Exploring the Biomethane Potential of Different Industrial Hemp (*Cannabis sativa* L.) Biomass Residues

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Abstract: Industrial hemp stands out as a promising candidate for clean and sustainable biomass-to-bioenergy systems due to its multipurpose, high biomass yield and resource efficiency features. In this study, different hemp biomass residues (HBRs) were evaluated as a potential feedstock for renewable biomethane production through anaerobic digestion (AD). The biochemical methane potential (BMP) of the raw and pretreated fibers, stalks, hurds, leaves and inflorescences was investigated by means of batch anaerobic tests. The highest BMP was obtained with the raw fibers (i.e., 422 ± 20 mL $CH_4 \cdot g$ VS^{-1}), while hemp hurds (unretted), making up more than half of the whole hemp plant dry weight, showed a lower BMP value of 239 ± 10 mL $CH_4 \cdot g$ VS^{-1}. The alkali pretreatment of unretted hurds and mechanical grinding of retted hurds effectively enhanced the BMP of both substrates by 15.9%. The mix of leaves and inflorescences and inflorescences alone showed low BMP values (i.e., 118 ± 8 and 26 ± 5 mL $CH_4 \cdot g$ VS^{-1}, respectively) and a prolonged inhibition of methanogenesis. The latter could be overcome through NaOH pretreatment in the mix of leaves and inflorescences (+28.5% methane production).

Keywords: anaerobic digestion; hemp; hurds; inflorescences; pretreatment

1. Introduction

Bioenergy and food security are strongly interlinked and globally relevant issues, both relying on limited resources such as arable land, fresh water and chemical fertilizers [1–3]. As a matter of fact, the production of first-generation biofuels to replace fossil fuels with geologically recent carbon (i.e., the carbon fixed into crop biomass) is challenging food security, generating concerns over the long-term sustainability of such an approach [4]. With the aim of rebalancing the energy–food nexus, non-food crops and agricultural residues have been proposed as a potential feedstock for the sustainable implementation of second-generation biorefineries [5,6]. In such context, the rediscovery of versatile and resource efficient crops such as industrial hemp (*Cannabis sativa* L.) offers the opportunity to reconcile agricultural food and bio-commodity supply with bioenergy production.

After almost ceasing in the second half of the 20th century, hemp production has been steadily increasing in the last decades thanks to the diffusion of its industrial variety, characterized by low concentrations of the psychotropic substance delta-9 tetrahydrocannabinol [7]. The high biomass productivity (up to 20 tons dry matter per hectare), together with the potential supply of seeds, fibers and other biobased raw materials to fit multiple applications is driving the rise of industrial hemp production [8–10]. Although hemp has thousands of applications, each specific production chain generates high amounts of hemp biomass residues (HBRs), which are often regarded as waste [11].

Indeed, the cultivation of specific hemp varieties is usually oriented to maximize the production of one or more portion of the plant (e.g., seeds, fibers or inflorescences). Consequently, the remaining plant biomass, less suited for the production of other bioproducts due to low quality and quantity, is usually wasted [12]. Even in the case of the dual-purpose production of hemp for seeds and fibers, hemp hurds (the inner lignocellulosic portion of hemp stalk), amounting to 52% (*w/w*) of the whole plant dry biomass, is most often discarded together with the leaves (Figure 1). Moreover, due to a contrasting legislation in some European countries like Italy, the commercialization of the plant components such as leaves and inflorescences has stopped, increasing the amount of residual biomass generated by hemp cultivation [13]. In view of the steep growth of the global industrial hemp market, the value of which is projected to increase six-fold by reaching 26.6 billion USD in 2025, HBRs will accumulate in ever larger quantities [14].
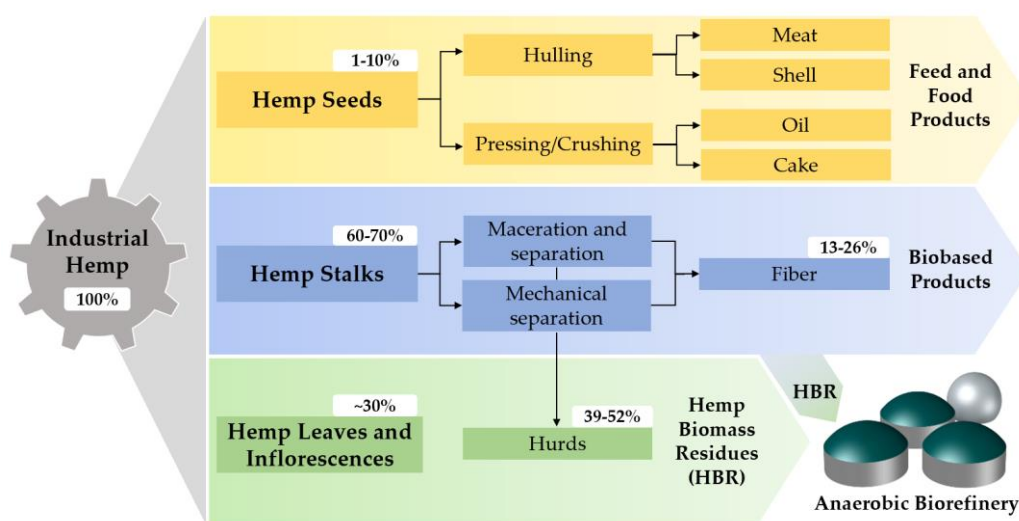
Figure 1. Breakdown of industrial hemp plant components, products and potential uses. Percentage values relate to the typical composition of the industrial hemp plant in terms of the dry weight of the whole plant biomass. Values for fibers (expressing the total of primary and secondary fibers) and hurds were calculated by considering an average of 65% of stalk dry weight [15]. Adapted from Ingrao et al. [7].

