Italian Journal of Agronomy 20 (2025) 100032

Contents lists available at ScienceDirect

Italian Journal of Agronomy

journal homepage: www.sciencedirect.com/journal/italian-journal-of-agronomy

Research Article

# Improving biomethane production from giant reed: effect of agronomic strategies and fungal pretreatment under semi-arid Mediterranean conditions

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ARTICLE INFO

*Keywords:*
*Arundo donax* L.
Lignocellulosic biomass
Long-term plantation
Anaerobic digestion
*Pleurotus ostreatus, Irpex lacteus*
Green energy

ABSTRACT

In recent years, lignocellulosic perennial grasses have attracted attention as a feedstock to produce biomethane through anaerobic digestion, considered a sustainable energy solution for reaching the RED II directive's aims. *Arundo donax* L., commonly known as giant reed, is a lignocellulosic perennial rhizomatous grass, widespread in the Mediterranean environment and a promising candidate for bioenergy production. Anaerobic digestion is one of the most environmentally friendly technologies to produce biomethane from lignocellulosic biomass. However, the use of lignocellulosic material as substrate requires a pretreatment to break down its recalcitrance structure and enhance biomethane yield. This study investigated the suitability of a long-term plantation of *Arundo donax* as a feedstock for advanced biomethane production. The effects of cropping cycle (23rd *vs* 24th *vs* 25th), harvest time (winter vs autumn), and nitrogen fertilization (non-fertilized *vs* 80 kg N $ha^{-1}$) on biomass yield and quality were evaluated. Additionally, biomethane production through anaerobic digestion was evaluated, as influenced by pretreatment duration (30 vs 50 days) with two white rot fungi (*Pleurotus ostreatus vs Irpex lacteus*). Nitrogen fertilization significantly increased biomass yield, with the highest value (13.9 t $ha^{-1}$) recorded for winter harvest in the 25th cropping cycle. *Pleurotus ostreatus* pretreatment of giant reed biomass improved anaerobic digestion, increasing the final biomethane yield compared to untreated biomass. The highest biomethane production (2122 $m^3$ $CH_4$ $ha^{-1}$) was recorded for winter harvest on fertilised crops pretreated by *P. ostreatus* for 30 days, as result of the positive effect of nitrogen fertilisation on biomass yield and biomethane production. These results indicate the potential of giant reed to provide high biomass and biomethane yield combining sustainable agronomic practices and an effective pretreatment process.

## 1. Introduction

The use of biomass as biofuel has been promoted to increase the rate of renewable energy, according to Renewable Energy Directive II (RED II) and its revised package 'Fit for 55' which set targets for the share of renewable energy in the EU's energy consumption to meet the objectives of reducing Europe's net greenhouse gas emissions by at least 55 % by 2030 (Directive (EU), 2018). In recent years, lignocellulosic perennial grasses have been attracting attention as a feedstock to produce biomethane through anaerobic digestion, considered a sustainable energy solution for reaching the RED II directive's aims. *Arundo donax* L., commonly known as giant reed, is a lignocellulosic perennial rhizomatous grass, distributed across the Mediterranean. Among different perennial grasses, giant reed is a promising candidate for bioenergy production because of its ability to thrive in a wide range of environmental conditions with low agronomic inputs (use of irrigation, fertilisers, and pesticides) (D'Imporzano et al., 2018; Lewandowski et al., 2003). Giant reed is able to produce high biomass in adverse conditions and degraded soils typical of marginal lands (Lino et al., 2023). Bonfante et al. (2017) obtained large biomass production from giant reed cultivation in marginal areas of Southern Italy, concluding that this crop may be a valid alternative to traditional crops of marginal non-irrigated areas that would increase the resilience of local farming. Moreover, giant reed, among perennial crops, shows interesting characteristics such as the ability to reduce erosion risk, improving soil fertility and the capability

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https://doi.org/10.1016/j.ijagro.2025.100032
Received 28 November 2024; Received in revised form 22 February 2025; Accepted 22 February 2025
Available online 26 February 2025

of keeping high and stable biomass yield over the years with low cultivation costs, thus avoiding competition with food crops for soil use (Bahrs and Angenendt, 2019). Visconti et al. (2020) reported that giant reed cultivation reduces soil losses (up to 78 %) compared to leaving soils fallow, improving ecosystem services of hilly cropping systems.

Lignocellulosic material from perennial grasses can be efficiently used for direct biomass combustion or for different biochemical and thermochemical conversion pathways to be transformed into several biofuels (e.g., biomethane, bioethanol) and value-added products (e.g., building material, phonic insulating material, biochar) (Yogalakshmi et al., 2023). One of the most important characteristics of perennial grass lignocellulosic structure is its cell wall; it is made up of cellulose, hemicellulose, lignin, and trace amounts of non-structural components, such as extractives, protein, lipids, pectin, and minerals (Dahadha et al., 2017). The process of converting giant reed biomass into biomethane involves anaerobic digestion, a well-established method for producing biomethane from perennial energy grasses as feedstock. (Song et al., 2023). This process is also gaining attention as a sustainable method for waste management and renewable energy production (Xu et al., 2019). However, the interactions of cell wall components create a complex biochemical structure, and the high lignin content of the biomass makes giant reed rather recalcitrant and resistant to the enzymatic hydrolysis phase during the anaerobic digestion process (Cianchetta et al., 2017; Sawatdeenarunat et al., 2015). Thus, to overcome this rate-limiting step and improve the efficiency of the conversion process, pretreatments are needed to break down the recalcitrant structure of lignocellulosic biomass, allowing a weakening of the crystallinity of cellulose, reducing the inhibiting effect of lignin, and increasing the accessible surface area for enzymatic attack (Sasmal and Mohanty, 2018).

