

Article

Co-Ensiling of Wheat Straw as an Alternative Pre-Treatment to Chemical, Hydrothermal and Mechanical Methods for Methane Production

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Abstract: Wheat straw without pre-treatment is only converted to methane to a low degree during anaerobic digestion for fuel production due to its low hydrolysis. Current pre-treatment technologies are challenged by high expenses to energy or chemical agents. We examined the low-tech co-ensiling pre-treatment as an alternative pre-treatment of wheat straw, and compared the results with hydrothermal, chemical and mechanical pre-treatment methods. The effects of co-ensiling duration and the mixing ratio between straw and sugar beet root on the methane yields, surface morphology and chemical composition were examined. It was found that co-ensiling could improve production of methane by 34.7%, while a combined hydrothermal and chemical pre-treatment could increase the production of methane by 25.4%. The study demonstrated that the effect of co-ensiling could overlap with hydrothermal and chemical pre-treatment by having similar effects to increase lignocellulosic hydrolysis and improve methane production.

Keywords: Anaerobic digestion; biofuels; sustainability; silage; lignocellulose; biogas

1. Introduction

The bio-economy strategy from European Union (EU) defined bioenergy as a key element in the green-sustainable transition in Europe [1]. To cope with the growing global population, rapid exhaustion of resources, climate change and increasing environmental problems, large quantities of biomasses are required. Low-cost and maintenance energy crops for biofuels can provide more biomass, but it competes with food production. It is therefore important to utilize residual biomass and apply principles of Biogasdoneright®, where the bioenergy production is integrated into the rest of the value chain [2]. An alternative, unexploited and abundantly available resource is the by-product straw from grain production, which is left in the field after harvesting, collected for fodder or incineration [3]. Exploration of using agricultural residues such as wheat straw has therefore been substantially accelerated due to its ecological and economic advantages [4], where the supply for both the food, feed and energy chain will be improved when the production of energy crops will be reduced [5].

Wheat straw has a lignocellulosic structure, which is a polymer with a heterogenous complex of lignin (15–20%) and carbohydrates like cellulose (30–40%) and hemicellulose (20–30%) [6]. The carbohydrates are potential sources of fermentable sugars used for biogas production, while the

hydrolysis of lignin is very tough because of its high degree of cross-linkages with various types of bonds [7]. Enzymatic hydrolysis of macromolecules to monomers for digestion is limited by several factors. Studies have concluded that these factors include available surface area, degree of polymerization, crystallinity of cellulose, moisture content and lignin concentration among others [8,9]. Hence, effective pre-treatment strategies are required to cope with the hydrolysis limitations.

A wide variety of pre-treatment methods have been proposed at lab-scale studies. These methods include physical methods such as milling [10], microwave [11], extrusion [12] or ultrasonication [13]; chemical methods such as alkaline or acid hydrolysis [14,15]; physiochemical methods such as steam explosion [16] or liquid hot water [17]; and biological methods with enzymatic pre-treatment [18] among others. In spite of being able to increase the biodegradability and methane (CH₄) yield from lignocellulosic biomasses, the majority of the lab-scale pre-treatments are technically challenged if upscaled and are unfavorable regarding the cost of additives and their energy consumption [19].

Studies have shown that an alternative technique is ensiling, which may function as a beneficial and cost-effective pre-treatment for lignocellulosic materials prior to anaerobic digestion [20]. Ensiling is a common and traditional storage procedure used in the livestock industry to provide animal feed throughout the year. The preservation with ensiling requires an anaerobic environment and occurs spontaneously by several stages. The first stage is an aerobic stage, where the organic matter is degraded by aerobic respiration until the trapped oxygen is completely consumed [21]. The second stage is an anaerobic stage, where a complex consortium of endogenous microbes, predominantly lactic acid bacteria, consumes the sugars from the feedstock to produce organic acids and alcohol from hydrolysis of both structural and non-structural carbohydrates [20]. These compounds accumulate and decline the pH to between 3.7–4.5 depending on many factors like the buffer capacity and lactic acid concentration [22,23]. At low pH, the third stage begins, which favors the growth of beneficial microbes such as *Lactobacillus* sp., while the undesired detrimental microorganisms such as *Clostridium* sp. are inhibited [23]. The inhibition of the undesired microorganisms is partially due to both the pH drop, the low water activity and the production by *Lactobacillus* sp. of antimicrobial compounds such as the acetic acid, lactic acid, antifungal peptides and antibacterial peptides [24]. The silage can be stable in this stage for an extended period until the silage is exposed to air, whereupon the aerobic processes continue. Previous studies have documented the effect of ensiling, where ensiling can increase the CH₄ yield by 18% [25] to 42% [26] depending on crop species, harvest time, additives, solid content, ensiling duration and efficiency. Ensiling is challenged by dry matter (DM) losses, which studies have reported to be up to 35% [26], and down to 1% loss for successful ensiling processes [27]. If the moisture content in the biomass is too high or if the pH drop rate is too low, risk for growth of clostridial microorganisms arise. The presence of butyric acid should therefore not be detected in well-fermented silages, because it indicates that metabolic activity from clostridial organisms has been occurring, and as a result of this, large losses of DM occur [23].

Pure straw does not ensile at the typically low moisture content, and even for moist straw, ensiling is challenged by a lack of easily degradable and water-soluble carbohydrates, which are required for reducing the pH by a rapid formation of organic acids. Contrary to mono-ensiling of straw, mono-ensiling of sugar beet root generates low pH values (3.3–3.5) and has limited buffering capacities [28]. This mono-ensiled sugar beet root contains high concentrations of sugars, organic acids and alcohols that have a high biodegradability. However, the acids can induce acidification of the slurry in a reactor and may result in inhibition of the microbial activities and growth [29]. Hence, co-ensiling of wheat straw with sugar beet root has multiple positive synergies, where the addition of sugar beet roots provides an adequate moisture level and the easily degradable water-soluble carbohydrates required for the ensiling. Additionally, studies have shown that sugar beet roots contain a natural microbial culture of both *Leuconostoc mesenteroides* subsp. *dextranicum* strains and *Lactobacillus* strains, which would promote the lactic acid fermentation during co-ensiling [30]. As a result of this, the organic acids will break down the waxy hydrophobic surface of straw and function as acid catalysts to disrupt the ester bonds in the biopolymers [19]. The high DM content in straw will minimize the

risk of effluent loss from the sugar beet roots, as straw can absorb excess liquid, and may prevent the clostridial growth, which is typically inhibited at DM contents above 30% [26].

We studied the effectiveness of co-ensiling of wheat straw and sugar beet root as a pre-treatment of straw and compared it with other pre-treatment methods such as hydrothermal, mechanical and chemical pre-treatments. Anaerobic digestion in batch mode, silage products, e.g., compositions and concentrations, scanning electron microscopic (SEM) analysis to observe structural changes were carried out. Synergistic effects affected by various ensiling durations and the mixture ratios between sugar beet root and straw were examined, and compared with hydrothermal, mechanical and chemical pre-treatment results.