A sustainable alternative to valorize the high carbon and energy content of largely available HBRs is offered by anaerobic digestion (AD). Nevertheless, the recalcitrant nature of HBRs as lignocellulosic materials (LMs) restrains the enzymatic hydrolysis, thereby limiting the AD performances. As a consequence, biological, physical or chemical pretreatments are required to increase both the rates and the yields of biomethane production from LMs [16–18]. In the last decade, the AD of industrial hemp has been the focus of several research works, which investigated the biomethane potential of the whole hemp plant as well as of hemp straw residues at both the lab and pilot scale [19–21]. In regard to hemp biomass pretreatment, the use of biological, enzymatic additives as a mean to increase the hydrolysis rate and enhance the biomethane production of hemp straw residues has been recently evaluated [21]. Physical pretreatments such as milling or steam treatment have also been employed to ameliorate the AD performances of hemp biomass [22]. Finally, chemical pretreatments have been widely investigated for the manufacturing of biofuels or biopolymers from hemp [23,24], but not yet for biomethane production.

The present study goes beyond the past research works performed on the whole hemp plant biomass by assessing the biomethane potential (BMP) of individual and multiple, potential HBRs: stalks, fibers, hurds (unretted) and leaves and inflorescences. Moreover, chemical and mechanical pretreatments were tested with the aim to investigate their effect on the AD of HBRs. Batch BMP tests were performed with untreated, NaOH- and H_2SO_4-pretreated (chemical pretreatments) and

ground (mechanical pretreatment) HBRs. Dilute alkali (i.e., NaOH) and acid (i.e., H_2SO_4) chemical pretreatments were chosen for their low capital and operational costs as well as for their ease of supply. In addition to biomethane production, the time evolution of the volatile fatty acids (VFA) concentration was also monitored under the different experimental conditions to evaluate the potential buildup of AD intermediate compounds.

2. Materials and Methods

2.1. Raw Hemp Biomass

The hemp biomass used in this study originated from an industrial hemp cultivation of *Cannabis sativa* L., cultivar "Eletta Campana", located in the Campania region (Italy). Eletta Campana is a widespread cultivar in southern Italy and was chosen due to its ability to produce high amounts of biomass, seeds and inflorescences. The hemp was cultivated from April to August on a one-hectare agricultural field, without the use of chemical fertilizers. The cultivation was aimed at the production of inflorescences, which could not be further harvested and processed due to the enactment of a law which banned their commercialization in Italy [13]. The samples of stems and a mix of leaves and inflorescences were randomly and separately collected from different parts of the hemp plants during the harvest performed in 2019. The collected samples were stored into closed plastic bags at room temperature (21 °C) to serve as a source of HBRs. Pure Eletta Campana inflorescences produced by another hemp cultivation located in the same region were collected and stored under the same conditions.

2.2. Hemp Biomass Residues Processing

Once in the lab, the hemp biomass was further processed to separate hurds and fibers from stalks by reproducing the two main processes used for fiber production: the separation from retted stems (after maceration) and the direct mechanical separation from unretted stems (Figure 1) [25]. Retting is the process of soaking hemp stalks in water, or leaving them on the field under the action of dew, in order to allow the microbial degradation of the bonding between the hurds and fibers [26]. For this study, retted stems were obtained through maceration into tap water for 6 days. Subsequently, the fibers and hurds were separated manually and placed to dry at 45 °C for 72 h in a TCN 115 laboratory oven (Argo Lab, Modena, Italy). The mechanical separation of the fibers and hurds from unretted stems was instead performed with the aid of a cutter. Prior to any pretreatment or AD test, all HBRs were ground and sieved (fibers and hurds) or directly sieved (mix of leaves and inflorescences) to isolate the fraction between 2 and 4 mm. The characteristics and the pictures of the HBRs used in this study are reported in Table 1 and Figure 2, respectively.

Table 1. Characterization of the anaerobic inoculum and the untreated and pretreated HBRs. TS: total solids; VS: volatile solids; N–NH$_4^+$: ammonium nitrogen. Values for the inoculum are given as the range of the mean values measured on the anaerobic digestate used for the biochemical methane potential (BMP) tests. Values for the HBRs are expressed as the mean and standard deviation calculated on three replicates.

