et al., 2016), facilitate the control of self-propagating plants (Büchi et al., 2020) and accumulate organic matter in the soil (Jian et al., 2020). However, the cost-effectiveness of the cultivation of CC has to be improved to ensure the economical relevance of their use for AD. If it mostly depends on the harvest yield that has to compensate the cultivation costs, the potential of the crop still has to be efficiently stored and preserved until its use in the anaerobic digester. The storage of wet harvested crops (in opposition to haymaking) is generally performed by ensiling. This process relies on the spontaneous lactic fermentation and acidification of the biomass under anaerobic conditions by the action of endogenous bacteria that ferment the accessible sugars contained in the plant into lactic acid and other compounds such as acetic acid and ethanol (Elferink et al., 1999). Once a low pH corresponding to acidic conditions is reached, the microbial activity can be inhibited over several months until the silo opening. Ensiling has been shown to be an efficient conservation method of crops for AD, but its success depends on several parameters, and significant degradations can occur in non-optimal conditions (Teixeira Franco et al., 2016).

Because of their fibrous composition, the degradability of CC into methane can however be low. The accessibility of the microorganisms involved in the anaerobic digestion to the fermentescible carbohydrates of hemicellulose and cellulose is limited by the complex structure of the lignocellulosic matrix, which lowers both yield and kinetic of biogas production from crops (Monlau et al., 2013). A large variety of pretreatment technologies has been reported in the literature in order to increase the degradability of lignocellulosic biomasses into methane. Due to their high efficiency to degrade lignin, the alkaline pretreatment constitutes a promising option for an application on lignocellulosic biomasses (Carrere et al., 2016). Significant improvements of the degradability of lignocellulosic biomasses into methane were obtained, even in mild conditions with low energy and reactive requirements (Khor et al., 2015; Thomas et al., 2018). In mild conditions, the extension of the pretreatment duration was shown to have a beneficial impact, allowing to compensate the slower kinetic of the reaction (Thomas et al., 2018). Considering the alkaline reagents, NaOH, KOH and CaO are among the most studied for alkaline pretreatment. However, NaOH and KOH are expensive and NaOH can additionally cause an increase in the digestate salinity, which may be harmful to the soil in the case of the use of the digestate as a fertilizer (Shahid et al., 2018). In contrast, CaO is already used in agriculture to control soil acidity with soil liming and presents the advantage to be less expensive. CaO is also available in high quantity as it is produced from the calcination of limestone, an abundant component of the earth crust. For these reasons, CaO constitutes an interesting option as a reagent for alkaline pretreatment, despite its lower efficiency when compared to NaOH and KOH (Jiang et al., 2017).

Since CC are commonly stored by ensiling for anaerobic digestion, applying an alkaline pretreatment after storage would no represent an optimized solution. Indeed, a larger amount of reagent would be necessary to elevate the pH of silage, which is rich in organic acids and buffered around pH 4. In order to bypass this problem, the alkaline pretreatment of crops could therefore be applied directly after harvesting. By extending the pretreatment duration until several months, a combined storage and pretreatment process could thus be developed, allowing to take advantage of the long term action of the alkaline pretreatment. Few examples of alkaline storage of crops are reported in the literature. Pakarinen et al. (2011) experimented the use of urea (30 and 60 $g.kg_{TS}^{-1}$) as an alkaline reagent on hemp for ethanol and biogas production over a 4 and 8 months storage period. Despite a final pH of 8.7 which is a low value in the context of alkaline pretreatment, significant increase of the enzymatic hydrolysability (+46%) and BMP (+21%) were found. However, the same increase was measured in the silage experiment with no additive and no mass balance was calculated during this alkaline storage experiment, which makes difficult to interpret such energy potential variations. Digman et al. (2010) studied an alkaline storage process for ethanol production using lime in anaerobic conditions on switchgrass and reed canarygrass. The CaO loading varied from 14.6 to 100 $g.kg_{TS}^{-1}$, with storage durations from 30 to 180 days. The authors found that all storage conditions allowed an efficient preservation of the biomass, even if no global energy balance including mass losses was evaluated. The best pretreatment efficiency was found with the highest lime loadings of 85 and 100 $g.kg_{TS}^{-1}$. The results also showed that only these high lime concentrations allowed to maintain a high pH of 9.1 ± 0.9 and 10.1 ± 0.4, respectively, until the end of the storage. Final pH was highly correlated with the CaO loading (r = 0.97) and with the efficiency of the pretreatment (r = 0.82). Interestingly, no correlation was found between the pretreatment duration and its efficiency. In addition, both chemical and microbial mechanisms involved during storage were not clarified. In a recent work, Van Vlierberghe et al. (2021) showed a fast pH decrease from 12 to 7 after a few days, using a relatively low CaO loadings (60 $g.kg_{TS}^{-1}$). The pH destabilization was attributed to two possible phenomena both abiotic (chemical deacetylation of hemicellulose) and biotic (microbial fermentation) phenomena. The deacetylation reaction consists in the solubilization and removal of the acetyl groups that are covalently bonded to the xylan backbone of hemicellulose (Chen et al., 2012), releasing acetic acid in the medium. In lignocellulosic materials like corn stover, acetyl groups esterified to the hemicellulose structure represent in average 2.2% of TS and were shown to increase the biomass recalcitrance (Humbird et al., 2011). Dilute alkaline pretreatments were shown to be efficient at solubilizing the acetyl groups of hemicellulose in mild conditions (Chen et al., 2014), making probable the occurrence of this phenomenon in alkaline storage. Considering the microbial fermentation, lactic, acetic and butyric fermentations were involved (Van Vlierberghe et al., 2021). However, the understanding of the contribution of both mechanisms was limited. In addition, even if the methane potential of the crops was efficiently preserved, the expected positive effect of the pretreatment was not achieved, which was attributed to the fact that the lignocellulosic substrate was not exposed to an alkaline pH for a sufficient period of time. Maintaining a high level of pH during the whole storage duration was consequently identified to be necessary for both inhibitions of undesirable microbial activity and increase of the pretreatment action on lignocellulose.

The present work aims to give an insight of the very short and long-term mechanisms involved in alkaline storage of crops with lime. In the very first stage of storage, the chemical deacetylation of hemicellulose and its contribution to the pH change were studied during the first hours of alkaline storage. In addition, the effect of the storage conditions on the physico-chemical characteristics of the crops were identified during the whole storage duration of six months, along with the evolution of the microbial communities present on the substrate which had never been studied to the authors' knowledge. A direct comparison of the energy potential balance between different alkaline storage conditions and the conventional silage process is discussed, in order to conclude on the relevance of alkaline storage process.

2 MATERIAL AND METHODS

2.1 Feedstock

Three cover crops were used as a substrate. For the first experiment which evaluated chemical deacetylation, a sample of rye (*Secale cereale, Rye 1*) harvested at BBCH59 (end of heading stage) and cultivated as a winter cover crop on an agricultural site in France (Biométhagri, Florensac, 34101, Hérault, France) was used. The rye was shredded using a garden shredder (AXT 2550TC, Bosch GmbH) directly after harvesting and stored at -20°C until the experiment.

