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Potassium Hydroxyde Pre-Treatment Enhances Methane Yield from Giant Reed (Arundo donax L.)

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Abstract: The biogas production through the anaerobic digestion (AD) of giant reed (Arundo donax L.) biomass has received increasing attention. However, due to the presence of lignin, a low CH4 yield can be obtained. Aiming to improve the CH4 yield from giant reed biomass, the effectiveness of a thermo-chemical pre-treatment based on KOH was evaluated in this paper. The usefulness of a washing step before the AD was also assessed. The pre-treatment led to a specific CH4 yield up to 232 mL CH4 g−1 VS which was 21% higher than that from untreated biomass; the maximum daily rate of production was improved by 42%, AD duration was reduced by 10%, and CH4 concentration in the biogas was increased by 23%. On the contrary, the washing step did not improve the AD process. Besides, washing away the liquid fraction led to biomass losses, reducing the overall CH4 production. The use of a KOH-based pre-treatment appears as a good option for enhancing the AD of giant reed, also presenting potential environmental and agronomical benefits, like the avoidance of salty wastewater production and the likely improvement of the digestate quality, due to its enriched K content.

Keywords: alkaline pre-treatment; anaerobic digestion; Arundo donax L.; biogas; giant reed; lignocellulosic biomass; methane

1. Introduction

The biogas produced through anaerobic digestion (AD) is one of the biofuels on which most attention is focused. Anaerobic digestion is considered an environmentally friendly fuel generation technology as it requires a lower energy input than that required for the production of other biofuels [1,2]. Besides, the AD process can rely on the utilization of residual biomass, by-products, or waste. Therefore, in addition to the production of biofuels, AD can contribute to the disposal of these biomasses, and the reduction of the emission of greenhouse gases and other pollutants, generating wastewater that can be used as a high-quality fertilizer [3,4].

The biogas production from lignocellulosic biomasses has received increasing interest due to possible economic and environmental advantages deriving from their use [2]. Lignocellulosic biomasses have a worldwide availability in the order of 1000 Gt per year [2]. Among them, perennial crops can present very high biomass yield and low agronomic input requirements, in terms of fertilizers, tillage, pesticides, and irrigation water [5].

Lignocellulosic biomasses are composed of cellulose, hemicellulose, and lignin; notably, cellulose and hemicellulose are structural carbohydrates that can be converted to CH4 during the AD process [6]. Lignin, on the other hand, is an aromatic polymer with low biodegradability, well suited to resist microbial attacks and oxidative stress [7]. The three constituents of the lignocellulosic biomass are inherently connected, resulting in a substrate
relatively recalcitrant to the AD process. Thus, the pre-treatment of these biomasses is a viable strategy for enhancing the bioconversion process [6].

The AD performances are strictly related to the effectiveness of the pre-treatments, which can be divided into mechanical, thermal, chemical, and biological, and can also be combined [6–8]. Among chemical pre-treatments, alkaline pre-treatments increase the porosity of lignocellulosic materials, dissolving lignin through the solvation and saponification of the ester bonds between lignin and holocellulose, releasing the structural carbohydrates and making them available for AD [9]. The thermal pre-treatment of lignocellulosic biomass has been extensively studied [1]. The combination of thermal and alkali pre-treatments can increase biomass degradation, however, thermo-alkaline pre-treatments can generate microbial inhibitors, such as polyphenols resulting from the degradation of lignin [7,10,11], dissolved in a salty alkaline solution. Commonly, the removal of the inhibitors before the AD process or the enzymatic hydrolysis is made by washing the biomass with concurrent organic matter loss and generation of liquid wastes that are generally discarded [12,13]. Although the washing step is considered necessary, to date, specific studies are lacking.