Substrate	Parameter	Condition			
		Untreated	Alkaline Pretreated (NaOH) [a]	Acid Pretreated (H$_2$SO$_4$) [b]	Mechanically Pretreated (Grinding) [c]
Inoculum	TS (%)	6.65–7.27	n.a.	n.a.	n.a.
	VS (%)	4.51–5.20			
	N–NH$_4^+$ (g N–NH$_4^+$/L)	0.86–1.12			
	pH	7.6–7.8			
Fibers	TS (%)	89.33 ± 0.18	n.a.	n.a.	n.a.
	VS (%)	87.43 ± 1.90			
Inflorescences	TS (%)	87.19 ± 0.55	n.a.	n.a.	n.a.
	VS (%)	79.44 ± 0.55			
Stalks	TS (%)	89.93 ± 0.44	93.14 ± 0.64	n.a.	n.a.
	VS (%)	85.77 ± 0.50	89.54 ± 0.17		
Unretted hurds	TS (%)	89.88 ± 0.04	94.25 ±1.76	n.a.	n.a
	VS (%)	88.38 ± 0.07	90.57 ±1.71		
Retted hurds	TS (%)	89.66 ± 0.19 *	92.03 ±1.16	96.66 ± 0.12	89.66 ± 0.19
	VS (%)	85.77 ± 0.50 *	88.68 ± 0.35	95.95 ± 0.06	85.77 ± 0.50
Mix of leaves and inflorescences	TS (%)	89.07 ± 0.18	92.03 ± 0.54	92.39 ± 0.54	89.07 ± 0.18
	VS (%)	71.61 ± 0.88	72.14 ± 0.48	79.80 ± 0.48	71.61 ± 0.88

[a] 1.6% (*w/w*) NaOH, incubation at 30 °C for 24 h; [b] 1% H$_2$SO$_4$, incubation at 121 °C for 1.5 h; [c] Particle size: 1–2 mm; * These values are taken from the study of Papirio et al. [27].

Figure 2. Pictures showing the different HBRs used in this study: stalks (**a**), the unretted hurds (**b**), the retted hurds (**c**), the fibers (**d**), the inflorescences (**e**) and the mix of leaves and inflorescences (**f**).

2.3. Chemical and Mechanical Pretreatments of Hemp Biomass Residues

To enhance the enzymatic hydrolysis of HBRs, chemical and mechanical pretreatments were performed prior to the BMP tests. Table 1 shows the various pretreatments used for each of the HBRs tested. The two chemical pretreatments were aimed at breaking apart the compact lignocellulosic structure by removing the lignin barrier and modifying the crystalline structure of cellulose. The mechanical pretreatment was instead aimed at increasing the surface area of the substrate.

An alkaline pretreatment was performed on hemp stalks, retted and unretted hurds and on the mix of leaves and inflorescences by using a diluted 1.6% (*w/w*) NaOH (98% purity, Merck, Darmstadt, Germany) solution. An amount of 16 grams of each of the substrates was mixed with 100 mL of the NaOH solution and placed at 30 °C for 24 h [28] in a TCN 115 laboratory oven (Argo Lab, Italy). An acid pretreatment was carried out on retted hurds and on the mix of leaves and inflorescences by using a diluted 1% H_2SO_4 (95% purity, VWR, Radnor, USA) solution and a total solid (TS) concentration of HBR of 7% (i.e., 7 g TS of HBR per 100 mL of H_2SO_4 solution) [29]. Therefore, 12 grams of retted hurds and 25 grams of the mix of leaves and inflorescences were placed in 153 and 318 mL of H_2SO_4 solution, respectively. The mixture of substrate and acid solution was kept at 121 °C for 1.5 h [30] in the oven. Upon the completion of each chemical pretreatment, the HBRs were distributed on a filter paper and washed with tap water until the eluate pH achieved values between 7.0 and 7.5. Subsequently, the washed substrates were dried at 45 °C for 72 h in the oven prior to being characterized in terms of total (TS) and volatile (VS) solids and used for the BMP tests. The mechanical pretreatment was performed on retted hurds and on the mix of leaves and inflorescences by further grinding and then sieving the substrates to obtain the fraction between 1 and 2 mm.

2.4. Biochemical Methane Potential Tests

The BMP tests of untreated and pretreated HBRs were performed in 100 mL serum bottles (Wheaton, USA) placed in a thermostatic water bath to guarantee a mesophilic temperature range, i.e., 37 ± 1 °C. As a source of anaerobic inoculum, a digestate from a full-scale AD digester treating cow and buffalo manure, located in the Campania region (Italy), was used. The physical–chemical characteristics of the anaerobic inoculum are reported in Table 1. Prior to being employed in the BMP tests, the inoculum was placed at 37 ±1 °C for 2 days. An amount of 40 grams of inoculum was poured into each bottle, and a corresponding quantity of HBRs was added to maintain an inoculum to substrate ratio of 2 g VS·g VS^{-1}. A final, constant, working volume of 50 mL was set in all the experiments by adding deionized water. The headspace of each bottle was flushed with analytical grade nitrogen gas (Rivoira, Milano, Italy) and sealed with a rubber septum and an aluminum crimp. Control bottles with inoculum and deionized water were also set to evaluate the biomethane production of the sole inoculum. The latter was used to calculate the net biomethane production of HBRs by subtracting it from the values measured in the other BMP tests.