Two other cover crops harvested on another agricultural site (Biométharn, Aiguefonde 81200, Tarn, France) were used to evaluate the long-term alkaline storage during the second set of experiments. One sample of rye (*Secale cereale, Rye 2*) cultivated as a winter cover crop, harvested at BBCH 73 (early milk stage) and one sample of sunflower (*Helianthus annuus*) cultivated as a summer cover crop and harvested at maturity stage BBCH 88 (end of the ripening stage). The two crops were stored overnight as a whole plant at 4°C in sealed plastic bags, avoiding moisture change before use, and used directly after for storage experiment.

2.2 Experimental set-up

Before all experiments, the crops were chopped using a garden shredder (AXT 2550TC, Bosch GmbH) until homogeneous particle size of approx. 1-2 cm was reached and further passed through a knife shredder (BB230, BLiK®) for better longitudinal cut of the crop pieces. The different experiments described below were made using the following setup. 2.6L glass flasks sealed with air-tight lids equipped with a rubber septum that allows pressure measurement and gas sampling were used. Replicates were prepared for each storage condition to be sacrificed after the determined durations to monitor the impact of different storage conditions on biomass conservation and on fermentation kinetics. 700g of sample were packed into the flasks which were then flushed with N_2, sealed and stored in a dark room.

2.3 Short-term experiment

The deacetylation experiment was divided into two stages. The first one aimed to evaluate the acetic acid production kinetic. The TS of the crop was adjusted to 25% by oven drying at 40°C, and a lime loading of 100g.kg_{VS}^{-1} was applied. Several replicates were prepared to be sacrificed after 2, 4, 8, 24 and 168 hours of storage (3 replicates for each storage time). The flasks were stored at 4°C in order to inhibit the microbial activity. This method was preferred to others, such as thermal treatment, in order to minimize the side effects on the substrate characteristics. The second stage focused on the influence of lime loading and moisture content on the acetic acid release during the first hours of the process. Four conditions were tested following a 2x2 factorial design. The different conditions are summarized in Table 1. TS values of 10 and 40% were chosen, as these extreme values can be found for cover crops at harvest time. The TS value of the crop was adjusted by adding distilled water or by oven drying at 40°C. CaO loadings of 25 and 200 g.kg_{TS}^{-1} were applied. The lower level was chosen as one of the lowest amounts found in the literature. The higher level was set as a large excess of CaO whose value is usually less than or equal to 100 g.kg_{TS}^{-1} (Digman et al., 2010; Khor et al., 2015).

Table 1: Pretreatment conditions applied for the second phase of deacetylation experiment and effect on pH

TS (%FM)	Loading ($g.kg_{VS}^{-1}$)	Initial pH	Final pH
10	25	10.7 ± 0.0	9.9 ± 0.1
10	200	13.2 ± 0.0	13.2 ± 0.1
40	25	9.8 ± 0.0	8.4 ± 0.1
40	200	13.1 ± 0.0	13.1 ± 0.1

2.4 Long-term experiment

A lime loading of 100 $g.kg_{TS}^{-1}$ was selected according to the results of Digman *et al.* (2010) and the deacetylation experiment presented above. Alkaline storage was applied on two different cover crops (i.e., sunflower and rye). For both crops, a “dry” alkaline storage was achieved by applying a dry powder of CaO directly, reaching a TS content of the mixture of 41 and 47% TS for rye and sunflower, respectively. For rye samples, an additional “wet” condition was experimented, as Digman *et al.* (2010) showed that a higher moisture content increased the pretreatment action. Deionized water was gradually sprayed on the mixture during homogenization in order to increase the lime diffusion into the biomass and adjust TS, until a total TS of 29% was reached. Water addition was limited to this extent because higher moisture are reported to cause undesirable effluent production in bunker silo during storage (Teixeira Franco et al., 2016). Ensiling assays were conducted as controls, since silage is still the most common method for crop storage prior to anaerobic digestion. The same storage protocol was used for silage experiments, except that no chemical additives were added. For each storage condition, five replicates were prepared to be sacrificed and analyzed after 2, 7, 21, 60 and 180 days of storage. The flasks were stored in a dark place where temperature was controlled at 22°C.

2.5 Silo monitoring and sampling

After the flasks were closed, gas production and weight losses were regularly monitored until flask opening. Volumetric gas production was quantified by pressure difference. The pressure was measured using a manometer (Keller LEO® 2) and gas was released when pressure exceeded 1.2 bar, after what the flasks were immediately weighted. The volume of gas inside the flask (headspace + pore space) was calculated by subtracting the volume of added substrate to the total volume of the flask. The volume of substrate was calculated using its theoretical density calculated as follows, adapted from McNulty *et al.* (McNulty and Kennedy, 1982):

$$\rho_t = (1 - C)\,[(M/\rho_l) + (1 - M)/\,\rho_s] + C/\rho_{CaO} \quad (1)$$

where M = fractional moisture content related to fresh matter (FM); C = fractional CaO content related to fresh matter; ρ_l = liquid or water density = 1000 kg/m^3; ρ_s = dry matter density = 1421 kg/m^3 and ρ_{CaO} = CaO density = 3345 kg/m^3. The composition of the gas was analyzed using a gas chromatography (Perkin Elmer Clarus 580) with a Rt-U-bond column (30m x 0.32mm x 10µm) for CO_2 separation, and a Rt-Molsieve 5Å column (30m x 0.32mm x 10µm) for H_2, O_2, N_2 and CH_4 separation. Argon was used as vector gas (350 kPa, 34 mL/min). Injector and detector temperature was set on 200°C, and oven temperature on 65°C. Detection was made by thermic conductivity. For each planned storage duration, one flask was opened after final gas and weight measurement. The whole sample was then crushed using a knife mill (Pulverisette 11, Fritsch). Total solids (TS) and volatile solids (VS) were measured immediately. Water extraction was performed for pH, water soluble carbohydrates (WSC), volatile fatty acids (VFA) and other metabolites measurements. 30 g of fresh sample were soaked in 150 mL of deionized water for 16 h to 20 h at 4°C in sealed plastic pots in triplicate, similarly as proposed by Porter & Murray (Porter and Murray, 2001). pH measurement was made directly after extraction on the mixture. Then, the liquid phase was separated by centrifugation (18750 g, 20 min, 4°C) and frozen in air-tightly closed tubes for further WSC, VFA and metabolites analysis. A fraction of the samples was separated and stored at -20°C for further BMP measurement. Finally, aprox. 100 g of fresh sample were freeze-dried and milled using a 1mm grid for fiber distribution analysis.