2. Materials and Methods

2.1. Biomasses

The biomasses in this study were sugar beet roots and winter wheat straw, with both biomasses produced on a farm near Thisted in Denmark. Sugar beets of the cultivar Gerty were de-topped by a forage harvester and harvested by use of a beet harvester (Thyregod, Denmark). The sugar beets were washed and stored in plastic bags for seven days and then chopped into a particle size of approximately 0.5–2 cm by use of a shredder. Samples of this sugar beet pulp were either frozen or used directly in the co-ensiling experiment with wheat straw. The harvested winter wheat straw was chopped using screens with a hole size of 32 and 36 mm, resulting in particle lengths of approximately 2–4 cm.

2.2. Co-Ensiling Setup

The co-ensiling experiment was conducted at lab-scale with a series of different mixtures of sugar beet pulp and chopped wheat straw and with various durations of ensiling. The ensiling experiment was done by the usage of a vacuum bag system as described by Johnson et al. (2005), which has been found to be an effective method for lab-scale ensiling [19,21,31]. Sugar beet pulp was either ensiled alone (mono-ensiling) or together with chopped wheat straw (co-ensiling) in sugar beet root:straw ratios of 100:0, 94:6, 88:12, 80:20 based on wet weight (WW). Mono-ensiling of straw was not conducted, as the moisture concentrations were too low to cause any fermentations. For each biomass ratio, 15 vacuum bags were prepared with 750 g of WW mixture, and after 0, 2, 6 and 8 months, two replicate bags were randomly chosen for each ratio and frozen at $-18\text{ }^{\circ}\text{C}$ to terminate the ensiling process after various durations. Bags frozen after 0 months correspond to fresh un-ensiled biomass. The samples were kept at $-18\text{ }^{\circ}\text{C}$ until sample analysis. Prior to the pre-treatments and analyzes, the two replicated bags were thawed and mixed. Samples were taken from the mixed replicates.

2.3. Pre-Treatment of Biomasses

Different sequential pre-treatment procedures were investigated on both un-ensiled straw and straw extracted from the co-ensiled samples to compare the effect of co-ensiling pre-treatment with more conventional pre-treatment methods. The pre-treatments included in this study were mechanical pre-treatments, namely, grinding, hydrothermal pre-treatment and a chemical pre-treatment. The pre-treatment procedures have been illustrated to provide a schematic overview in Figure 1.

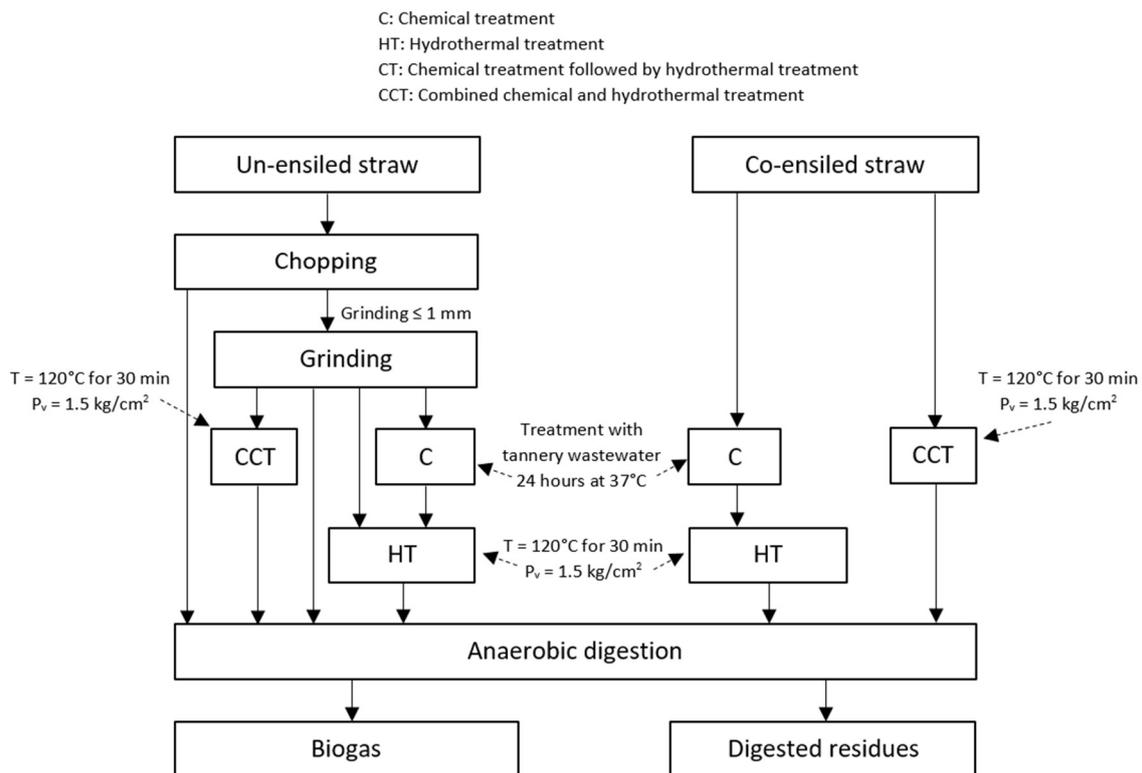


Figure 1. Schematic overview of pre-treatment procedures.

The mechanical pre-treatment (M) involved chopping followed by grinding of the un-ensiled straw to a size of (maximum) 1 mm. Tannery wastewater was used as a replacement of conventional alkaline pre-treatment medium [32]. Vazifekhoran et al. [32] reported that the use of tannery wastewater enhanced hydrolysis of wheat straw for biogas production, due to its content of lime (CaCO_3) and enzymes such as proteases, lipases, amylases and other industrial enzymes added during the processing of the hides at the tannery [32]. The tannery wastewater was collected at a local tannery on Funen in Denmark. Volatile solid (VS) content of the tannery wastewater was 0.9 g kg^{-1} and pH was 9.2. Three different procedures of pre-treatment were conducted on two different samples, un-ensiled straw and straw from co-ensiled samples (ratio of 80:20 and 8 months of ensiling). The 1st pre-treatment procedure was a hydrothermal pre-treatment (HT), where the straw was heated up to 120° for 30 min with a vapor pressure of 1.5 kg cm^{-2} in an autoclave while accompanied by demineralized water. A method recognized by other studies too [33]. The 2nd pre-treatment was a chemical pre-treatment (CT), where the straw was submerged in tannery wastewater for 24 h at 37°C in 500 mL infusion bottles followed by a washing of the straw and a hydrothermal pre-treatment. The 3rd pre-treatment was a combined chemical and hydrothermal pre-treatment (CCT), where the demineralized water in the hydrothermal pre-treatment was replaced by tannery wastewater. All samples were conducted in duplicates and an overview of the types of samples with their pre-treatment can be observed in Table 1.

Table 1. Overview of samples and their respective types of pre-treatments.

Mixing Ratio (WW-Based) (Sugar Beet, Root:Straw)	100:0	94:6	88:12	80:20	0:100
Ensiling duration (2, 6 or 8 months)	2 6 8	2 6 8	2 6 8	2 6 8	0
Pre-treatments (M, HT, CT or CCT)				8-HT 8-CT 8-CCT	0-M 0-HT 0-CT 0-CCT

WW, wet weight; M, mechanical pre-treatment; HT, hydrothermal pre-treatment; CT, chemical pre-treatment; CCT, combined chemical and hydrothermal pre-treatment.