2.5. Analytical Methods and Sampling

Biomethane production was monitored over a 42-day period and quantified by means of a volumetric displacement system, consisting of a 12% NaOH carbon dioxide trap (400 mL) and a biomethane collection vessel (400 mL) containing deionized water to be displaced. Samples of the digestate were withdrawn six times in the first two weeks for the quantification of VFAs and immediately frozen at −20 °C. Prior to the VFA analysis, the samples were centrifuged with a Multispin 12 mini centrifuge (Argo Lab, Italy) at 10,000 rpm for 5 min, and the supernatant was diluted with deionized water and filtered through 0.20 µm polypropylene membranes (VWR, Italy). The procedures here used for TS and vs. determination as well as the VFA measurement protocol and equipment were reported by Bianco et al. [31].

2.6. Statistical Analysis

All BMP tests were run in triplicate, and the experimental data were expressed as the mean ± standard error. The statistical significance of the differences between the biomethane production measured with the untreated and pretreated HBRs was assessed through one-way ANOVA. A value of $p \leq 0.05$ was considered to be statistically significant. The Microsoft Excel (version 1908) (Office 365, Microsoft Corporation, Redmond, USA) statistical package was used to perform the statistical analysis.

3. Results and Discussion

3.1. Biomethane Potential of Untreated Hemp Biomass Residues

The specific cumulative biomethane production of the untreated HBRs is reported in Table 2. Among the substrates tested in this study, the untreated hemp fibers showed the highest BMP value, reaching a final biomethane production of 422 ± 20 mL $CH_4 \cdot$g VS^{-1}. The latter was 53% higher than the 275 ± 7 mL $CH_4 \cdot$g VS^{-1} produced by the untreated stalks, and about 76% higher than the 239 ± 10 mL $CH_4 \cdot$g VS^{-1} produced by the unretted hurds. The mix of leaves and inflorescences achieved a lower BMP of 118 ± 8 mL $CH_4 \cdot$g VS^{-1}, while the inflorescences alone produced only 26 ± 5 mL $CH_4 \cdot$g VS^{-1}.

Table 2. Mean specific final cumulative biomethane production (mL $CH_4 \cdot$g VS^{-1} of HBRs added) measured through the BMP tests on the untreated and pretreated HBRs. Values are expressed as the mean and standard deviation calculated on three replicates. The percentage values within brackets represent the cumulative biomethane production surplus or deficit of pretreated substrates against that of the untreated ones.

HBRs	Condition			
	Untreated	**Alkaline (NaOH) [a]**	**Acid (H_2SO_4) [b]**	**Mechanical (Grinding) [c]**
Fibers	422 ± 20	n.a.	n.a.	n.a.
Inflorescences alone	26 ± 5	n.a.	n.a.	n.a.
Stalks	275 ± 7	249 ± 33 [ns] (-9.5%)	n.a.	n.a.
Unretted hurds	239 ± 10	277 ± 13 * $(+15.9\%)$	n.a.	n.a.
Retted hurds	242 ± 13 [d]	255 ± 1 [ns] $(+5.7\%)$	245 ± 13 [ns] $(+1.3\%)$	280 ± 4 * $(+15.9\%)$
Mix of leaves and inflorescences	118 ± 8	151 ± 8 * $(+28.5\%)$	98 ± 10 [ns] (-16.6%)	118 ± 17 [ns] $(+0.3\%)$

[a] 1.6% (*w/w*) NaOH, incubation at 30 °C for 24 h; [b] 1% H_2SO_4, incubation at 121 °C for 1.5 h; [c] Particle size: 1–2 mm; [d] These values are taken from the study of Papirio et al. [27]; [ns] Not statistically significant: *p*-value > 0.05; * Statistically significant: *p*-value ≤ 0.05; n.a.: Not applicable.

In recent years, a limited number of studies reported the BMP of hemp leaves and stalks [22,32] and more recently, also that of retted hurds [27]. To the authors' best knowledge, this is the first scientific report characterizing the BMP of each specific portion of the hemp plant: fibers and hurds (unretted), the mix of leaves and inflorescences as well as the inflorescences alone. The high biomethane of hemp fibers is in agreement with the already reported predominance of cellulose (57–77%) and hemicellulose (9–14%) over lignin (5–9%), as compared to the more bio-recalcitrant woody core of the stalk, i.e., hurds, having a higher lignin content (19–30%) [33,34]. Despite their high BMP, fibers make up only a small portion of the whole hemp plant, ranging between 13 and 26% (*w/w*) of the plant dry biomass (see Figure 1). Moreover, once separated from the stalk, they represent a valuable source of natural fibers for the bio-based industry. Given the above, hemp fibers were not tested further in this study.

Concerning the other HBRs, the biomethane production obtained with the Eletta campana stalks in this study is higher than that achieved with Futura 75 cultivar by Kreuger et al. [22], who reported

a BMP value of 207 mL CH_4·per gram of finely ground (<1 mm) stalk dry matter (DM). If a VS/TS ratio of 95% (Table 1) is used to better compare the value obtained by Kreuger et al. [22], the resulting 218 mL CH_4·g VS^{-1} is still approximately 20% lower than that observed in this study.