2.6 Physicochemical analyses

TS were measured in triplicate by drying 30 g of sample at 105°C for 24 h. Volatile Solids (VS) were then measured by calcination of the dry residue (550°C, 3 h). Silage and other substrates obtained from crops transformation may content volatile fatty acids (VFA), lactic acid (LA) and diverse alcohols that evaporate during oven drying causing underestimation of TS. For this reason, the TS content value measured by oven drying was corrected using the equation proposed by Porter and Murray (2001):

$$TS_C = TS_M + 0.375\,LA + 0.892\,VFA + 0.975\,Alcohols \quad (2)$$

where TS_C = corrected TS; TS_M = measured TS; LA = lactic acid concentration; VFA = total VFA concentration; Alcohols = total alcohols concentration. All concentrations are in g/gFM. TS_C was further used for VS calculation.

pH was measured in triplicate using WTW® SenTix® 41 probe on a WTW® inoLab® pH7110. WSC and metabolites concentration was measured from the centrifuged liquid phase after filtering (0.2 µm nylon filter) by High Performance Liquid Chromatography on Aminex 4PX-87H column (Bio-Rad) at 45°C. Sulfuric acid (0.005 M; 0.3 mL/min) was used as mobile phase. WSC content was calculated as the sum of glucose, xylose, arabinose, fructose. Fiber distribution was analyzed in triplicate on previously freeze dried and milled samples using Van Soest method (Van Soest and Wine, 1967). Water extract (*W.EX*), neutral detergent fiber (*NDF*), acid detergent fiber (*ADF*), acid detergent lignin (*ADL*) and calcination residue (*CAL*) contents were determined. Water soluble compounds (*W.SOLU*), neutral detergent soluble compounds (*SOLU*), hemicellulose (*HEMI*), cellulose (*CELL*) and lignin content (*LIGN*) were calculated as follow : *W.SOLU* = 1 – *W.EX* ; *SOLU* = *WEX* – *NDF* ; *HEMI* = *ND*F – *ADF* ; *CELL* = *ADF* – *ADL* ; *LIGN* = *ADL* – *CAL*. Prior to the fiber extraction, a preliminary extraction of fat was performed on sunflower samples, as recommended by Van Soest *et al*. Lipids were extracted with an ASE instrument (Dionex ASE-200) with heptane:ethanol (2:1, vol:vol) for solvent, as described by Yang *et al.* (Yang et al., 2019). No fat extraction was made on rye samples, whose lipid content was expected to be negligible (Herrmann et al., 2011). The total carbon (TC) and total nitrogen (TN) were measured with an elemental analyzer (FlashSmart®, Thermo Fisher Scientific®) on finely grounded freeze dried samples. TC and TN analyses were not replicated. BMP measurements were made following the recommendations of Holliger et al. (2016) . Samples were previously prepared for BMP test by freezing a certain amount of substrate containing around 2 g_{TS} of sample (exact TS and VS were calculated later) soaked in $NaHCO_3$ buffer. The buffer was added to minimize the difference of pH between ensiled and alkali-stored samples, avoiding additional pretreatment effect of alkaline conditions before BMP test. Gas measurement was made using an automatic batch test system (AMPTS II, Bioprocess Control, Sweden). The methane potential value was expressed as the volume of methane produced per initial amount of VS estimated after taking into account the mass losses that occurred during storage. This was made to evaluate the global energy balance of the different storage processes. The generated sequencing datasets are registered in the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under the BioProject accession number PRJNA788459.

2.7 Microbial community analysis

For each sampling time of the storage experiment, a 300 mg sample was collected and stored into a 2 mL sterile Eppendorf tube and stored at -20°C until DNA extraction. DNA extraction, sequence data analysis and quantitative PCR were performed as described by Dauptain et al. (2020). Sequencing was made at the technology platform Genome and Transcriptome (GeT) of the Génopole Toulouse, France. Only OTUs with a relative abundance of 1.5% or more in at least one sample were selected for further data analysis.

2.8 Statistical data analysis

One-way analysis of variance was performed on fiber distribution and BMP after verifying normality (Shapiro-Wilk test) and variance homogeneity (Levene test) with R package “rstatix”. Pair-wise t-test adjusted with Bonferroni correction was further performed to assess the significance of the difference in mean between two samples. The package “ggplot2” was used for all graphical representations.

3 Results and discussions

3.1 Raw material characterization

The main characteristics of the different crops are presented in Table 2. The higher maturity crops (i.e., rye 2 and sunflower) were characterized by higher solid content and lower water-soluble carbohydrates (WSC). Cover cops are usually characterized by a TS value lower than 30%, with an average of 17 ± 5 (Molinuevo-Salces et al., 2013). The TS content at harvest greatly depends on the crop maturity stage, related to sowing and harvesting dates, climate and crop species (Kaiser and Piltz, 2004). WSC were mostly composed of fructose and glucose, the main primary soluble carbohydrates in temperate forages (Downing et al., 2008). These carbohydrates are essential for the lactic fermentation and acidification of silages (Elferink et al., 1999), but also represent a high amount of easily accessible carbohydrates for undesirable fermentation in alkaline storage.

The three crops had C/N ratios of 50, 54 and 23 for rye 1, rye 2 and sunflower, respectively, which corresponds to low (rye) and medium (sunflower) values when compared to other crops (Molinuevo-Salces et al., 2014).

Sunflower samples were characterized by a higher lignin content than in rye samples. Rye samples presented a higher hemicellulose content, that is a more easily accessible fiber for biogas production (Monlau et al., 2013).

Table 2 : Main physicochemical characteristics of the studied cover crops.

	Rye 1	Rye 2	Sunflower
TS (%FM)	21.8 ± 0.0	37.1 ± 0.1	42.5 ± 0.5
VS (%FM)	20.4 ± 0.0	35.9 ± 0.1	40.1 ± 0.2
TN (%VS)	0.9	0.8	1.9
TC (%VS)	44.6	43.3	44.8
pH	6.2 ± 0.0	6.0 ± 0.1	6.8 ± 0.0
VFA ($g.kg_{VS}^{-1}$)	< d.l.	< d.l	< d.l
Fructose ($g.kg_{VS}^{-1}$)	79.3 ± 11.5	33.6 ± 2.8	44.8 ± 0.2
Glucose ($g.kg_{VS}^{-1}$)	60.1 ± 6.5	20.4 ± 1.4	21.9 ± 0.4
WSC ($g.kg_{VS}^{-1}$)	147.0 ± 13.3	54.0 ± 3.1	66.9 ± 0.7
EtOH ($g.kg_{VS}^{-1}$)	< d.l.	3.1 ± 0.3	< d.l
Lipids ($g.kg_{VS}^{-1}$)	n.d.	n.d.	5.6

n.d. : not determined; <d.l. : below the detection limit

3.2 Short-term experiment

The first short-term experiment allowed to investigate the kinetics of acetic acid production that occurs during the early stages of the alkaline storage (Figure 1). Despite the reaction temperature (4°C) which strongly limits the microbial fermentation activity, a large amount of acetic acid (AA) was produced immediately after the lime addition. After only 2 h, 80% of the final AA concentration had already been released. The maximum value of 24 $g.kg_{VS}^{-1}$ was reached after 4 h and remained stable for one week. In the literature, similar amounts of acetyl groups were released for lignocellulosic biomasses. Humbird et al (2011) reported an average value of 22 $g.kg_{VS}^{-1}$ in corn stover, while Castro et al. (2017) and Chen et al. (2014) measured acetyl content of 26 and 27 $g.kg_{VS}^{-1}$ in rice straw and corn stover, respectively, which suggests that a high deacetylation yield was obtained in these experiments. In addition, the AA production was not associated to WSC consumption nor fermentation gas production or other accumulation of metabolites. The pH was maintained at a value of 12.8 during this period. As a consequence, the production of AA during the first hours of alkaline treatment (lime loading of 100 g.kg_{VS}^{-1}) is related to a chemical deacetylation mechanism.