2.4. Biochemical Methane Potential Assays

The biochemical methane potential (BMP) measurements were conducted on the ensiled and pre-treated samples listed in Table 1 and were conducted in accordance with the German standard method VDI 4630 [34]. The inoculum for the BMP tests was collected from a commercial biogas plant (37 °C). The inoculum was pre-incubated for 8 days for degassing, where residual biodegradable organic material was depleted. This reduced the impact of its CH₄ production on the BMP tests as recommended in [35]. During the pre-incubation, preliminary tests, i.e., DM and VS, were conducted. The DM and VS for degassed inoculum were 83.9 g kg⁻¹ and 48.4 g kg⁻¹, respectively.

The BMP assays were conducted in infusion bottles (500 mL working volume) enclosed with rubber stoppers and metal clamps. The inoculum and substrate were mixed with a ratio (I/S) of 3:1, respectively, based on VS content. The bottles filled until a headspace of 70% were available in the infusion bottles. Subsequently, the BMP bottles were flushed with N₂ for 3 min before incubation to change the atmosphere inside the bottle to an anaerobic environment. The BMP assays were incubated at 37 °C to ensure optimal temperature for mesophilic anaerobic digestion for 30 days. In addition to the substrate samples, duplicate samples of inoculum were included to assess the methanogenic activities related to the inoculum and microcrystalline cellulose was included as a known reference material.

A quantitative assessment of the CH₄ production was conducted according to the study of Filer et al. [36], where the syringe method was used. A glass syringe was to be connected to the BMP bottle by a needle, and the overpressure would cause the piston to be pushed back until atmospheric pressure was measured on a manometer also connected to the BMP bottle. This method provided a quantitative measure for the volume of the produced biogas. The collected biogas was collected in vacuum vials and its gas composition was analyzed using a gas chromatograph (GC) (7890A, Agilent Technologies), which was equipped with a 30 m × 0.320 mm column (J&W 113-4332, Agilent Technologies) and a thermal conductivity detector. The carrier gas was helium and had a flow of 30 cm s⁻¹. The injection temperature was 110 °C, while the oven and detector temperatures were 250 °C. An injection volume of 0.4 mL with a split ratio of 1:100 was configured. The GC was calibrated for CO₂ and CH₄ with certified gas standards.

The volumetric measured biogas was corrected to dry biogas at standard conditions of 273.15 K and 101.325 kPa following the method from the German standard VDI4630 [34]. The cumulative produced CH₄ was normalized by the VS content to provide the biochemical methane potential of the samples. The BMP of the inoculum was also determined to correct the results from its contribution to the CH₄ production.

2.5. Sample Analysis

The solid contents of the substrates were analyzed, prior to the BMP tests, in relation to its DM and VS content. The DM and VS were determined according to the EPA standard method [37]. The measured DM and VS were adjusted according to the method in the study of Vahlberg et al. [38] to

avoid underestimation when volatile substances, including alcohols and volatile fatty acids (VFA), were vanished during drying. The VFA and alcohols were therefore added to the measured VS content to avoid overestimation of the BMP [39].

The VFA concentration (C2–C5) of the substrates were analyzed on a GC (7890B, Agilent Technologies), which was equipped with a flame ionization detector and a 30 m × 0.25 mm × 0.25 μm column (HP-INNOWax, Agilent Technologies,) with helium as carrier gas. The injection temperature 150 °C, while the initial column temperature was 95 °C with a holding time of 0.5 min. A temperature gradient was used for the column, where the temperature was ramped up to 140 °C with a rate of 10 °C min⁻¹. After reaching 140 °C, the temperature gradient was changed to a rate of 40 °C min⁻¹ until 200 °C was reached, where it was kept for 5 min. Prior to the GC analysis, the substrates had been acidified with ortho-phosphoric acid 85% until a pH of 2 had been reached. Subsequently, the fraction for analysis was collected by removal of the solids with centrifugation for 15 min at 13,000 RPM (Eppendorf MiniSpin) followed by filtration with a 0.5 μm cellulose filter. Calibration of the GC was conducted with triplicates of standard solutions in the range of 3.5 mM to 105 mM. The concentrations of the alcohols; sorbitol, glycerol and ethanol, and sugars; glucose, maltose and fructose in the substrates were determined using a high-performance liquid chromatography (HPLC) (Agilent 1100, Agilent Technologies Deutschland GmbH & Co. Kg, Waldbronn, Germany) equipped with a refractive index detector. The alcohols and sugars were separated with a Phenomenex Rezex RHM-Monosaccharide H+ (8%) column with ultrapure water (Milli-Q) as eluent. The flow rate through the column was 0.6 mL min⁻¹ and the column was kept at 80 °C. Before the samples were injected into the HPLC, they were diluted in Milli-Q water and centrifuged at 5000 RPM for 15 min followed by filtration through a 0.45 μm cellulose filter prior to analysis.

The elementary analysis was conducted to determine C, H, O, N and S atomic content in the substrate using a CHNS analyzer (Vario MACRO cube, Elementar analysensysteme GmbH, Germany). The samples of biomasses were packed, and 5 mg was weighed in small tin packages for C, H, N and S-analysis and silver packages for O-analysis and loaded into the autosampler carousel. A ball valve allowed the sample to be purged by the carrier helium gas. Then it was let into the combustion tube with a temperature of 1150 °C and afterwards into a reduction tube at 850 °C. The gas mixture was separated in its components via purge and trap chromatography and the detector for the system was a thermal conductivity detector (TCD).

2.6. Data Analysis

Based on the atomic composition of the samples, the theoretical biochemical methane potential (TBMP) was calculated to provide a stoichiometric estimate of the CH₄ potential. To access the TBMP, Buswell equation was used to describe the conversion of different substrates, C_aH_bO_cN_dS_e, into a biogas composition with varying concentrations of carbon dioxide (CO₂), CH₄, ammonia (NH₃) and hydrogen sulphide (H₂S) [40]. This equation was rewritten into Equation (1), as described in the study of Nielfa et al. [41].

$$\text{TBMP} \left[\frac{\text{NL CH}_4}{\text{kg VS}} \right] = \frac{\left(\frac{a}{2} + \frac{b}{8} - \frac{c}{4} - \frac{3d}{8} - \frac{e}{4} \right) \cdot 22.4 \frac{\text{L}}{\text{mol}}}{12a + b + 16c + 14d + 32e} \cdot 1000 \quad (1)$$

Knowing the elemental composition could thereby provide a measure for the TBMP. The biodegradability of the biomasses was accessed with Equation (2) [42].

$$\text{Biodegradability} [\%] = \frac{\text{BMP}}{\text{TBMP}} \cdot 100 \quad (2)$$

The synergistic effects of co-ensiling of different ratios of sugar beet sugar beet root and straw were evaluated by comparing the measured BMP of the different mixtures, with a calculated BMP

based on the data from fresh sugar beet roots and fresh straw and their respective VS proportions in the different ensiling mixture.