In the case of hemp hurds, the fiber separation process did not influence the AD performance in terms of the final biomethane production (Table 2). In one of our recent studies, the AD of untreated retted hurds showed, in fact, a final BMP value of 242 ± 13 mL CH_4·g VS^{-1} which is in line with the one obtained with unretted hurds in this study [27]. Overall, the BMP of the hemp hurds is in accordance with those reported for the AD of the whole hemp plant biomass (i.e., stalks, leaves and inflorescences). Adamovičs et al. [32] obtained a BMP value of 216 and 246 mL CH_4·g VS^{-1} for finely ground (1–5 mm) hemp biomass from USO 31 and Futura 75 cultivars, respectively. Cumulative biomethane productions ranging between 259 and 301 mL CH_4·g VS^{-1} for Fedora19 cultivar were instead obtained by Heiermann et al. [19]. Finally, Kreuger et al. [20] reported a BMP value of 234 mL CH_4·g VS^{-1} for the Futura 75 biomass digested under thermophilic conditions (i.e., 50 °C).

Figure 3A shows the cumulative biomethane profiles obtained with the untreated mix of leaves and inflorescences. The low BMP values obtained with this specific HBR is not directly supported by the previously published data. Adamovičs et al. [32] reported biomethane yields as high as 365 mL CH_4·g VS^{-1} for USO31 leaves, outscoring the BMP measured for the whole hemp plant biomass. As pertinently observed by the authors, leaves alone should be less recalcitrant to hydrolysis and hence, this should result in higher biomethane yields. Although not as high, the BMP values of 200 mL CH_4 per g DM^{-1} (i.e., 250 mL CH_4 per g VS^{-1}, considering a 80% vs. content) reported by Kreuger et al. [22] for Futura 75 leaves is about the double of that observed in this study.

The poor biomethane potential here observed with the mix of leaves and inflorescences can possibly be explained with the presence of a consistent amount of hemp inflorescences, which were harvested and mixed together with leaves. Inflorescences alone, in fact, showed an extremely low biomethane production (Figure 3A), and almost completely inhibited methanogenesis. After a net positive biomethane production of 26 ± 5 mL CH_4 g VS^{-1} obtained in the first two days (see final cumulative biomethane production in Table 2), the methanogenesis in the presence of inflorescences showed a strong and unrecoverable inhibition for the whole duration of the BMP test. This led to a 78% lower cumulative biomethane production as compared to that of the untreated mix of leaves and inflorescences.

The potentially inhibiting effect of apolar extractive compounds from hemp biomass has been previously discussed by Kreuger et al. [20]. The lag phase and transient methanogenic inhibition in early harvested hemp observed by those authors was explained with the possible presence of volatile terpenes, which normally accumulate in wood resin [35]. The adverse effects of terpenoids over AD were already investigated in a previous study, reporting a 95% decrease in the biomethane production in the presence of 0.5% terpenoids from fruit flavors [36]. Hemp inflorescences, and leaves to a lower extent, are rich in resinous compounds, and more than 150 terpenes (mono- and sesquiterpenes) have been identified in hemp resin [37]. In view of the above, the high concentration of such compounds in inflorescences may have thus been responsible for the inhibition of the methanogenesis observed in this study. Overall, this finding suggests that resin-containing leaves and inflorescences are not a suitable AD substrate.
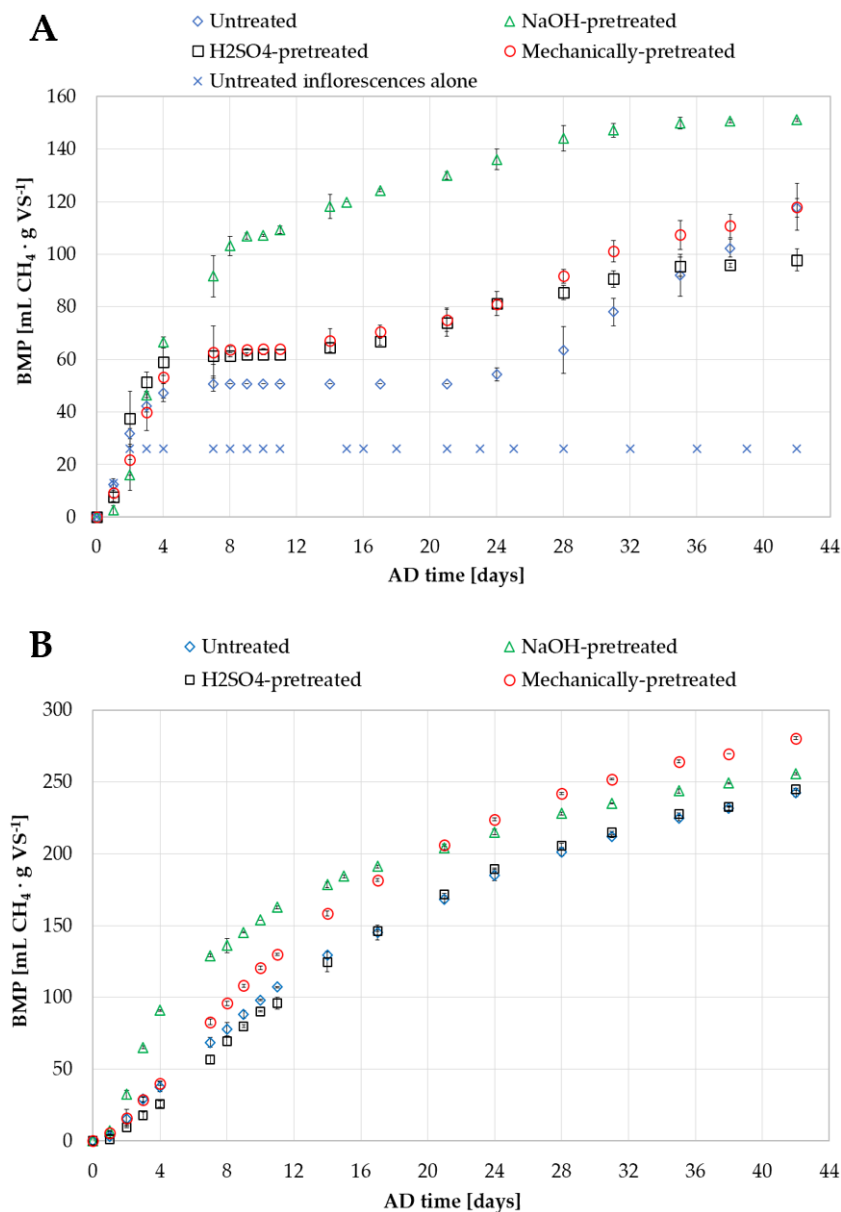
Figure 3. (**A**): Evolution of the cumulative biomethane profiles obtained with the untreated (◇), NaOH-pretreated (△), H_2SO_4-pretreated (□) and mechanically pretreated (○) mix of leaves and inflorescences. The cumulative biomethane profile obtained with the untreated inflorescences alone is also shown (×). (**B**): Cumulative biomethane profiles obtained with the NaOH-pretreated (△), H_2SO_4-pretreated (◇) and mechanically pretreated (○) retted hurds. The cumulative biomethane profile of untreated retted hurds (□) refers to that already reported by Papirio et al. [27]. The lines on the symbols indicate the standard error bars. AD time: anaerobic digestion time.