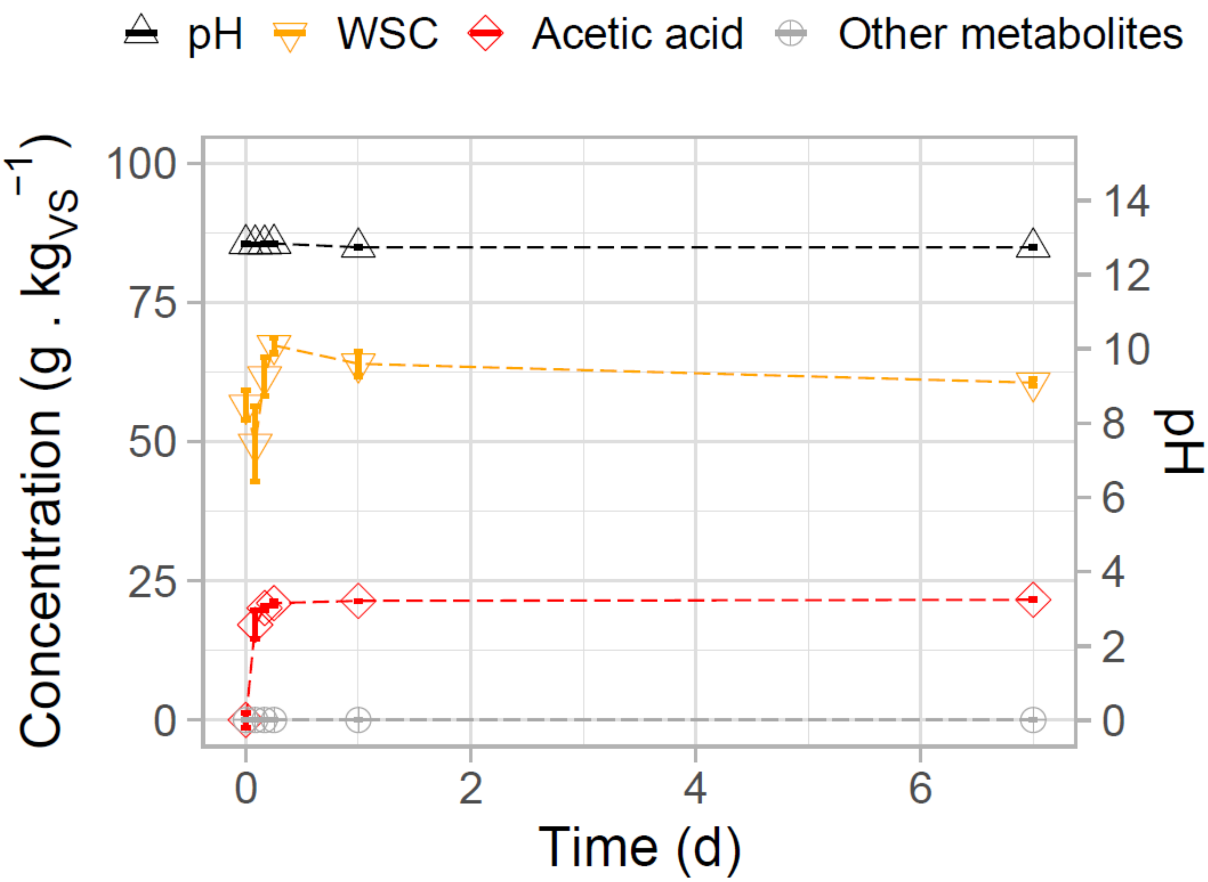

Figure 1: Variation of acetic acid, WSC and pH during the first days of alkaline storage. Dots represent averaged values of the sacrificed triplicates. Error bars indicate standard deviation within triplicates.

The effect of lime loading and TS content on the final AA accumulation and pH was investigated in a second set of experiments. The reaction duration was set at 48 h. Since deacetylation kinetics have been shown to be rapid, a longer experimental time is not necessary to investigate this phenomenon. The main effects of CaO and TS are shown in Figure 2. Results from the first experiment (lime loading of 100 g.kg_{VS}^{-1}) after 24h of reaction were also added for comparison. The CaO loading was shown to have the highest effect since the AA release doubled between lime loadings of 25 and 200 gCaO.kg_{TS}^{-1}. Interestingly, few additional AA was released for the highest load of 200 gCaO.kg_{TS}^{-1} in comparison to the previous experiment (100 gCaO.kg_{TS}^{-1}). This may be explained by the fact that high deacetylation yield was already obtained at a lime loading of 100 $gCaO.kg_{TS}^{-1}$. Consequently, it is not expected that an increase of lime loading over 100 $gCaO.kg_{TS}^{-1}$ induces an additional effect on pretreatment efficiency, since previous studies showed that when enough lime has been added to remove acetyl groups, further lime addition is not beneficial to pretreatment action on lignocellulosic biomass (Chang et al., 1998).

The moisture content was also shown to have an effect on deacetylation yield, which is in agreement with the results of Digman *et al.* (2010) who observed a higher pretreatment effect in wet conditions. The higher deacetylation efficiency in wet conditions may be explained by the better solubilization and diffusion on CaO in the medium.

Lime loading had an important effect on initial and final pH (Table 1). At lime loading of 25 $gCaO.kg_{TS}^{-1}$, the initial pH was close to 10, while higher values of 13 were found with lime loadings of 100 and 200 $gCaO.kg_{TS}^{-1}$. Most importantly, the lowest lime loading was not sufficient to ensure a high pH stability, since pH drops of of 0.8 and 1.4 were observed for TS content of 10 and 40%, respectively. In such storage environments the microbial activity would not have been totally inhibited, and undesirable fermentations could start (Van Vlierberghe et al., 2021). The final pH for two highest lime loading of 100 and 200 $gCaO.kg_{TS}^{-1}$ were similar and stable at a value of 13, and thus no beneficial effect on pH was found when increasing the lime loading over 100 $gCaO.kg_{TS}^{-1}$.

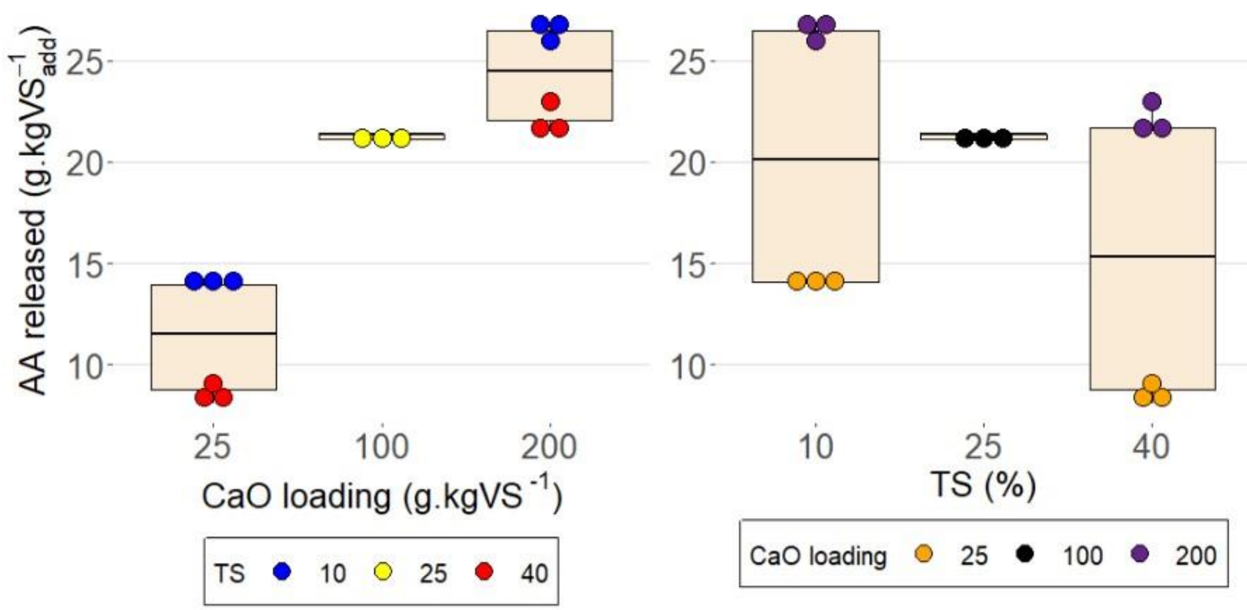

Figure 2: Effect of CaO loading and TS on acetic release after 2 days of reaction. Each dot represents a value from a single replicate.