The biogas production kinetics were modelled with first-order kinetics for ensiled samples [43] and modified Gompertz model for pre-treated samples [44] to assess the effects of the treatments. The first-order kinetic model hypothesises that the rate of the biogas yield is increasing linearly with the digestion time and is expressed in Equation (3).

$$y(t) = y_0 \cdot (1 - e^{-k \cdot t}) \quad (3)$$

where:

- $y(t)$ = The cumulative methane yield after a specific digestion period, t , in days (NL CH₄ kg⁻¹ VS)
- y_0 = The ultimate methane potential (BMP) (NL CH₄ kg⁻¹ VS)
- k = The hydrolysis constant (day⁻¹)
- t = Cumulative digestion time for biogas production (days)

For more complex biomasses with higher lignocellulosic fractions, the first-order kinetic model may not precisely simulate the whole biogas production [45]. The rates of the biogas production for lignocellulosic straw samples with their associated pre-treatments were therefore expressed with the modified Gompertz model, which includes the duration of the lag phase and thereby imply that the biogas production does not increase linearly with time. The modified Gompertz model is expressed in Equation (4) and is usually depicted as a sigmoidal curve.

$$y(t) = A \cdot e^{\frac{\mu_m \cdot t}{\lambda} \cdot (\lambda - t) + 1} \quad (4)$$

where:

- $y(t)$ = The cumulative methane yield after a specific digestion period, t , in days (NL CH₄ kg⁻¹ VS)
- A = The biogas production potential (NL CH₄ kg⁻¹ VS)
- μ_m = The maximum biogas production rate (NL CH₄ kg⁻¹ VS day⁻¹)
- λ = Lag phase period (days)
- t = Cumulative digestion time for biogas production (days)

Two-way analysis of variance (ANOVA) tests were applied to examine the effect of the ensiling duration and mixture in relation to the DM and BMP, while one-way ANOVA tests were applied to examine the effect of pre-treatment on the BMP. All with a confidence level of 95% ($p < 0.05$). The average of the duplicates was calculated and reported in this paper with the standard deviation.

3. Results and Discussion

3.1. Dry Matter Losses

The results of the chemical analyzes of the different mixtures of sugar beet root and wheat straw are summarized for mixtures in Table 2 and pure biomasses in Table 3. The un-ensiled wheat straw and the un-ensiled sugar beet root used for the different mixtures had a DM of 885.6 ± 0.1 g kg⁻¹ and 210.8 ± 0.3 g kg⁻¹, respectively. The fresh sugar beet root and straw were mixed with wet weight ratios of 94:6, 88:12 and 80:20, acquiring a DM concentration of 258.0 ± 0.3 g kg⁻¹, 298.5 ± 0.2 g kg⁻¹ and 345.8 ± 0.2 g kg⁻¹, respectively, with all lying in the favorable range of 250–350 g kg⁻¹ for ensiling [27]. During ensiling a significant loss of DM for all the silage samples was observed ($p < 0.05$) (Figure 2).

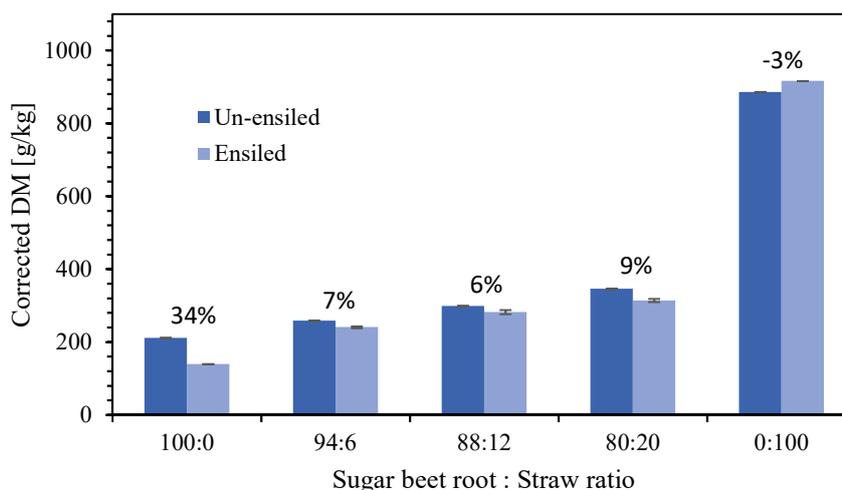


Figure 2. Corrected dry matter (DM) concentration of mixtures of sugar beet root and wheat straw at different ratios (WW) before and after ensiling (8 months). Wheat straw would not ensile due to its high dry matter concentration and is therefore only stored for 2 months. The relative DM loss during ensiling is listed above the columns. The error bars indicate the standard deviation.

A great loss of dry matter for mono-ensiled sugar beet roots was observed (34% of DM loss after 8 months of ensiling), whereas co-ensiling had far lower DM losses (<10%). Much of the same results in terms of DM loss during the ensiling have been observed by previous studies [26]. Consequently, previous studies have reported that the co-ensiling resulted in lower DM losses, highlighting that co-ensiling was effective for conserving DM when biomass long-term storage was unavoidable [19]. The DM losses during ensiling were mainly attributed to the deterioration of readily available carbohydrates from unfavorable fermentation processes. These unfavorable fermentations could occur from microorganisms as *Clostridium* and other enterobacteria generating H_2 and CO_2 among others. Increasing the DM concentration to around 300 g kg^{-1} had been reported to inhibit the clostridial fermentation [46]. This was accomplished by mixing sugar beet root with wheat straw, and thereby ensuring that the main fermentation was caused by lactic acid bacteria. These lactic acid bacteria would contribute to inhibition of the growth by unfavorable microorganisms by producing both antimicrobial peptides and organic acids that also induced the pH drop, which would result in the lowest losses of DM [23,24].

Table 2. Characterization of co-ensiled substrates with a mixing ratio (WW) of sugar beet root and wheat straw at 94:6, 88:12 and 80:20 at different ensiling periods.