In recent years, the perennial grass giant reed (Arundo donax L.) has received great attention due to its high biomass yield [14,15]. Despite the high degree of recalcitrance to enzymatic digestibility [16], little is known about the most suitable pre-treatments for this species. One of the best options to improve CH4 yield from giant reed is the pre-treatment with NaOH, followed by a washing step [17]. However, the wastewater generated is poorly suited to agronomic use due to the presence of Na, which may lead to soil degradation in the long term [18]. A KOH-based pre-treatment could be more appropriate mainly because the resulting wastewater could be utilized as fertilizer in agriculture [19]. To date, the production of CH4 from giant reed has been studied by applying hydrothermal pre-treatments at high temperatures (up to 230 °C), or at low temperatures (24–25 °C) with an alkali [12,17,20]; information on the effectiveness of combined thermo-alkaline pre-treatments is still lacking. Notably, the use of KOH to pre-treat giant reed to enhance CH4 yield has never been reported. This study aimed at evaluating the effect of the thermo-chemical pre-treatment of giant reed biomass with KOH on CH4 yield. To our knowledge, this is the first study exploring this combination on giant reed. The actual need for a washing step before AD was also evaluated by comparing the CH4 production in reactors fed with washed or unwashed pre-treated biomass of giant reed.

2. Materials and Methods

2.1. Feedstock

Representative samples of giant reed aboveground biomass were collected in January 2017 from a two-year crop stand at the experimental station of CREA located at Anzola dell’Emilia (Bologna, northern Italy, Lat. 44°32’ N, Long. 11°11’ E, 38 m a.s.l), then they were oven-dried at 65 °C until constant weight (dry matter, DM), milled at 1.5 mm, and sieved to pass a 1 mm screen. Samples were collected and stored at room temperature in plastic bags.

The fiber composition and other quality traits of the dry milled giant reed biomass are reported in Table 1 (see Section 2.7 for the analytical methods).

2.2. Experimental Design

The following treatments were compared: K: 0.135 g KOH g−1 of volatile solids (VS) at 121 °C for 20 min; T: 121 °C for 20 min; Kw: K followed by a washing step, Tw: T followed by a washing step; GR: untreated control. Therefore, the milled and sieved giant reed biomass was hydro-thermally pre-treated with or without KOH. Reactors without KOH were included to estimate the temperature and pressure contribution to the pre-treatment effectiveness. Then, the pre-treated materials were either washed or left unwashed before inoculation and biogas production monitoring.
Table 1. Fiber composition and other quality traits of the dry milled giant reed biomass.

| Trait                  | Mean (SD)   |
|------------------------|-------------|
| Total solids (TS), %FW | 94.81 (0.02) |
| Volatile solids (VS), %FW | 89.62 (1.80) |
| Ash, %FW               | 5.19 (0.01) |
| pH in water            | 5.40 (0.30) |
| Total N, %DM          | 0.39 (0.05) |
| Total C, %DM          | 46.70 (0.60) |
| Total P, %DM          | 0.05 (0.00) |
| C/N, mol/mol          | 142 (21)    |
| Hemicellulose, %DM    | 43.1 (1.5)   |
| Lignin, %DM           | 23.6 (0.8)   |
|                       | 11.4 (0.5)   |

1 Percentage of fresh weight; 2 Percentage of dry matter.

The experiments were performed according to a completely randomized block design with 5 treatments and 3 replicates, for a total of 15 experimental units.

The pre-treatments and the washing step were carried out as described below.

2.3. Pre-Treatments without Washing Step

The K and T pre-treatments were performed in triplicates at 121 °C for 20 min in 100-mL glass reactors closed with rubber stoppers and aluminum seals. The reactors contained an amount of giant reed powder corresponding to 1.00 g VS and 9.00 g of distilled water (T reactors) or 9.00 g of a titrated KOH solution suitable to obtain a final concentration of 0.135 g of KOH per g VS of giant reed (K reactors). The reactors were left closed to cool at room temperature until the inoculation for the AD experiments.

2.4. Pre-Treatments with Washing Step

The Kw and Tw pre-treatments were performed in triplicates at 121 °C for 20 min in 250 mL Erlenmeyer flasks. The flasks contained giant reed powder corresponding to 10.0 g VS and 90.0 g of distilled water or KOH solution at the same final concentration reported above. The flasks were maintained closed and left to cool at room temperature before the washing step. The pre-treated insoluble fractions were separated from the liquid fractions by vacuum filtration (pore size 0.45 µm) and washed with distilled water until neutrality to eliminate possible inhibitors (hereinafter “the washing step”). The solid fractions were oven-dried at 60 °C until constant weight and aliquots were used for the AD experiments, after VS determination, whereas the remaining parts were frozen for further analysis.