The short-term experiment confirmed the hypothesis of the chemical deacetylation of hemicellulose to be responsible for the AA production at the beginning of alkaline storage. However, if the acetic acid release seems to have an effect on pH at a low lime loading, no effect on pH related to this phenomenon was observed for a CaO loading higher than 100 $g.kg_{VS}^{-1}$ even after a week of storage at 4°C, suggesting that this lime loading was sufficient to maintain a high pH in absence of microbial fermentation and to reach a high deacetylation rate.

3.3 Long-term experiment

During the whole storage experiment, replicates were periodically sacrificed and analyzed in order to monitor the evolution of biomass transformation over the storage period. pH, metabolites accumulation and gas production (Figure 3) together with identification of the microbial populations (Figure 4) allowed to understand the mechanisms involved during alkaline storages of 180 days. Depending on the cover crops and storage conditions (alkali-stored and ensiled samples), three main steps can be identified and are described below. In addition, the analysis of the microbial populations indicated that different microorganisms were involved in each step.

3.3.1 Dynamical changes during storage

3.3.1.1 First phase (0 to 21 days)

In all alkaline storage experiments, high pH of 12.6 ± 0.1 were reached just after the addition of lime to the shredded crop. These values are comparable to those observed in the previous section **Erreur ! Source du renvoi introuvable.** (Figure 1 and Table 1). By applying a CaO load of 100 $g{\cdot}kg_{VS}^{-1}$, pH was therefore maintained in an alkaline range, since pH did not vary significantly during this phase. It is noteworthy that, at a lower CaO load of 60 $g{\cdot}kg_{VS}^{-1}$, Van Vlierberghe *et al.* (2021) reported a significant pH drop during the same period from 12 to 7 in less than one week. Acetic acid concentrations of 26 and 30 $g.kg_{VS}^{-1}$ were released from the hemicellulose of rye and sunflower, respectively, suggesting that a high rate of deacetylation occurred. Low productions of other metabolites and fermentation gases were measured, suggesting an absence or low microbial activities. In fact, during the first three weeks of storage, microbial abundance remained low in the alkaline samples, due to the inhibitory action of high pH (Figure 4). During the same period, for silage conditions, a lactic fermentation was responsible for the acidification of the medium and the pH dropped quickly from 6.0 (rye) and 6.8 (sunflower) to 4.0. This fermentation is also obviously linked to a strong production of CO_2. Both crops underwent a conventional silage fermentation pathway and presented the characteristics of high quality silages according to Bureenok et al. (2016) with a pH lower than 4.5, a lactic acid concentration higher than 30

$g \cdot kg_{TS}^{-1}$ and butyric acid concentration representing less than 10% of total VFA concentration. In silage samples, a bacterial growth was observed and the initial abundances were multiplied by 19 and 5.2, growing from $1.05 \cdot 10^9$ to $2.10 \cdot 10^{10}$ and from $1.08 \cdot 10^9$ to $6.74 \cdot 10^9$ 16S rRNA gene copy number$\cdot g_{FM}^{-1}$ for rye and sunflower, respectively. This bacterial growth was mostly composed of bacteria from Lactobacillales and Enterobacteriales orders. Among the most represented lactic acid bacteria (LAB) after 2 days of fermentation, *Weissella sp.* (OTU5) and *Leuconostoc sp.* (OTU3) were dominant for rye while *Weissella* and *Lactococcus* (OTU10) prevailed for sunflower. These heterofermentative bacteria are known to be predominant in the epiphytic microflora of plants and to be responsible of a fast lactic acid production during the early fermentation phase (Gharechahi et al., 2017). From day 2 to 21, *Weissella*, *Leuconostoc* and *Lactococcus* decreased and were progressively replaced by different OTU of more acid tolerant *Lactobacillus* sp., as commonly observed in silage fermentation (Gharechahi et al., 2017). Additionnaly, *Enterobacteriaceae* (OTU4) were observed during this early phase. *Enterobacteriaceae* are commonly considered as undesirable in silage fermentation since they ferment amino acids into NH_3, compete with LAB for nutrients and reduce WSC into acetic acid and components that do not decrease pH. Below 4.5, pH prevented *Enterobacteriaceae* multiplication, consequently their number was considerably reduced as the medium acidified. During this phase, mostly lactic acid was produced, suggesting predominant homofermentation mechanisms.