Parameters Ensiling Period	Units Months	94:6			88:12			80:20		
		2	6	8	2	6	8	2	6	8
<i>Solid analysis:</i>										
Dry matter	g kg ⁻¹	246.5 ± 16.4	177.7 ± 7.6	240.5 ± 2.4	260.5 ± 8.8	271.2 ± 6.9	282.0 ± 5.7	350.0 ± 1.2	338.8 ± 9.2	313.9 ± 4.4
Volatile solids	g kg ⁻¹	232.9 ± 15.8	163.5 ± 6.4	226.8 ± 3.0	244.5 ± 8.0	252.2 ± 4.4	262.8 ± 5.2	324.5 ± 0.7	313.4 ± 9.9	279.8 ± 16.6
<i>Volatile fatty acids*:</i>										
Acetic acid	g L ⁻¹	0.66 ± 0.01	0.91 ± 0.01	0.85 ± 0.03	0.68 ± 0.02	0.77 ± 0.02	0.74 ± 0.01	0.68 ± 0.02	0.84 ± 0.02	0.92 ± 0.00
Butyric acid	g L ⁻¹	0.13 ± 0.01	0.00 ± 0.00	0.10 ± 0.00	0.12 ± 0.00	0.07 ± 0.00	0.11 ± 0.00	0.11 ± 0.00	0.10 ± 0.01	0.09 ± 0.00
<i>Alcohols:</i>										
Ethanol	g L ⁻¹	0.03 ± 0.00	0.54 ± 0.00	0.43 ± 0.00	0.12 ± 0.00	0.09 ± 0.00	0.07 ± 0.00	0.12 ± 0.00	0.34 ± 0.00	0.36 ± 0.00
Glycerol	g L ⁻¹	0.11 ± 0.00	0.94 ± 0.05	0.43 ± 0.01	0.12 ± 0.00	0.09 ± 0.00	0.07 ± 0.00	0.12 ± 0.00	0.34 ± 0.00	0.36 ± 0.00
Sorbitol	g L ⁻¹	11.89 ± 0.01	6.20 ± 0.06	14.50 ± 0.06	11.58 ± 0.02	9.54 ± 0.03	14.26 ± 0.00	11.93 ± 0.01	12.55 ± 0.02	10.91 ± 0.00
<i>Sugars:</i>										
Maltose	g L ⁻¹	0.89 ± 0.00	0.37 ± 0.05	0.98 ± 0.00	1.01 ± 0.00	0.75 ± 0.00	1.21 ± 0.00	1.19 ± 0.00	1.01 ± 0.00	0.96 ± 0.00
Glucose	g L ⁻¹	5.11 ± 0.00	2.14 ± 0.03	5.88 ± 0.06	5.93 ± 0.01	4.24 ± 0.03	7.13 ± 0.00	7.17 ± 0.00	6.52 ± 0.00	6.23 ± 0.00
<i>Elemental composition:</i>										
Carbon	wt%	58.38	69.63	58.63	56.37	60.73	61.36	56.38	56.90	61.88
Nitrogen	wt%	1.43	1.57	1.51	1.20	1.02	0.95	1.21	0.96	1.00
Oxygen	wt%	33.83	23.37	34.35	35.09	31.57	30.46	34.84	35.61	30.21
Sulphur	wt%	0.97	0.85	0.77	0.74	0.53	0.43	0.44	0.43	0.35
Hydrogen	wt%	5.39	4.59	4.75	6.60	6.14	6.80	7.13	6.20	6.56

* Propionic-, Isobutyric-, Valeric- and Isovaleric acid were below the detection limit.

Table 3. Characterization of mono-ensiled substrates of sugar beet root (100:0) and wheat straw (0:100) at different ensiling periods.

Parameters Ensiling Period	Units Months	100:0			0:100
		2	6	8	2
<i>Solid analysis:</i>					
Dry matter	g kg ⁻¹	196.1 ± 7.1	162.4 ± 3.8	139.0 ± 0.4	915.6 ± 0.5
Volatile solids	g kg ⁻¹	187.1 ± 6.6	153.5 ± 3.6	130.6 ± 0.2	848.6 ± 2.9
<i>Volatile fatty acids*:</i>					
Acetic acid	g L ⁻¹	0.82 ± 0.04	0.76 ± 0.02	1.26 ± 0.03	N/A
Butyric acid	g L ⁻¹	0.08 ± 0.01	0.03 ± 0.01	0.00 ± 0.00	N/A
<i>Alcohols:</i>					
Ethanol	g L ⁻¹	0.10 ± 0.00	0.40 ± 0.00	0.68 ± 0.00	N/A
Glycerol	g L ⁻¹	0.11 ± 0.00	0.20 ± 0.01	0.75 ± 0.01	N/A
Sorbitol	g L ⁻¹	10.23 ± 0.07	6.14 ± 0.00	3.66 ± 0.00	N/A
<i>Sugars:</i>					
Maltose	g L ⁻¹	0.40 ± 0.06	0.37 ± 0.00	0.22 ± 0.00	N/A
Glucose	g L ⁻¹	1.66 ± 0.10	1.69 ± 0.05	1.17 ± 0.02	N/A
<i>Elemental composition:</i>					
Carbon	wt%	49.37	51.32	54.11	64.24
Nitrogen	wt%	1.21	1.37	1.68	1.09
Oxygen	wt%	41.35	41.12	38.71	28.51
Sulphur	wt%	3.19	1.78	1.52	0.16
Hydrogen	wt%	4.87	4.41	3.98	5.98

* Propionic-, Isobutyric-, Valeric- and Isovaleric acid were below the detection limit.

3.2. Effect of Co-Ensiling

The un-ensiled wheat straw, which was characterized by a slow hydrolysis with a first-order hydrolysis constant of 0.04 day⁻¹, a high DM concentration and a low biodegradability of 28.3% (Table 4) had a BMP of only 186.7 ± 12.0 NL CH₄ kg⁻¹ VS. Wheat straw has insufficient concentrations of fermentable sugars and too low water content to facilitate lactic acid fermentation. However, a previous study has shown that lignocellulosic biomasses such as wheat straw can be ensiled by applying different strategies: (1) ensure optimal water activity by adding water, (2) add fermentable sugars to promote the growth of *Lactobacillus*, (3) inoculate the ensiling of wheat straw with a lactic acid bacteria culture, (4) decrease the pH by using organic acids [47]. Additionally, sugar beet roots could be a co-ensiling biomass to facilitate the ensiling of wheat straw.

Multiple positive synergistic effects were achieved by mixing the wheat straw with sugar beet roots containing a natural culture of lactic acid bacteria, a high concentration of moisture and easily degradable carbohydrates that could accelerate the generation of organic acids during the co-ensiling. A higher biodegradability was observed due to the acid hydrolysis by the produced acids from the sugar beet root carbohydrates.

The cumulative methane production trend for the co-ensiled samples of wheat straw and sugar beet root is illustrated in Figure 3, where a rapid methane formation can be observed for the first 5 days of anaerobic digestion, which constituted the majority of the total methane produced.