3.2. Effect of Alkaline, Acid and Mechanical Pretreatment on the Biomethane Potential of Hemp Biomass Residues

The final, cumulative biomethane production obtained with chemically and physically pretreated HBRs is shown in Table 2. Figure 3 displays the evolution of biomethane production observed with the pretreated mix of leaves and inflorescences (Figure 3A) and with pretreated retted hurds (Figure 3B).

The dilute alkaline NaOH-pretreatment significantly enhanced the BMP of the mix of leaves and inflorescences (+28.5%) (Table 2 and Figure 3A) and of unretted hurds (+15.9%) (Table 2) (*p*-values: 0.0403 and 0.0312). A 5.7% biomethane production increase was observed with the NaOH-pretreated retted hurds (Table 2 and Figure 3B), while the same pretreatment caused a 9.5% decrease in the BMP

of the pretreated stalks (Table 2). The latter differences were, however, not statistically significant (*p*-values: 0.2238 and 0.3380).

The chemical pretreatments have been widely applied to all types of LMs, aiming at altering the structural characteristics that limit the enzymatic hydrolysis and consequently, the overall biomass digestibility [17]. Alkaline pretreatments are known to increase cellulose digestibility mainly by solubilizing the lignin fraction of LMs, while pretreatments based on acidic solutions are more effective towards hemicellulose dissolution [38]. Khattab and Dahman [24] reported a higher efficiency of NaOH- and H_2SO_4-based pretreatments on hemp hurds. This study, investigating the polyhydroxybutyrate (PHB) production from carbohydrates recovered through hemp hurds' pretreatment, showed that a 2% NaOH pretreatment for 1 h at 135 °C decreased the lignin content of hurds to 4.0 ± 0.4%. In the same work, pretreating hurds with a 2% H_2SO_4 solution resulted in a residual lignin content as high as 15.0 ± 0.5%. The above discussed data seem to support the findings of this study, although the alkali pretreatment used here had a longer duration (24 h) and was carried out at milder temperatures (30 °C). In fact, a high lignin solubilization could justify the enhancement of the BMP observed for the NaOH-pretreated unretted hurds. The positive effect of the alkaline pretreatment on the AD of the mix of leaves and inflorescences points instead towards a potential removal of inhibitory compounds. The latter observation is supported by the different cumulative biomethane profile (Figure 3A) obtained with the NaOH-pretreated mix of leaves and inflorescences, showing no sign of methanogenic inhibition as compared with the other experimental conditions. Conversely, the NaOH-pretreated hemp stalks showed lower BMP values. Despite being not statistically significant, this negative trend was read as a possible loss of highly biodegradable sugars from the outer fiber layer surrounding the stalk. Therefore, pretreatments were not further tested with this HBR.