2.5. Anaerobic Digestion

After the pre-treatments, all the reactors (K, T, Kw, Tw, in triplicates), as well as 3 reactors fed with untreated giant reed (GR), were prepared as follows: each reactor contained 1 g VS and 41 mL of sterile potassium phosphate-buffered medium (based on ATCC medium #2825) without carbon sources as hydration medium (HM) in a final volume of 50 mL. When necessary, an appropriate amount of distilled water was also added to reach 50 mL of final volume (Kw, Tw, and GR reactors).

Before inoculation, the pH was checked in all the reactors and it resulted equal to 7.3 ± 0.1, on average, due to the buffering effect of the addition of the relatively large volume of HM. Thus, it was not necessary to adjust it. The reactor headspace was gassed using 100% N₂ throughout all the preparation steps before inoculation. Each reactor was inoculated with 5 mL of inoculum. The inoculum used (52.9 ± 0.3 g VS L⁻¹) was prepared according to Vasmara et al. [21], using pig slurry as raw material.

Anaerobic digestion was carried out in 118.5 mL reactors as previously described [22]. The reactors were plugged with butyl rubber stoppers and aluminum seals and they were incubated at 35 °C for 91 days. Biogas production and composition were monitored during the whole incubation period. The cumulative CH₄ volume was calculated as the sum of
the methane collected in the syringe and that accumulated in the headspace of the reactors and expressed in standard conditions (STP) of temperature (273 K) and pressure (101 kPa). The incubation period was considered completed when the biogas production stopped in all the reactors. Reactors containing only inoculum and HM were also included as blanks. In the blank reactors, methane production was not observed.

2.6. Parameters of the Cumulative Methane Production Curves (Gompertz Parameters)

A modified three-parameter Gompertz equation (Equation (1)) was adopted in this study to describe the time course of cumulated CH₄ production [21]:

\[
M(t) = M_{\text{max}} \exp \left\{ - \exp \left[ \left( \frac{e R_{\text{max}}}{M_{\text{max}}} \right) (\lambda - t) + 1 \right] \right\}
\]

where \(M(t)\) (mL CH₄(STP)) is the total quantity of CH₄ produced at the culture time \(t\) (d) in standard conditions (STP) of pressure (101 kPa) and temperature (273 K); \(e\) is the Euler’s number; \(M_{\text{max}}\) (mL CH₄) is the maximum cumulative CH₄ production; \(R_{\text{max}}\) (mL CH₄ d⁻¹) is the maximum daily rate of CH₄ accumulation; and \(\lambda\) is the lag time duration (d), that is the time of microbial adaptation before the exponential phase of CH₄ production begins. This function is often used to interpolate microbial growth curves [23].

Since in each reactor 1 g VS was present, \(M_{\text{max}}\) corresponds to the specific methane yield (SMY) which represents the CH₄ yield obtainable from a specific substrate, expressed as mL CH₄ g⁻¹ VS. The actual methane production (AMP), which accounts for biomass losses, was calculated as the value of SMY multiplied by the percentage of retained VS. This percentage refers to VS still present in the sample after the washing step. The AD duration was calculated as the time required to reach \(M_{\text{max}}\) (Time \(M_{\text{max}}\)), by adding the \(M_{\text{max}}/R_{\text{max}}\) value to the length of the lag phase (\(\lambda\)).

2.7. Analytical Methods

Biogas composition in the reactor headspace was measured by means of a MicroGC Agilent 3000 gas chromatograph, equipped with 2 columns: Molsieve and Plot U; carrier gas: argon; detector: TCD.

Total solids (TS), VS, ash, total P concentration, and pH were determined according to standard methods [24] on the oven-dried, milled, and sieved biomass of giant reed and on washed pre-treated materials. TS were determined gravimetrically by thermal treatment at 105 °C at a constant weight. VS were determined as the difference between TS and ash. Ash was determined by incineration in a muffle furnace at 550 °C for 10 h.