3.3.1.2 Second phase (21 to 60 days)

After the third week of storage, silage conditions were stabilized and few additional fermentative microbial activity was observed. Almost no additional accumulation of metabolite nor gas production occurred. The total bacterial abundance was strongly reduced due to the acid conditions. $1.04 \cdot 10^9$ and $1.75 \cdot 10^9$ rRNA gene copy number$\cdot g_{FM}^{-1}$ were measured for silage of rye and sunflower, respectively. In alkali-stored conditions, two different behaviors took place. For the two experiments performed under dry conditions (41 and 47 %TS for rye and sunflower, respectively), the stable state obtained in phase 1 was maintained. The absence of fermentative activity can be identified by the lack of gas and metabolites production, which is confirmed by the low bacterial abundance (Figure 3 and Figure 4). In the wet condition however, a destabilization of the alkaline condition occurred after the third week of storage. Indeed, an heterolactic fermentation suddenly started and induced a pH drop down to 7.4. In this storage condition, WSC were consumed and converted into lactic acid, acetic acid, propionic acid and ethanol that accumulated in the medium. Fermentation gases (CO_2 and H_2) were also released. Unlike in silage conditions, a significant production of H_2 occurred. During this fermentation step in wet alkaline rye, H_2 production was likely linked to acetic and propionic fermentation (Hillion et al., 2018) since no butyric acid was found in the medium at this time. In storage processes prior to anaerobic digestion, H_2 production has to be avoided because of the high energy potential of this molecule (Kreuger et al., 2011). During this fermentation phase, the microbial concentration increased significantly from $1.28{\cdot}10^8$ to $9.60{\cdot}10^9$ rRNA gene copy number$\cdot g_{FM}^{-1}$ from day 21 to 60. The bacterial bloom was mostly due to the growth of Lactobacillales and Enterobacteriales in a similar way as during the early fermentation phase of silage. Clostridiales were also found in a smaller proportion. However, despite similarities in the order of the most represented bacteria in the fermentation of wet alkaline and ensiled samples, the actual microbial populations were different, as can be seen in Figure 4. In wet alkaline rye, the bacteria from Lactobacillales order were mostly composed of *Enterococcus sp.* (OTU6), with also *Carnobacterium sp.* (OTU8) present in a smaller proportion. *Enterococcus* bacteria are tolerant to pH until 9.6, but not below 4.5 (Cai, 1999). *Enterococcus* are mostly known as homofermentative bacteria. However high amounts of ethanol and acetic acid were found, indicating that other metabolic pathways took place. Although being LAB, *Carnobacteria* grow at pH range of 7-9, and even until 10.4 for *Carnobacterium maltaromaticum* that are found in the cold and alkaline tufa columns (Leisner et al., 2007). This bacteria genus is mostly related to food spoilage of chilled fish, meat and dairy products (Lorenzo et al., 2018). However, C*arnobacteria* can also be found in silage and are reported to participate in the primary lactic acid fermentation following heterolactic mechanisms (Pahlow et al., 2003). Bacteria from the Enterobacteriales order were mostly represented by a member of *Enterobacteriaceae* family (OTU4). This *Enterobacteriaceae* may develop on WSC and amino acids to produce acetic acid and NH_3 in destabilized alkaline storage in the same way as in silage, however the absence of further acidification could not allow their inhibition. *Enterobacteriaceae* are also reported to be H_2 producers, which is consistent with the observations that were made from day 21 to 60 (Cabrol et al., 2017). Additionally, Clostridia from *Lachnospiraceae* family were observed (OTU41). Clostridia are highly reported as undesired in silage fermentation. Clostridia proliferate mostly at pH > 5 and grow on amino acids, sugars and lactic acid to produce mostly butyric acid, CO_2 and H_2; they thus cause BMP reduction (Kreuger et al., 2011; Pahlow et al., 2003; Teixeira Franco et al., 2016). More specifically, *Lachnospiraceae* bacteria are largely represented in the gut or rumen of mammals where pH conditions close to neutrality are met (Evans et al., 1988). They are able to ferment diverse plant carbohydrates to VFA (butyrate, acetate, propionate) and alcohols like ethanol (Vacca et al., 2020).

The transformations that occurred in wet alkaline rye during this storage phase shows the complexity of inhibition mechanisms in alkaline storage. Despite a seemingly previous stable period of three weeks, microbial bloom finally happened with fermentation patterns implying H_2 production and Clostridia development that may lead to energy loss during the rest of storage. This shows that, in addition to CaO loading and initial pH, moisture content of the medium plays a crucial role in alkaline stability.

3.3.1.3 Third phase (60 to 180 days)

During this last phase of storage, no changes were observed in the silage conditions, nor in the alkaline storage of sunflower in dry condition. In dry alkaline storage of rye, a slow heterolactic fermentation was observed, similar to the one that was observed previously in wet alkaline rye. OTU8, OTU6 and OTU4 grew and were responsible for a low heteroloactic fermentation, without causing a destabilization comparable to the one observed in wet condition during phase 2. The bacterial development was seemingly slowed down by the water availability that was lower than in wet alkaline samples. In wet alkaline storage of rye, a secondary butyric fermentation took place. Most of the metabolites (i.e. lactic acid, acetic acid and ethanol) released during the previous phase were converted into butyric acid until a high concentration of 78 $g \cdot kg_{VS}^{-1}$ was reached, similarly as observed by Van Vlierberghe et al. (2021). The butyric fermentation was explained by a significant increase in the abundance of Clostridiales bacteria: *Caproiciducens sp.* (OTU29) and *Clostridium sp.* (OTU13), replacing OTU41 (*Lachnospiraceae*) that was found earlier. Concerning the bacteria from Lactobacillales order, no significant changes in abundance nor composition of their population was observed.

From day 100, gas production and mass losses kinetics started to slow down in wet alkaline storage, suggesting a decrease in the microbial activity. This final relative stable state could be explained by the very high concentration of butyric acid that accumulated in the medium and inhibited microbial activity. In wet alkaline rye, considering a pH 7.7 of the medium at this time, butyric acid was present at 99.9% in the dissociated form according to the Henderson-Hasselbalch equation (Equation (1).

$$pH \ = pK_a + log\frac{A^-}{AH} \qquad (1)$$

where A^- = concentration of acid under its dissociated form, AH = concentration of acid under its undissociated form. Butyric acid is known to have a strong inhibitory effect on fermentation, mostly in its undissociated form that can cross the cell membrane. However, the dissociated form is also reported to have an inhibitory effect from a high concentration due to the ionic strength that may result in the cell lysis of hydrogen-producing bacteria (Van Niel et al., 2003). A strong inhibitory effect of butyrate on fermentation for hydrogen production was reported in diverse studies. Zheng and Yu (2005) observed an almost total inhibition of H_2 production with 25 $g \cdot L^{-1}$ butyrate at initial pH 6.0 (93% of dissociated form). Similar results were obtained in another study at 26.4 $g \cdot L^{-1}$ butyrate at initial pH 7.0 (99.3% of dissociated form) (Wang et al., 2008). In the present study, the final butyrate concentration of 78 g·kg_{VS}^{-1} is equivalent to 26.6 ·g.L^{-1} when reported to the water fraction of the samples. The high amount of butyrate present in the medium at the end of wet alkaline storage could therefore be responsible for the inhibition of the microbial activity and final stabilization of the biomass.

Despite the similar initial properties of the different alkali-stored conditions, very different variations of the biomass properties occurred during storage as consecutive fermentations took place in some samples. This experiment confirmed that microbial fermentations were mostly responsible for the pH drop that can be observed in alkaline storage. Interestingly, if an elevated pH was shown to allow the inhibition of the microbial activity, the bacteria present in the medium were able to restart their activity from a high pH. As fermentation restarted, the pH decreased, which limited the inhibitory action of lime and allowed a fast bacterial growth. High pH (>12) and TS content (>40%) were shown to provide the best anaerobic alkaline stability. In silage experiment, the lactic fermentation allowed an efficient acidification of the substrate that stopped the bacterial growth. As a result, no secondary fermentations occurred in silage. A third inhibition mechanism at neutral pH was observed, due to the high butyric acid concentration. This inhibition allowed to prevent further degradations of the substrate such as methanogenesis to happen, thus avoiding energy losses and greenhouse gas emissions during storage.