The dilute H_2SO_4-pretreatment led to a 16.6% lower biomethane production for the mix of leaves and inflorescences (*p*-value: 0.0906) (Table 2 and Figure 3A) and a 1.3% higher BMP for retted hurds (*p*-value: 0.8246) (Table 2 and Figure 3B), which were not significantly different from the BMP of the untreated substrates. Although, as already mentioned, acid pretreatments are known to solubilize hemicellulose and remove more sugars than alkali pretreatments [38], so no further conclusion can be drawn on the basis of the data here discussed as no significant difference with the untreated substrates was observed.

Finally, the mechanical pretreatment through grinding enhanced the biomethane production of retted hurds by 15.9% (*p*-value: 0.0177) (Table 2 and Figure 3B). This is in line with the 15.6% BMP enhancement measured by Kreuger et al. [22] on milled hemp stalks (<1 mm size). Contrarywise, the mechanical grinding did not affect the BMP of the mix of leaves and inflorescences. Only a 0.3% increase in the biomethane production was obtained, which was not statistically significant (*p*-value: 0.9787) (Table 2 and Figure 3A). The latter can probably be explained with the structural composition of this substrate, which is already characterized by a high specific surface area and, thus, does not benefit from additional grinding.

3.3. Evolution of Volatile Fatty Acids

The production, accumulation and degradation of VFAs during the AD of the various untreated and pretreated HBRs was also investigated in this study. VFA evolution was monitored up to day 14 by measuring the concentration of acetic, propionic, iso-butyric, butyric and iso-valeric acids. The sum of the latter, expressed as a concentration of acetic acid equivalent (mg HAc·L^{-1}), was used to express the total VFA concentration and compare the results from the different operational conditions (Figure 4). On average, more than 95% (*w/w*) of the detected total VFAs were made up by acetic, propionic, iso-butyric and butyric acids in all the experiments (data not shown).
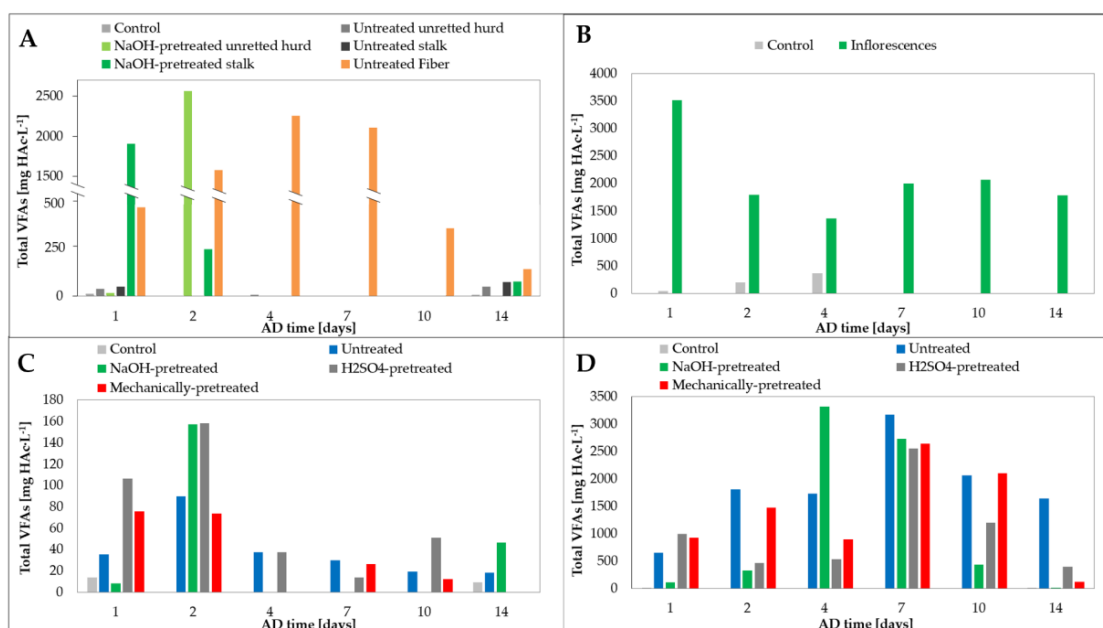
Figure 4. Evolution of the total volatile fatty acids observed during the anaerobic digestion of the untreated unretted hurds, stalks and fibers and of the NaOH-pretreated unretted hurds and stalks (**A**), the untreated inflorescences (**B**), the NaOH-, H_2SO_4- and mechanically pretreated retted hurds (**C**) and the untreated, NaOH-, H_2SO_4- and mechanically pretreated mix of leaves and inflorescences (**D**). The volatile fatty acids concentrations of the untreated retted hurds shown in panel C refer to those already reported in the study of Papirio et al. [27].