Total C and total N were determined in duplicate by means of CHN Truspec (Leco). The pH was determined after suspension, 2-h stirring and sedimentation of 1.1 g dry matter in 50 mL distilled water, by means of a Crimson Titromatic 1S pH meter.

Fiber fractions were assessed on samples dried at 65 °C until constant weight, determining neutral detergent fiber (NDF), acid detergent fiber (ADF), and lignin (ADL), according to Van Soest et al. [25]. The hemicellulose content was estimated as the difference between NDF and ADF, while cellulose was estimated as the difference between ADF and ADL.

To calculate the retained fraction of the various organic fractions of interest (VS, hemicellulose, cellulose, and lignin) they were normalized based on TS losses after the washing step as follows (Equation (2)):

\[
\text{Retained fraction} = \frac{O_{\text{F}} \times T_{S_{r}}}{O_{\text{F}_{\text{ini}}}}
\]

where \(O_{\text{F}}\) is the organic fraction of interest in % dry matter (DM), \(T_{S_{r}}\) is the retained percentage of TS after the washing step, \(O_{\text{F}_{\text{ini}}}\) is the initial organic fraction of interest in % DM.
2.8. Enzymatic Saccharification of the Pre-Treated Samples and Reducing Sugar Determination

The samples of the pre-treated materials, obtained from 5 g of milled giant reed, were saccharified in 250 mL Erlenmeyer flasks (final volume of 50 mL, in triplicates). Each flask contained the washed or not washed lignocellulosic materials, citrate buffer 50 mM, pH 4.8, sodium azide (0.2 mg mL\(^{-1}\)) and a mix of commercial cellulase enzymes (300 \mu L/flask) (SAE0020, Sigma Aldrich). The flasks were continuously mixed in an orbital shaker (180 rpm, 50 °C, 72 h). Samples of 20 \mu L were withdrawn at 0 h and 72 h, kept in ice during manipulation, then stored at -20 °C before analysis for reducing sugars. Reducing sugars were quantified by the 3,5-dinitrosalicylic acid (DNS) method [26] adapted for 96-well microplates, in duplicates [27]. Dilutions of glucose and the enzyme mix were included, as standards.

2.9. Statistical Analysis

All the statistical analyses were performed using the SAS package procedures [28]. The fitting of the Gompertz model to measurements was done using the PROC NLIN. The Gauss-Newton method was used to estimate parameter values. The PROC MIXED procedure [29] was used to test the significance of the treatment effects on AD parameters. The SAS LSMEANS statement was used for multiple comparisons of the means. Factors and factor interaction effects were considered significant at \(p < 0.05\). To compare the treatment mean values, the Tukey Honestly Significant Difference (HSD) at \(p = 0.05\) was used.

3. Results and Discussion

The pre-treatment aims to deconstruct lignocellulosic biomass to make the holocellulose available for attack by microbial enzymes. Therefore, an effective pre-treatment enhances the biomass hydrolysis, which can be more or less accentuated depending on the pre-treatment severity, as well as on the initial biomass composition. The KOH rate, and pre-treatment temperature and duration used in this work are based on previous observations when pre-treating giant reed with NaOH at 120 °C [30]. These authors reported that both delignification and sugar yield (after enzymatic hydrolysis) steadily increased with raising NaOH concentration up to 1% \(w/w\), while a plateau was finally reached at slightly higher concentrations (1.2–1.5% \(w/w\)). Thus, the pre-treatment conditions applied in this study were chosen to guarantee an effective fiber deconstruction, being KOH molarity comparable to that of NaOH 1% \(w/w\), and time and temperature being the same as those used in the previous study.

3.1. Pre-Treatment Efficiency and Effects on the Biomass

To have a confirmation of the effectiveness of the pre-treatments applied, an enzymatic saccharification test had been performed on the differently pre-treated samples (Table 2). T pre-treatment yielded 129 mg of reducing sugars per gram of input biomass compared to 72 mg obtained with untreated GR, meaning that hot water and pressure alone were able to produce a significant pre-treatment effect. However, KOH addition was determined to enhance the pre-treatment efficiency since K reactors reached, on average, a yield of 336 mg \(g^{-1}\) (Table 2). A similar sugar yield (380 mg \(g^{-1}\)) after enzymatic hydrolysis following an alkaline pre-treatment of giant reed with comparable NaOH concentrations has been recently reported [31]. This proves that NaOH can be effectively replaced by KOH to pre-treat giant reed. The significantly lower sugar yields of washed compared to unwashed materials (54 and 301 mg \(g^{-1}\) for Tw and Kw, respectively) are due to the washout of the solubilized dry matter and VS in particular (Tables 2 and 3). Obviously, in the reactors where the biomass was pre-treated and not washed, there was no biomass loss.