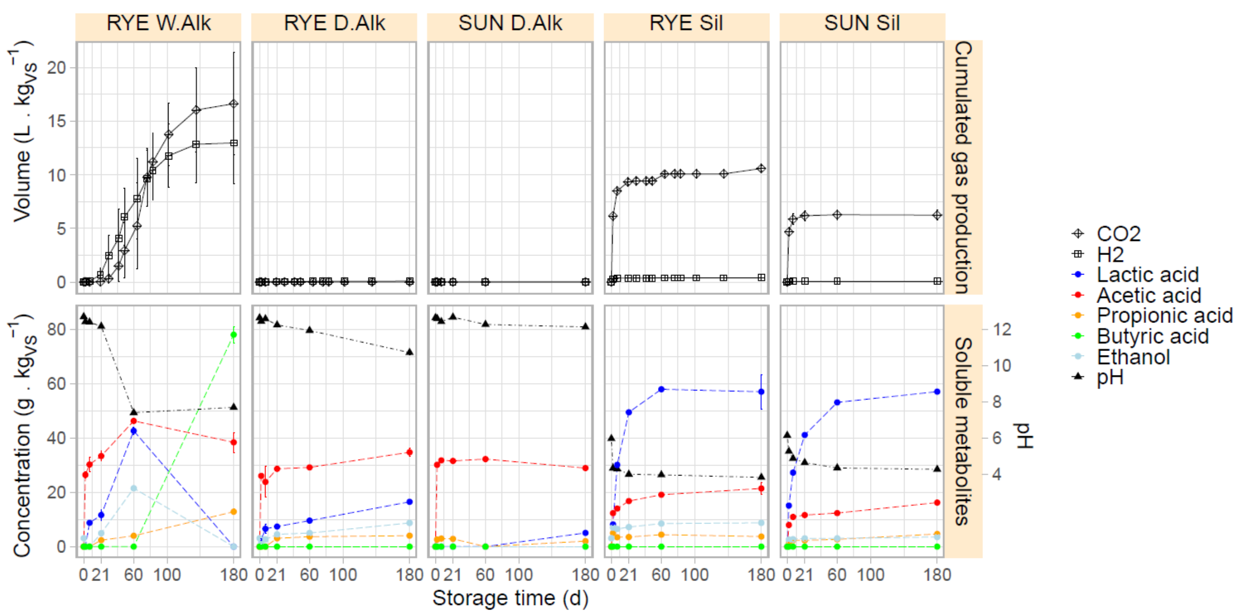

Figure 3: pH, metabolites and gaz variation during storage. CO_2 and H_2 are expressed in cumulated L.$kgVS_{init}^{-1}$. Metabolites are expressed in g.$kgVS_{added}^{-1}$. W.Alk, D.Alk and Sil stand for wet alkaline, dry alkaline and silage, respectively. SUN stands for sunflower. Error bars indicate standard deviation.

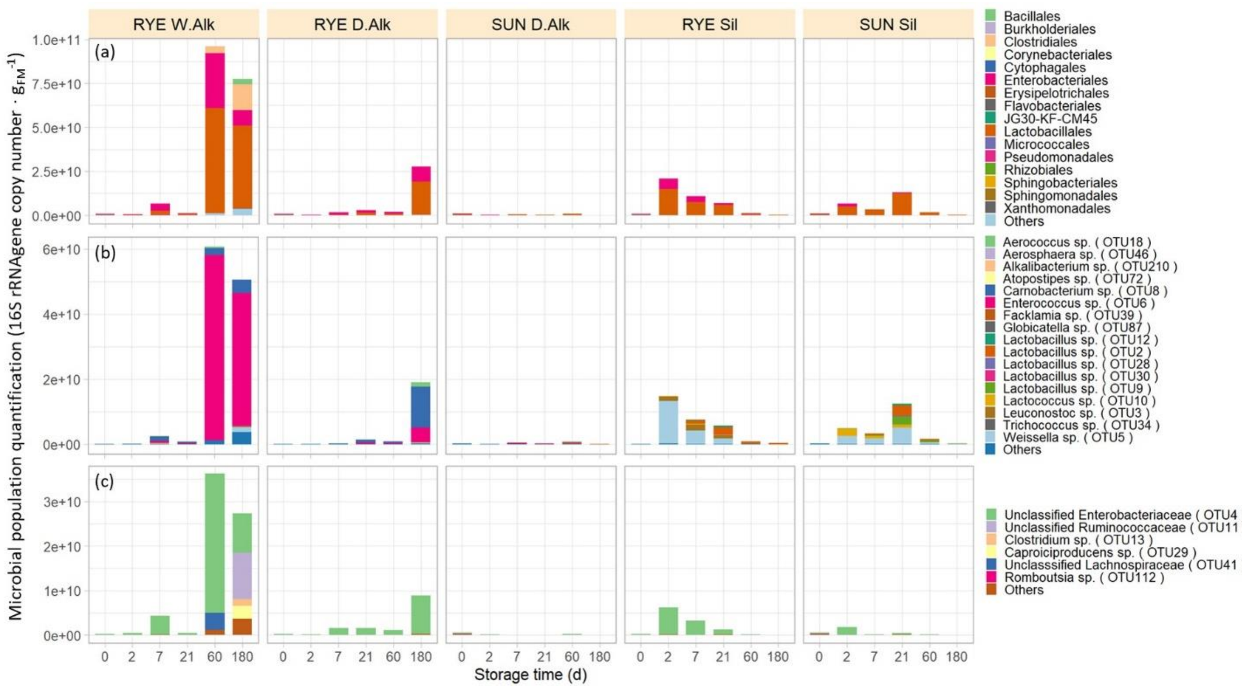

Figure 4: Major OTUs abundance (relative abundance >1.5% for at least one storage time) expressed in 16S rRNA gene copy.g_{FM}^{-1}. (a): Principal orders, (b): bacteria affiliated to Lactobacillales order, (c): bacteria affiliated to Enterobacteriales and Clostridiales orders. W.Alk, D.Alk and Sil stand for wet alkaline, dry alkaline, respectively. SUN stands for sunflower.

3.3.2 Impact of storage conditions on biomass characteristics and methane potential

The impact of storage conditions on fiber distribution characteristics was evaluated according to the Van Soest and Wine method (Van Soest and Wine, 1967) after 180 days of reaction (**Erreur ! Source du renvoi introuvable.**). The alkaline storage had mostly a strong impact on the hemicellulose fraction, that was significantly reduced by 53, 57 and 29% for rye storage in wet and dry conditions, and sunflower in dry condition, respectively. The hemicellulose reduction could not be only explained by the deacetylation mechanisms associated with the measured acetic acid release that represents 10% of the raw material hemicellulose content in wet and dry alkaline storage, and 19% in sunflower. Consequently, an actual dissolution of hemicellulose occurred due to the pretreatment action. As the hemicellulose was solubilized, both soluble fractions W-SOLU and SOLU increased. Alkaline storage had little effect on cellulose that was significantly different only between the dry alkaline conditions and the silage of the same crop. These results are in agreement with other experiments from the literature, where lime pretreatment was shown to partly solubilize hemicellulose, while the cellulose was not degraded (Kim and Holtzapple, 2005). Considering the lignin fraction, a reduction of 10% and 21% was measured in dry alkaline storage of rye and sunflower, respectively. However, due to the high variability of the raw material characteristics, these changes were not considered as significant by the pair-wise t-test adjusted with Bonferroni correction. In the literature, alkaline pretreatment with lime is reported to partly solubilize lignin. Delignification rates until 30-50% can be obtained with non-oxidative lime pretreatments at mild temperature (25 to 55°C) (Kim and Holtzapple, 2006; Wyman et al., 2005). The limited delignification obtained in the present experiments may be explained by the low moisture content applied combined with the use of CaO as a dry powder. Due to the low solubility of CaO (1.65 $g \cdot L^{-1}$ at 20°C), only a small fraction of the added lime may have been solubilized and was actually available for the pretreatment. In silage experiments, no significant changes in the share between the different fractions were observed. As reported in the literature, the lignocellulosic matrix undergoes few modifications in well preserved silages (Feng et al., 2018).