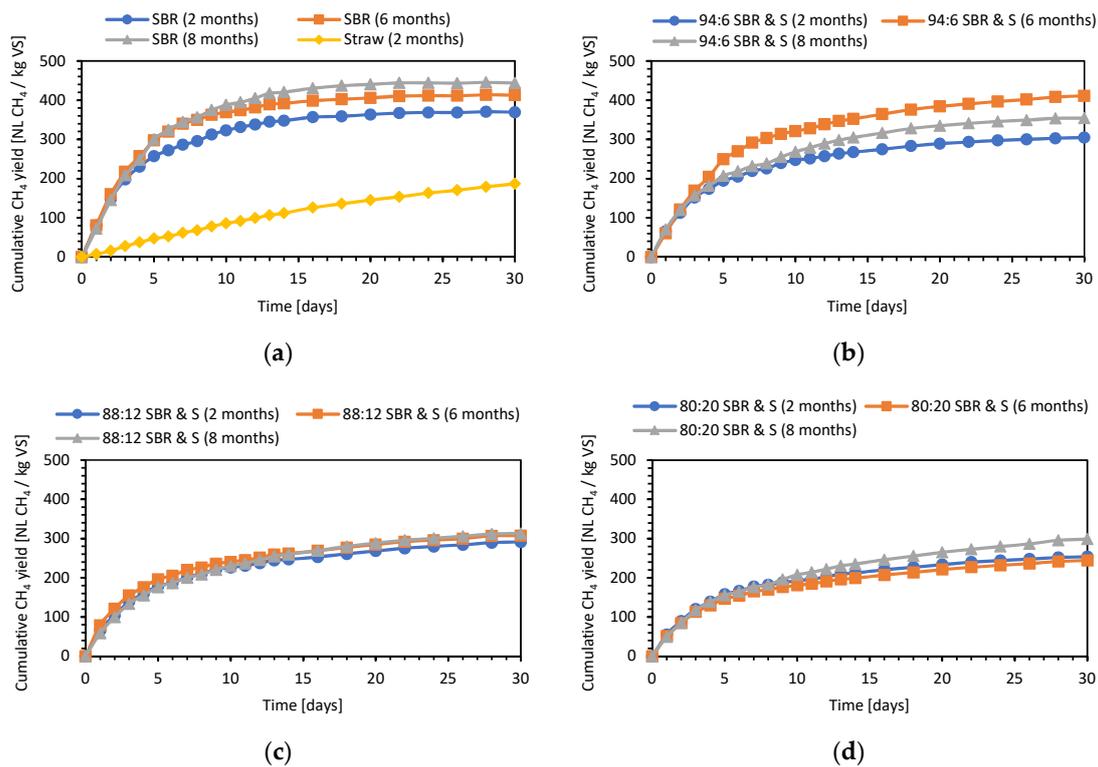


Figure 3. Cumulative CH₄ yield of (a) mono-ensiled samples of sugar beet root (SBR) and dry-stored straw (S), (b) co-ensiled SBR and S (94:6), (c) co-ensiled SBR and S (88:12), (d) co-ensiled SBR and S (80:20), at different ensiling periods.

A significant increase ($p < 0.05$) in the BMP was found as the co-ensiling duration increased, which is in accordance with increases in biodegradability along with longer co-ensiling duration (Table 4). It was found that the biodegradability of the biomasses changed from 44.41% to 64.43% for the 94:6 samples, 46.86% to 48.14% for the 88:12 samples and 42.60% to 46.64% for the 80:20 samples. The synergistic effect of co-ensiling on the BMP varied from -3.15% to 34.7% , in which the largest effect was found for 8 months of co-ensiling (co-ensiling ratio of 80:20). The synergistic effect was found to significantly increase as the straw ratio increased in the co-ensiled samples ($p < 0.05$). The increasing synergistic effect may partly be attributable to the reduced DM loss introduced by the wheat straw, and the increased biodegradability of straw during co-ensiling due to the acid production that breaks the wax layer and the acid hydrolysis that breaks ester bonds in the straw, which also has been reported by other studies [19].

Table 4. Methane yield, biodegradability and kinetic constants for ensiled and pre-treated samples with a mixing ratio (wet weight based) of sugar beet root and wheat straw at 100:0, 94:6, 88:12, 80:20 and 0:100 at different ensiling periods.

Sample	Ensiling Duration	Additional Pre-Treatment	BMP [NL CH ₄ kg ⁻¹ VS]	Biodegradability [%]	Synergistic Effect of Co-Ensiling and Pre-Treatment [%]	k [day ⁻¹]	Kinetic Constants		
	[Months]						A [NL CH ₄ kg ⁻¹ VS]	μ _m [NL CH ₄ kg ⁻¹ VS day ⁻¹]	λ [day]
100:0	2		369.4 ± 7.4	84.0		0.24			
100:0	6		412.5 ± 5.6	92.2		0.25			
100:0	8		443.6 ± 11.4	94.7		0.21			
94:06	2		304.5 ± 10.2	53.7	-3.2	0.21			
94:06	6		411.1 ± 2.6	60.0	21.8	0.18			
94:06	8		354.1 ± 15.1	64.4	1.2	0.17			
88:12	2		291.2 ± 12.8	50.2	8.8	0.20			
88:12	6		307.4 ± 0.4	49.5	10.6	0.22			
88:12	8		312.9 ± 11.8	48.1	11.6	0.16			
80:20	2		253.9 ± 11.9	42.6	13.8	0.19			
80:20	6		244.3 ± 9.3	42.6	8.5	0.18			
80:20	8		298.6 ± 18.0	46.0	34.7	0.14			
80:20	8	HT	310.5 ± 8.3	47.9	4.0		309.32	20.28	0.00
80:20	8	CT	298.2 ± 24.9	46.0	-0.1		298.03	20.88	0.00
80:20	8	CCT	312.6 ± 2.6	48.2	4.7		308.91	20.88	0.00
0:100	0		186.7 ± 12.0	28.3		0.04	190.99	8.83	0.64
0:100	0	M	186.6 ± 6.2	28.2	-3.2		174.55	14.98	2.14
0:100	0	HT	214.8 ± 6.5	32.4	15.1		205.50	15.48	1.94
0:100	0	CT	223.4 ± 5.8	33.9	19.7		214.04	16.81	2.19
0:100	0	CCT	234.1 ± 5.9	35.5	25.4		224.33	16.81	1.87

The best mixing ratio of the investigated ratios appears to be 80:20, as it induced a higher synergistic effect of the CH₄ yield while maintaining low DM losses. The enhanced methane yields in cases of co-ensiling are reported to be due to the breakage of inter- and intra lignocellulosic polymers caused mainly by the acidification of the substrate [19]. This is in accordance with previous studies, which reported that co-ensiling gave the high pre-treatment effect for the cases of lignocellulosic concentrations like hemp and straw [19–21], while other biomasses, e.g., sugar beets, had no effect [48]. This indicates that co-ensiling may function as a biological pre-treatment of lignocellulosic biomasses. Chemical pre-treatments are usually preferred over biological pre-treatments, as biological pre-treatments often are limited by their relative long treatment times required. Nevertheless, the concept of co-ensiling as a biological pre-treatment still has high potential, as the technique also functions as a method to store wet and dry biomass together for longer periods to allow an available feedstock for biogas plants throughout the year.

3.3. Mechanical, Hydrothermal and Chemical Pre-Treatment Effects

Un-ensiled wheat straw had a high unused CH₄ potential (BMP of 186.7 ± 12.0 NL CH₄ kg⁻¹), as only a biodegradability of 28.3% was achieved during these studies (Figure 4). Methane production rate by the first-order kinetic analysis, the degradation constant (k) was 0.04 d⁻¹, and when applying the modified Gompertz model, the maximum methane production rate (μ_m) was 8.83 NL CH₄ kg⁻¹ VS d⁻¹ (Table 4). The first-order kinetic analysis shows that only 3.5% of the remaining organic materials are converted to methane per day, which explains the speed of degradation is quite low, therefore pre-treatments were applied.

Mechanical pre-treatment of the straw yielded a BMP of 186.6 ± 6.2 NL CH₄ kg⁻¹ VS, which approached an almost similar value as the BMP of un-ensiled straw. However, it was evident that the mechanically pre-treated straw was more exposed to a higher rate of hydrolysis compared to the un-ensiled straw (Figure 4). Mechanical pre-treatment has been found to rupture the cell walls, decrease the viscosity, increase the accessible surface area and disrupt the crystallinity of cellulose, which would be beneficial for both the digestion and handling of biomass [49]. However, the mechanical pre-treatment was not able to disrupt the lignocellulosic structure in the biomass, which inhibited the hydrolysis and lessened the access of the enzymes to the carbohydrates [50]. This observation was underlined by accessing the maximum biogas production rate from the Gompertz modified model, which was 8.83 NL CH₄ kg⁻¹ VS d⁻¹ for fresh wheat straw, and 14.98 NL CH₄ kg⁻¹ VS d⁻¹ for mechanical pre-treated wheat straw. The applied mechanical pre-treatment therefore mainly increased the rate of hydrolysis, but not the overall CH₄ yield, which indicated that the pre-treatment did not have any severe effect on the crystallinity of the cellulose and reduction in the degree of polymerization.