The KOH addition caused more intense solubilization of the biomass than without addition, as indicated by the percentage of biomass retained (Table 3). The washing step of the thermo-chemical pre-treated biomass (Kw), in particular, led to a loss of 19% of the TS and 16% of the VS, compared to 5% losses without alkali (Tw).
Table 2. Reducing sugar yields after enzymatic hydrolysis of differently pre-treated giant reed biomass.

| Pre-Treatment | Reducing Sugar Yield |
|---------------|----------------------|
|               | Pre-Treated Dry Biomass | Initial Dry Biomass |
| GR            | 72 (d)                | 72 (d)              |
| T             | 129 (c)               | 129 (c)             |
| Tw            | 54 (d)                | 49 (d)              |
| K             | 336 (a)               | 336 (a)             |
| Kw            | 301 (b)               | 277 (b)             |

1 GR: untreated control; T: 121°C, 20′; K: 0.135 g KOH g⁻¹ VS, 121°C, 20′; w: washing step after pre-treatment.
2 Means sharing common letters are not significantly different at p < 0.05 according to Tukey Honestly Significant Difference test.

Table 3. Fiber composition of differently pre-treated giant reed after the washing step.

| Pre-Treatment | Trait               | Tw (Mean (SD)) | Kw (Mean (SD)) |
|---------------|---------------------|----------------|----------------|
|               | TS, % retained ²    | 94.67 (0.19)   | 81.20 (0.06)   |
|               | VS, % retained      | 95.00 (0.23)   | 83.90 (0.13)   |
|               | Cellulose, %DM ³    | 45.23 (0.03)   | 52.68 (0.45)   |
|               | Hemicellulose, %DM ³| 22.58 (0.23)   | 21.81 (0.06)   |
|               | Lignin, %DM        | 11.32 (0.06)   | 11.77 (0.22)   |

1 Tw: 121°C, 20′ + washing; Kw: 0.135 g KOH g⁻¹ VS, 121°C, 20′ + washing. ² Percentage still present in the sample after the washing step. ³ Percentage of dry matter.

As for the composition of the washed biomass, in the presence of alkali (Kw), there was a relative enrichment in cellulose fraction compared to Tw (Table 3), whereas the concentrations of hemicellulose and lignin were similar. Likewise, in comparison to the untreated biomass of giant reed (Table 1), the retained biomass showed enrichment in holocellulose, whereas lignin concentration remained unchanged (Table 3). However, in any case, the washing step resulted in losses of biomass, as discussed in more detail below.

3.2. Washing Effects

Thermo-chemical pre-treatments can generate inhibitors that could negatively affect the AD process. Therefore, generally, before AD the pre-treated solid biomass is recovered and washed, while the liquid fraction is discarded. However, washing the biomass, besides removing inhibitors and residual alkali, also takes away the solubilized organic fraction of the pre-treated biomass consisting mostly of hemicellulose (Figure 1), which instead could be conveniently utilized during the AD process. The washing step after the T pre-treatment led to a remarkable loss of hemicellulose and barely affected lignin (Figure 1), while the washing step after the K pre-treatment resulted in a more evident loss of both lignin and hemicellulose (Figure 1) indicating that K pre-treatment was more effective than T in fiber destructuring. The cellulose losses were negligible both for Tw and Kw. However, since the losses of lignin in Kw were almost triple if compared with those in Tw (Figure 1), it is clear that in Kw the fraction of cellulose available to the enzymatic attack was higher than in Tw. This explains the higher saccharification yield in Kw than in Tw shown in Table 2, despite the larger hemicellulose losses. In particular, the washing step following KOH-based pre-treatment allowed to remove 16% of initial lignin and also led to a loss of 25% of initial hemicellulose (Figure 1).
Alkaline pre-treatments cause the solubilization of the hemicellulose since they break the hydrogen bonds [32]. Some authors reported 10–44% lignin losses and 10–33% losses of hemicellulose for giant reed pre-treated with 0.15–0.5N NaOH at 24–25 °C for 24 h [12,17].