The gas production related to the different fermentation phases induced mass losses, mostly related to the production of CO_2. For rye, mass losses of 7.2 ± 0.6, 0.5 ± 0.1 and 2.9 ± 0.1 % of initial VS were measured in wet alkaline, dry alkaline and silage, respectively. For sunflower, the VS losses were limited to 0.3 ± 0.0 and 1.9 ± 0.1 % in dry alkaline and silage, respectively. Similar VS losses from 2 to 4% are normally observed in well preserved silage fermentation (Kaiser and Piltz, 2004; Kreuger et al., 2011).

BMP tests were performed on the fresh rye and sunflower used in storage experiment, along with samples stored during 7, 60 and 180 days. The results are presented in Figure 5. In most conditions, the biomass was efficiently stored and no significant BMP reduction was found after storage, excepted in wet alkaline rye. The unstable wet alkaline storage of rye caused a significant BMP loss of 13%, probably due to H_2 production (Kreuger et al., 2011), unlike previously observed by Van Vlierberghe et al. (2021) where the gas productions did not cause any measurable BMP loss, even with a 70% higher H_2 production due to fermentation.

The stable alkaline storage experiments (e.g. dry alkaline rye and sunflower) induced a BMP increase of respectively 10 and 21%. These changes were however considered as non-significant by the pair-wise t-test adjusted with Bonferroni correction, probably due to the high variability of BMP test. A significant increase of 15.7% was only observed between dry alkaline sunflower and sunflower silage. The results show that a slight increase in BMP was obtained due to the long-term action of the pretreatment, since a growing trend in BMP was only observed after 60 (sunflower) to 180 days (rye).

In the literature, BMP improvements of 17 to 37% (Thomas et al., 2018), 4 to 37% (Khor et al., 2015) and 7 to 34 % (Jiang et al., 2017) were obtained by pretreating similar lignocellulosic biomasses in mild conditions. The lower methane yield obtained in this experiment could be explained by the fact that drier conditions were applied, since moisture was pointed to have an impact on pretreatment efficiency (section 3.2 and (Digman et al., 2010)). TS of 29 to 47% were used here, while more wet conditions (around 10 %TS) were set in the literature. For both crops, silage storage allowed an efficient storage of the energy potential of the crop, showing the relevance of this process for crop storage for anaerobic digestion. As shown in the literature, a good management of silage allows to preserve efficiently the methane potential of crops (Teixeira Franco et al., 2016; Villa et al., 2020).

When stable alkaline conditions were obtained, alkaline storage showed a potential to provide a higher BMP of the crop, in comparison with storage by ensiling. However, a large amount of lime was required to obtain alkaline stability and may compromise the economical balance of the process. In a previous study, Jiang et al. (2017) found a positive net benefit of pretreatment when applying an alkaline pretreatment at 70 and 120 g $Ca(OH)_2.kgTS^{-1}$ (eq. 68 and 90 g $CaO.kgTS^{-1}$). Since alkaline stability is reached more easily at high TS, lower CaO loadings may be applied on dry crops like sunflower, which could increase the process sustainability. In order to conclude on the relevance of alkaline storage, the overall impact of lime addition on biomass valorization including aerobic stability at silo opening and continuous digestion of the crop should be investigated.

Table 3: Fiber distribution of the fresh and 180 d stored crops expressed in %VSinit. Within each column, different letters (abc) express a statistical difference between two samples of the same crop species stored under different conditions.

		W-SOLU	**SOLU**	**HEMI**	**CELL**	**LIG**
Rye	Fresh	29.5 ± 0.9^{a}	3.3 ± 1.1^{ac}	26.1 ± 1.5^{a}	31.6 ± 3^{a}	9.2 ± 3.6^{a}
	Wet Alkaline	29.4 ± 4.5^{a}	8.5 ± 1.3^{b}	12.2 ± 1.7^{b}	33 ± 0.9^{a}	9 ± 0.9^{a}
	Dry Alkaline	40.6 ± 7.8^{b}	5.8 ± 3.2^{ab}	11.2 ± 1.2^{b}	30.1 ± 0.9^{a}	8.3 ± 1^{a}
	Silage	29 ± 2.1^{a}	0.4 ± 1.3^{c}	22.8 ± 1.8^{a}	35.9 ± 3.1^{a}	10 ± 2.4^{a}
Sunflower	Fresh	21.4 ± 5.5^{a}	13 ± 4.6^{ab}	15.6 ± 0.2^{a}	27.8 ± 1.7^{ab}	16.5 ± 2.9^{a}
	Dry alkaline	23 ± 0.5^{a}	19.7 ± 1.2^{a}	11 ± 0.4^{b}	24.8 ± 0.1^{a}	13.1 ± 0.7^{a}
	Silage	20.7 ± 0.4^{a}	11.7 ± 0.4^{b}	13.5 ± 1.4^{a}	29.8 ± 1^{b}	15.2 ± 0.7^{a}

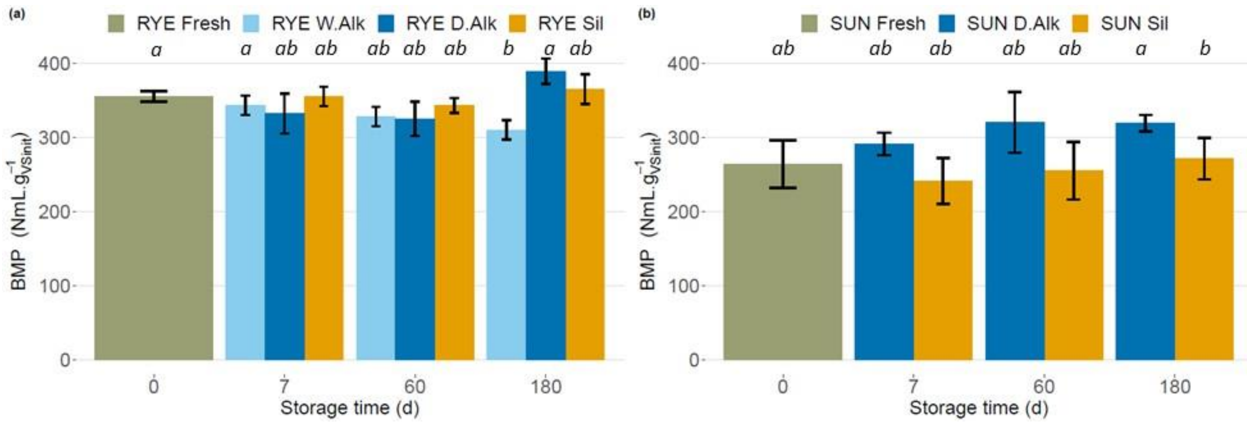

Figure 5: Impact of storage condition and duration on BMP. The error bars indicate standard deviation. W.Alk, D.Alk and Sil stand for wet alkaline, dry alkaline, respectively. SUN stands for sunflower.

4 Conclusion

Both alkaline and acid pH related to alkaline storage and silage allowed to inhibit the microbial activity over a long period. In alkaline storage, besides the initial lime loading, moisture was shown to influence storage stability. Despite of a stability period of 3 weeks, the bacteria present in the wet condition were able to restart their activity from an elevated pH of 12, causing a drop in pH and allowing a succession of fermentations to take place and limited but significant BMP losses. In successful alkaline storage, the long term pretreatment action allowed a BMP increase until nearly 16%.

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