Applying a hydrothermal pre-treatment on the wheat straw increased both the biodegradability and the hydrolysis rate of the straw and had a BMP yield of 214.8 ± 6.5 NL CH₄ kg⁻¹ VS, which was an increase of 15.1% compared to un-ensiled straw. The hydrothermal pre-treatment partly solubilizes the substrate and uses high temperatures, pressure and autoionization of water to generate hydrogen ions, which transforms the structure of lignin and reduces the pH to acidic levels [51]. This benefited the hydrolytic reactions, and the maximum biogas production rate, μ_m , was thereby increased to 15.48 NL CH₄ kg⁻¹ VS d⁻¹. Addition of a chemical alkaline agent, tannery wastewater, before (CT) or during the hydrothermal pre-treatment (CCT) increased the cumulative CH₄ yield additionally to 223.4 ± 5.8 NL CH₄ kg⁻¹ VS and 234.1 ± 5.9 NL CH₄ kg⁻¹ VS, respectively, which both were significantly different ($p < 0.05$) from un-ensiled wheat straw. The highest synergistic effect was found for CCT pre-treatment, where an effect of 25.4% was found. These results agreed with the study of Vazifehkhoran et al. [32], where an increase of 35% in BMP was achieved with tannery wastewater pre-treatment.

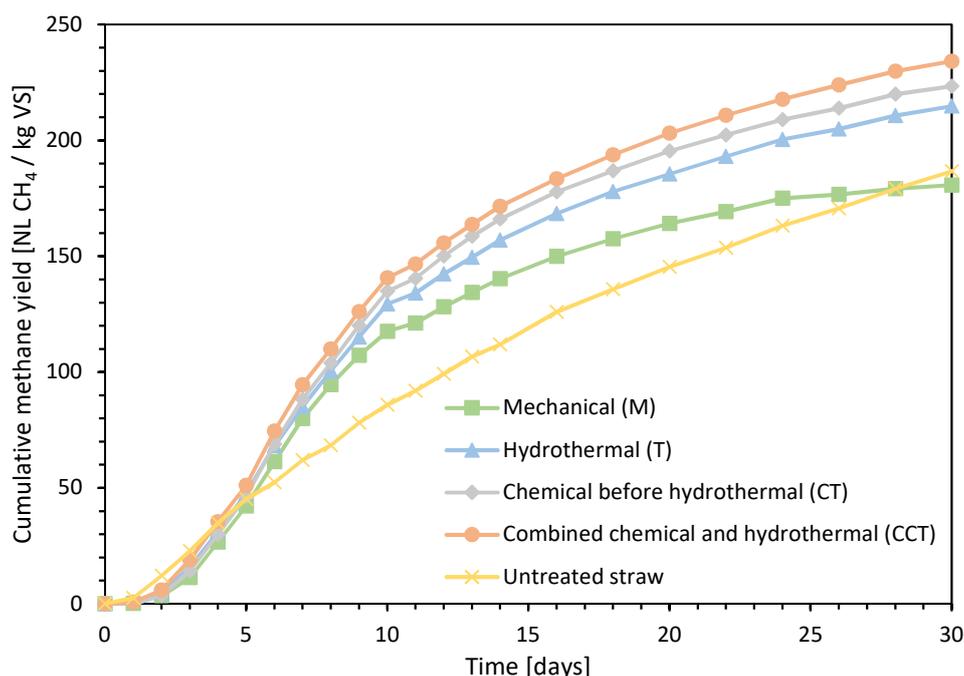


Figure 4. Cumulative methane yield of untreated straw, mechanical- (M), hydrothermal- (HT), Chemical- before hydrothermal (CT), combined chemical- and hydrothermal- (CCT) pre-treated straw.

3.4. Competitiveness of Co-Ensiling as a Pre-Treatment

Pre-treatments with co-ensiling, hydrothermal and chemical techniques were all able to increase the biodegradability of straw significantly ($p < 0.05$). However, when applying chemical and hydrothermal pre-treatment to co-ensiled samples, it was found that the effect of co-ensiling and the effect of chemical and hydrothermal pre-treatment were intersecting (Figure 5). Applying HT, CT and CCT pre-treatments to the co-ensiled samples did not increase the BMP significantly ($p > 0.05$). The co-ensiled sugar beet root and straw (8 months), 80:20, had a BMP of 298.6 ± 18.0 NL CH₄ kg⁻¹ VS, while pre-treatment of co-ensiled sugar beet root and straw, 80:20, with HT pre-treatment provided a BMP of 310.5 ± 8.3 NL CH₄ kg⁻¹ VS, with CT pre-treatment providing a BMP of 298.2 ± 24.9 NL CH₄ kg⁻¹ VS, and with CCT pre-treatment providing a BMP of 312.6 ± 2.6 NL CH₄ kg⁻¹ VS (Table 4). An effect of 4.6% was achieved by applying combined chemical and hydrothermal pre-treatment to the co-ensiled sugar beet root and straw (8 months), 80:20. Additionally, an effect of 34.0% was achieved by applying the combined chemical and hydrothermal pre-treatment to an un-ensiled mixture of sugar beet root and straw, 80:20, which had an estimated BMP of 233.26 NL CH₄ kg⁻¹ VS.

These results indicate that further pre-treatment of co-ensiled samples provided only a low effect. It could be beneficial to replace expensive and energy-consuming pre-treatments with a co-ensiling pre-treatment, which was cheaper in relation to energy and chemicals while providing a familiar traditional method for storing the crops. Additionally, the results indicated that a similar altering in the structure and physiochemical composition would occur when either co-ensiling or hydrothermal pre-treatment were conducted.