As far as KOH-based pre-treatments are concerned, information on giant reed is not available in the literature. Wheat straw pre-treated with KOH 9% w/w at room temperature for 3 days showed 31% and 45% losses for lignin and hemicellulose, respectively [19]. On the other hand, when pre-treating wheat straw with KOH 50% w/w at 20 °C for 24 h, losses of 54.7% and 52.7% for lignin and hemicellulose, respectively, were recorded [33]. Asghar and colleagues [34] reported a delignification rate of over 60% pre-treating Kallar grass and cotton stalk with KOH 3% w/v for 48 h at room temperature, whereas a delignification rate of 55.6% was obtained pre-treating switchgrass with KOH 2% w/v at 121 °C for 1 h [35]. Therefore, lignin and hemicellulose losses after the washing step are generally substantial, although strongly influenced by both the type of biomass and pre-treatment severity.

In this study, differently from hemicellulose and lignin, cellulose solubilization was limited, and losses after the washing step were not detected (about 1%) (Figure 1). Cellulose, indeed, under alkaline conditions is not easily hydrolyzed [11,33]. However, previously, cellulose losses between 16 and 20% had been reported, following a NaOH pre-treatment of giant reed [12,17]. Using KOH on wheat straw, cellulose losses between 12.6 and 13.5% had also been observed [19,33]. These larger losses could be due to differences in the solid/liquid separation procedure.
3.3. Effects of the Pre-Treatments on Anaerobic Digestion

The highest SMY values (231 and 232 mL CH\textsubscript{4} g\textsuperscript{−1} VS) were obtained from K pre-treated biomass, without any substantial difference between washed and unwashed samples (Figure 2; Table 4).

Figure 2. Measurements (points) and estimates (dashed curves) of the cumulative CH\textsubscript{4} production from giant reed during anaerobic digestion. Circles: KOH-based pre-treatments; squares: thermal pre-treatments; triangles: untreated giant reed. Empty symbols: washed samples; solid symbols: unwashed samples. Vertical bars represent the standard deviation of the mean (n = 3).

Table 4. Anaerobic digestion parameters of the differently pre-treated giant reed biomass.

| Pre-Treatment | SMY | R\text{max} | \lambda | Time\text{\textsubscript{max}} | CH\text{\textsubscript{4}}\text{max} | AMP |
|---------------|-----|-------------|--------|------------------|-----------------|-----|
|               | mL CH\textsubscript{4} g\textsuperscript{−1} VS | mL CH\textsubscript{4} d\textsuperscript{−1} | d      | d                | %    | mL CH\textsubscript{4} g\textsuperscript{−1} VS |
| GR            | 192 b \textsuperscript{1} | 4.26 b | 1.70 b | 46.8 a | 47 c | 192 b |
| T             | 198 b | 4.95 b | 2.38 b | 42.4 b | 49 bc | 198 b |
| Tw            | 203 b | 5.02 b | 2.66 b | 43.5 ab | 48 bc | 193 b |
| K             | 231 a | 6.03 a | 4.20 a | 42.5 b | 58 a | 231 a |
| Kw            | 232 a | 5.23 ab | 1.69 b | 46.1 a | 52 b | 195 b |

\textsuperscript{1} GR: untreated control; T: 121\degree C, 20'; K: 0.135 g KOH g\textsuperscript{−1} VS, 121\degree C, 20'; w: washing step after pre-treatment. \textsuperscript{2} SMY: specific methane yield; R\text{max}: maximum daily rate production; \lambda: lag phase duration; Time\text{\textsubscript{max}}: time to reach the maximum cumulative CH\text{\textsubscript{4}} production; CH\text{\textsubscript{4}}\text{max}: maximum content of CH\textsubscript{4} in the biogas; AMP: actual methane production = SMY multiplied by the percentage of retained VS. \textsuperscript{3} Means sharing common letters do not differ significantly at p < 0.05 according to Tukey Honestly Significant Difference test.