Among the tested hemp substrates, the highest VFA accumulation of approximately 3.5 (Figure 4B), 3.3 and 3.2 g HAc·L^{-1} (Figure 4D) was observed with the untreated inflorescences, NaOH-pretreated and untreated mix of leaves and inflorescences, respectively. Compared to the NaOH-pretreated substrates, where the VFAs were completely converted to biomethane by day 14, more than 1.6 g HAc·L^{-1} was observed with the untreated mix of leaves and inflorescences after two weeks of operation. Accordingly, the cumulative biomethane production measured on day 14 for the NaOH-pretreated mix of leaves and inflorescences was about 230% higher than the corresponding untreated substrate, which recovered the methanogenic activity only after day 24 (Figure 3A). This emphasizes how the NaOH pretreatment did not improve the final BMP value through an accelerated hydrolysis, but rather by decreasing the extent of methanogenic inhibition, likely through the solubilization and removal of part of the resinous compounds from the leaves and inflorescences. For the same substrate, the cumulative biomethane profiles obtained after the H_2SO_4-based and mechanical pretreatments (Figure 3A) indicate a recovery of the methanogenic activity already on day 14. This is confirmed by the lower residual VFA concentrations of around 0.4 and 0.1 g HAc·L^{-1} (Figure 4D), respectively.

Although the monitoring of VFA evolution over time during the AD of hemp was not performed in previous studies, similarly high VFA concentrations were observed by Kreuger et al. [20]. In their study on the effect of harvest time on the AD of hemp, the only VFA measurement performed by the authors allowed to observe up to 3.5 g HAc·L^{-1} in BMP tests with early harvested Futura 75 hemp biomass. The accumulation of VFAs and the inhibition of acetoclastic methanogens in early harvested hemp biomass was explained by the presence of potentially toxic extractive compounds, which concentrate in higher quantities in leaves and inflorescences (see Section 3.1). This observation finds evidence in the high and persistent accumulation of VFAs (Figure 4D), concurring with the strong inhibition of methanogenesis observed with the untreated inflorescences tested in this study (Figure 3A). These data point once more towards a possible inhibitory effect on AD from resinous-rich hemp substrates. In view of the past indications and the findings presented herein, future research

should be done to shed light on the origins and the extent of the inhibitory effects that hemp extracts from leaves and inflorescences have on methanogenic microorganisms.

Apart from leaves and inflorescences, also untreated fibers, NaOH-pretreated unretted hurds and stalks reached VFA concentrations of about 2.3, 2.6 and 1.9 g HAc·L^{-1} (Figure 4A), respectively. Although 2.0 g HAc·L^{-1} is generally recognized as the threshold concentration above which VFA accumulation starts to negatively affect methanogenic activity [39,40], none of the aforementioned substrates showed actual signs of inhibition. This might be due to the high alkalinity of the anaerobic inoculum (>12 g CaCO$_3$·L^{-1}), which allowed to keep a low amount of protonated VFAs by sustaining pH values above 7 [20]. Overall, the high VFA accumulation observed for the untreated fibers is in line with the lower lignin and high cellulose content of such substrate. Similarly, the enhancement of biomethane production obtained through alkaline NaOH pretreatment on unretted hurds is matched by the high VFA accumulation observed on day 2, pointing towards a higher hydrolysis rate and, hence, acidogenic activity. NaOH-pretreated stalks, showing higher VFA concentrations as compared to the untreated substrate, confirm the positive effect of the alkaline pretreatment on the hydrolysis rate, although the final BMP value was similar to that of the untreated substrate (*p*-value > 0.05) (Table 2).

Finally, the VFA concentrations measured with pretreated retted hurds did not exceed 160 mg g HAc·L^{-1} (Figure 4C). Compared to the NaOH-pretreated unretted hurds, the same pretreatment prompted an approximately 16 times lower VFA accumulation with retted hurds. Overall, the lower VFA levels, observed even in the presence of the BMP enhancement obtained with mechanical grinding, indicate that hydrolysis still restrains acidogenesis rates with retted hurds. As further evidence of this, the synergistic effects obtained during the co-digestion of retted hemp hurds and cheese whey, as reported in one of our recent studies, was most likely due to the enhanced enzymatic hydrolysis of the lignocellulosic structure of hurds [27]. In view of the above, future studies on HBRs could aim at investigating the potential of combining technically- and economically-feasible lignocellulosic biomass pretreatments, such as the ones used in this study, with versatile and alternative AD strategies such as co-digestion.

4. Conclusions

This study investigated, for the first time, the biomethane potential of various hemp biomass residues (HBRs). Despite the high BMP (i.e., 422 ± 20 mL CH$_4$·g VS^{-1}), hemp fibers have multiple market applications and are, thus, not primarily destined to AD. However, a yet untapped renewable bioenergy pool was identified in hurds and in the mix of leaves and inflorescences, usually regarded as waste materials. With the aim of unlocking the biomethane potential of such biorecalcitrant substrates, mild chemical and mechanical pretreatments were successfully implemented. To this extent, mechanical grinding allowed to increase the BMP (+15.9%) of untreated retted hurds, which reached 280 ± 4 mL CH$_4$·g VS^{-1}. Major positive effects were obtained with the use of a dilute NaOH pretreatment at ambient temperature. This allowed to improve the biomethane production of raw unretted hurds (i.e., 239 ± 10 mL CH$_4$·g VS^{-1}) and of the mix of leaves and inflorescences (i.e., 118 ± 8 mL CH$_4$·g VS^{-1}) by 15.9 and 28.5%, respectively. The BMP enhancement achieved with the mix of leaves and inflorescences is of particular interest in view of the unprecedented high methanogenic inhibitory effect showed by hemp inflorescences alone. The latter aspect calls for more specific studies.

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