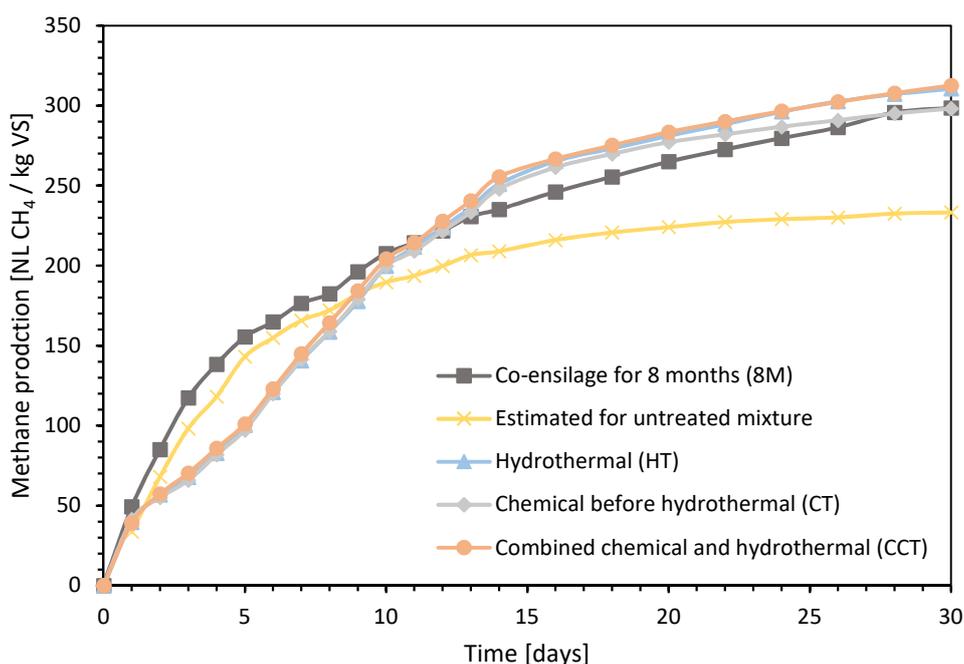


Figure 5. Cumulative methane yields of the co-ensiled sugar beet root and straw (80:20), hydrothermal- (HT), chemical- before hydrothermal- (CT), combined chemical- and hydrothermal- (CCT) pre-treated straw. Additionally, as a reference of the effect, the estimated biochemical methane potential (BMP) of the co-ensiling mixture, based on the BMP in the pure un-ensiled biomasses, was also added.

3.5. Surface Topography Analysis of Pre-Treated Straw

The physicochemical and structural changes from the different pre-treatment methods on the surface topography was studied by using SEM microscopy in the magnitude of 10–100 μm (Figure 6). The surface of the wheat straw was characterized by a compact, ordered and smooth surface structure with a display of the cell walls in the straw, which have been reported to have a diameter of 3.96 μm (Figure 6a) [52]. Additionally, the surface of the straw was encased in a thick cuticular waxy layer, which has been reported by other studies to decrease the biodegradability of the biomass, because it would reduce the accessibility to the surface area of cellulose [53].

A distinct difference was observed in the microstructure of the surface after both CCT pre-treatment and co-ensiling. The cell walls had become fractured, cracked and loosened, and the cuticular waxy layer was removed (Figure 6b,c). As such, the opened structure during the co-ensiling and CCT pre-treatment provided a higher accessible surface area that could undergo hydrolysis and be digested, which may partly be the cause for the increment in the CH_4 yield and biodegradability after pre-treatment. Similar disruptions in the surface structure were reported by Zheng et al. after both alkali, acid and hot water pre-treatment of wheat straw [54] and by Barman et al. after alkali pre-treatment on wheat straw [55].

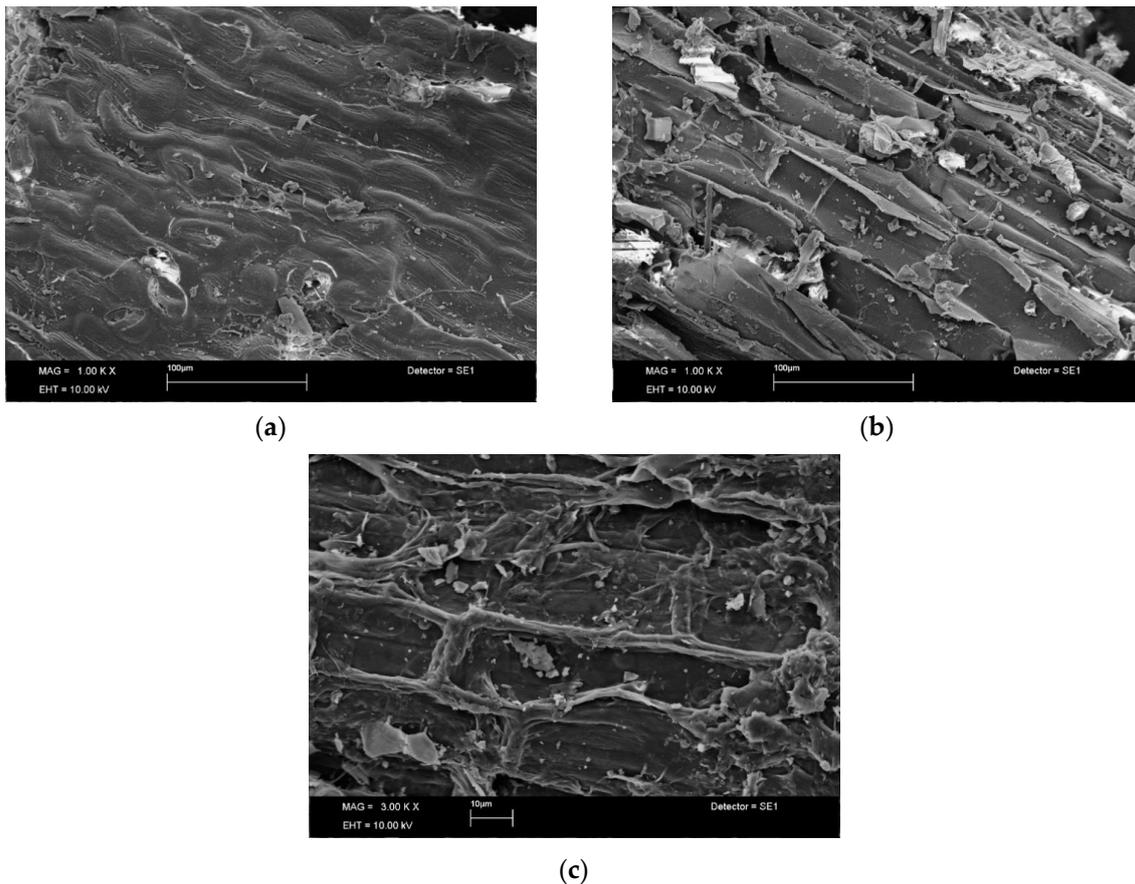


Figure 6. SEM of (a) un-ensiled wheat straw; (b) co-ensiled wheat straw (from the 80:20, sugar beet root: wheat straw, samples co-ensiled for 6 months); (c) wheat straw pre-treated with hydrothermal pre-treatment at 121 °C/1.2 bar for 30 min and chemically pre-treated with tannery wastewater in 24 h at 37 °C.

4. Conclusions

This study demonstrated co-ensiling as a combination of storage and pre-treatment for lignocellulosic biomasses. Co-ensiling of wheat straw and sugar beet roots induced structural changes in the straw, which increased the biodegradability and BMP of the biomasses. Additionally, the initial dry matter concentration was found to be the most important factor to suppress *Clostridium* fermentations, which caused dry matter losses. In comparison, a mechanical pre-treatment was found to increase the hydrolysis rate of straw. However, when combining hydrothermal and chemical pre-treatment, both the hydrolysis rate and the BMP increased significantly. Similar trends in BMP, productivity and surface morphology were observed for both the co-ensiling pre-treatment and the conventional hydrothermal and chemical pre-treatments. As such, these results highlight that co-ensiling had an overlapping effect with hydrothermal and chemical pre-treatments. Co-ensiling could therefore be an alternative to energy dense pre-treatments of lignocellulosic biomasses with low hydrolysis. However, further studies on pilot scale level are required to clarify the effect of the co-ensiling, to evaluate and improve the ensiling duration and to create an economical assessment to increase the feasibility of this technology.

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