These SMY values are in line with the CH\textsubscript{4} yield obtained from other substrates commonly used in AD such as, for example, cattle slurry, wheat straw, vegetable waste, garden waste [3,36] or from Brassica napus which recently was reported to yield up to
259 mL CH$_4$ g$^{-1}$ VS when irrigated with abattoir wastewater [37]. In this study, untreated giant reed (GR) reached 192 mL CH$_4$ g$^{-1}$ VS, which is consistent with what was reported by other authors for this crop [17,38] and is comparable with the SMY of miscanthus, which varied between 166 and 202 mL CH$_4$ g$^{-1}$ VS, depending on the genotype [18].

Therefore, the chemical pre-treatment of giant reed is necessary to achieve the CH$_4$ yields obtained with other kinds of biomass commonly used to produce biogas, as SMY from just thermally pre-treated samples (T) did not significantly differ from that of GR (Figure 2; Table 4).

On average, the SMY obtained from K reactors increased by 21% compared to the untreated GR (Table 4). Such an increase is consistent with that reported by Di Girolamo and colleagues [17] for giant reed pre-treated with 0.15N NaOH at 25 °C for 24 h. By using higher NaOH concentrations, Jiang and colleagues [12], obtained up to 63% CH$_4$ yield increase with 20 g L$^{-1}$ NaOH, at 24 °C for 24 h, while the pre-treatment of giant reed with 20 g L$^{-1}$ CaOH$_2$ at 25 °C for 24 h allowed to increase CH$_4$ yield by 34% compared to untreated [20]. This suggests that alkali type and concentration as well as pre-treatment conditions can affect the giant reed response in terms of CH$_4$ yield.

Data referring to the effect of KOH-based pre-treatments on CH$_4$ yield from giant reed are not available in the literature. Previously, KOH was used to pre-treat agricultural residues: on wheat straw, concentrations of KOH g$^{-1}$ TS between 1 and 9% at room temperature for 3 days were tested, obtaining a maximum increase of CH$_4$ yield of 40% for KOH at 6% [19] whereas, Liu and colleagues [33] testing concentrations between 2 and 50% g KOH g$^{-1}$ dry straw at 20 °C for 24 h, reported that the pre-treatment with 20% KOH was the most favorable allowing a 44% increase in SMY, while KOH concentration of 14%, very similar to that used in this study, increased CH$_4$ production by 26%. For corn stover, KOH was reported as more effective than Ca(OH)$_2$: the maximum increase in terms of CH$_4$ yield was 95% for KOH 2.5% w/v and only 40% for 2.5% Ca(OH)$_2$ w/v [39]. KOH also outperformed NaOH on wheat straw: pre-treating wheat straw at 19 °C for 24 h with 0.5% KOH w/w increased CH$_4$ yield by 23%, as with 2% NaOH w/w [40].

An effective pre-treatment also influences the daily production rate of CH$_4$ ($R_{max}$). This is because the pre-treatment makes the holocellulose polymers more readily attackable by the hydrolytic enzymes produced by bacteria involved in AD. In this study, the thermochemical pre-treatment led to a significant increase in $R_{max}$ especially from the unwashed biomass (Table 4). Significant differences were not detected between T and GR reactors in terms of $R_{max}$ value. The $R_{max}$ value obtained from the K reactors was 42% higher than that from GR (6.03 VS 4.26 mL CH$_4$ d$^{-1}$, respectively). Using a higher alkali concentration, Jiang and colleagues [12], reported an almost 100% increase of $R_{max}$ compared to the control for giant reed pre-treated with NaOH 20 g L$^{-1}$. For wheat straw pre-treated with KOH concentration similar to that used in this study, Liu and colleagues [33] reported a 67% increase of $R_{max}$, compared to the control.

Overall, AD had a regular course in all reactors (Figure 2); CH$_4$ production started simultaneously in all reactors except for K where the lag phase ($\lambda$) was significantly the longest (Table 4). However, in these reactors, the significantly highest value of $R_{max}$ was also recorded, so that the overall duration of the AD ($Time_{R_{max}}$) was significantly shorter in K compared to its washed equivalent (Kw) and GR (Table 4). The presence of the pre-treatment liquid fraction did not negatively affect CH$_4$ production: the unwashed reactors (K), indeed, provided SMY and $R_{max}$ values similar to the washed reactors. Besides, the highest CH$_4$ concentration in the biogas (CH$_4$ max) was obtained in K reactors and reached 58%. The only negative effect attributable to the presence of the pre-treatment liquid fraction can be recognized in the significantly longer lag phase duration of AD ($\lambda$) in K reactors (4.2 days), which was almost 2.5 times longer than that of the Kw reactors (Table 4). Among the inhibitors that can accumulate in the residual liquid fraction of the thermo-alkaline pre-treatment, there are the polyphenols released due to lignin destructuring [2,6,41]. Some authors have described the toxic effects of polyphenols on methanogens [42,43]. In a previous study, polyphenols were responsible for a delay in the start of AD of the spent
coffee ground [44], thus the lengthening of the lag phase observed for K in this study can be due to polyphenols presence. However, the inhibitory effect finished within the first 4 days and the time required to complete the AD was even found to be significantly shorter in the unwashed reactors than in the washed ones, which had an AD duration identical to that of untreated giant reed (Table 4).

The washing step, in addition to removing inhibitors and lignin, also led to the loss of material suitable for AD as demonstrated by the higher overall methane yield (AMP) obtained from K reactors compared to Kw (231 vs. 195 mL g\(^{-1}\) VS, respectively; Table 4). Indeed, due to the biomass losses following the washing step, the AMP from Kw reactors was not significantly different from that of GR, reducing the pre-treatment advantages (Table 4). This evidence is consistent with the literature: taking into account the biomass losses due to the removal of the liquid fraction, Liu and colleagues [33] did not find significant differences between 14% KOH pre-treated or untreated wheat straw in terms of overall CH\(_4\) produced, despite a significant specific methane yield increase.

Our results demonstrate that the liquid fraction derived from the pre-treatment not only did not inhibit AD but also contributed to CH\(_4\) production. This is supported also by the observation that the unwashed KOH pre-treated materials displayed the highest saccharification yield (Table 2), meaning that the liquid fraction contained readily digestible carbohydrates.

In conclusion, avoiding the washing step not only permits to increase CH\(_4\) production per initial biomass input unit but also alleviates the problem of disposing of saline wastewater, reducing the overall pre-treatment costs. Besides, the pre-treatment with KOH instead of NaOH would result in a digestate enriched in potassium, that could be utilized as fertilizer [19].

4. Conclusions

The thermo-chemical pre-treatment with KOH caused an efficient deconstruction of lignocellulosic biomass of giant reed, allowing an increase of CH\(_4\) yield by 21%. Besides, the maximum daily rate of production increased by 42%, and CH\(_4\) concentration in the biogas by 23%, while AD duration was reduced by 10%, compared to thermal pre-treatment or untreated control. The washing step resulted unnecessary since the liquid fraction derived from the pre-treatment negatively affected only the lag phase. Methane production from the washed pre-treated biomass did not differ from that of the untreated, due to the biomass loss that occurred after the washing step. Thus, the use of a KOH-based pre-treatment without washing appears as a good option for enhancing the methane yield of giant reed. Further studies are necessary to optimize pre-treatment conditions and KOH concentration.

Author Contributions: Conceptualization, C.V., S.C., R.M., and S.G.; methodology, C.V., S.C., R.M., and S.G.; validation, C.V. and S.C.; formal analysis, R.M.; investigation, C.V. and S.C.; resources, S.G.; data curation, C.V.; writing—original draft preparation, C.V. and S.C.; writing—review and editing, C.V., S.C., R.M., E.C., and S.G.; visualization, C.V.; supervision, R.M., E.C., and S.G.; project administration, R.M. and S.G.; funding acquisition, R.M. and S.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Italian Ministry of Agricultural, Food and Forestry Policies (MiPAAF) under the AGROENER project (D.D. n. 26329, 1 April 2016) http://agroener.crea.gov.it.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: Authors wish to thank Anna Orsi for laboratory analyses and assistance.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.
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