Pretreatment techniques are classified into four main categories: physical, chemical, physico-chemical, and biological pretreatments, based on the tools used for the process (mechanical, thermal, chemicals, and microorganisms) (Rodriguez et al., 2017). Biological pretreatment is one of the most environmentally friendly and sustainable pretreatment methods, as it is a mild process that does not necessitate high energy, cost, equipment inputs, and does not require chemical reagents (Shukla et al., 2023). Fungal pretreatment is one of the biological methods considered interesting for the pretreatment of lignocellulosic biomass and improving biomethane production (Mishra et al., 2018). This pretreatment involves the use of ligninolytic fungi, commonly white-rot fungi, that are able to selectively degrade lignin from holocellulose by producing oxidative ligninolytic enzymes, such as laccase, manganese peroxidase, and lignin peroxidase (Wu et al., 2022).

This study aims to evaluate, on a long-term *Arundo donax* plantation, the effects of two harvest times and two nitrogen fertilisation levels on biomass production during three growing seasons. Moreover, on biomass harvested in the 25th cropping cycle, the effect of a fungal pretreatment and its duration using two white-rot fungi (*Pleurotus ostreatus* and *Irpex lacteus*) on biomethane production was evaluated.

## 2. Material and methods

The following factors were studied: (I) harvest time (2 levels: autumn (A) and winter (W)), (II) nitrogen fertilisation (2 levels: N0 - 0 kg N ha$^{-1}$ and N80 - 80 kg N ha$^{-1}$), (III) fungal pretreatment (2 levels: *Pleurotus ostreatus* and *Irpex lacteus*) and (IV) pretreatment duration (2 levels:30 and 50 days), resulting in a total of 16 combinations.

### 2.1. Field experiment

The field trial was carried out during the 2020/2021, 2021/2022 and 2022/2023 growing seasons, corresponding respectively to the 23rd, 24th and 25th year of plantation at the Experimental Farm of the University of Catania, Italy (10 m a.s.l., 37°25′ N lat., 15°03′ E long.) in a typical xerofluvent soil. The giant reed plantation was established in 1997 from stem cuttings of the “Fondachello” clone transplanted at a density of 2.5 plants m$^{-2}$. Irrigation was applied after transplant and only during the first summertime to ensure a successful establishment. The irrigation was differentiated in the successive three years as 100 % of maximum evapotranspiration (ETm) restoration, 50 % of ETm restoration and rainfed conditions. Nitrogen fertilisation was applied at different rates at transplanting and for the following three years (0 kg N ha$^{-1}$, 60 kg N ha$^{-1}$, 120 kg N ha$^{-1}$) as reported by Cosentino et al. (2014). From 2001 the plantation was maintained without applying fertiliser, irrigation, or other agronomic inputs. From 2011, plots were maintained in rainfed conditions differentiating two factors: nitrogen fertilisation at two levels (N0- 0 kg N ha$^{-1}$, and N80- 80 kg N ha$^{-1}$) and harvest times (autumn and winter) on three replicated plots of 134 m$^2$ (8 × 17 m) for each treatment. Nitrogen was applied as ammonium sulphate after the autumn harvest to allow the crop to reach the stage of senescence in winter. After the winter cut, ammonium nitrate was used since crop regrowth begins in the spring. The autumn harvests were performed on 18th September 2020, 20th September 2021 and 13th September 2022, and the winter harvests on 9th February 2021, 14th February 2022 and 10th February 2023.

At harvest, the edge plants were removed from each plot, and the aboveground biomass from a designated sampling area of 6 m$^2$ (3 × 2 m) was harvested by hand. The fresh harvested biomass was weighed, and the results were reported in Mg ha$^{-1}$. A sub-sample was collected to separate the stems from the leaves, and fresh samples were randomly collected, weighed, and dried at 65 °C until a constant weight was achieved. The dry biomass percentage was calculated as the ratio between dry biomass weight and wet biomass weight which was multiplied by 100. Dry Biomass percentage was multiplied by the fresh biomass yield to obtain the dry biomass yield referred to the unit land area (Mg ha$^{-1}$).

### 2.2. Meteorological conditions

Meteorological conditions (maximum and minimum air temperatures and rainfall) for autumn and winter growing seasons were continuously measured using a weather station connected to a data logger (Delta-T, WS-GP1 Compact) starting from the 12 previous months for each harvest date. The potential evapotranspiration (ET0) was estimated according to Allen et al. (1998).

During the three-year field trial, the weather conditions were typical of the Mediterranean climate. Mean air temperatures throughout the autumn harvest cropping cycles (September-September) were slightly higher than during the winter harvest cropping cycles (February-February) every year, with the following average temperature recorded: 24.0 °C for the maximum, 13.2 °C for the minimum, and 18.4 °C for the mean temperature. For the winter season, the maximum, minimum and mean air temperatures were 23.3 °C, 12.2 °C, and 17.5 °C, respectively. The rainfall average during the autumn period was higher than the winter growing season (784.1 mm and 703.8 mm, respectively). An extreme precipitation event occurred at the end of October 2021, while every year the area was characterized by hot summers and severe drought conditions. The mean evapotranspiration during the autumn harvest growth cycle (1496 mm) was higher than during the winter harvest growth cycle (952 mm) (Fig. 1).

### 2.3. Characterisation of feedstock

Biomass samples of giant reed were oven-dried at 65 °C, then milled and stored for further analysis. The total solids (TS), volatile solids (VS), and fibre composition were determined. Total solids content, expressed as a percentage of the sample initial weight, was calculated by drying the sample at 105 °C to a constant weight, and VS content was determined after incineration in a muffle furnace at 550 °C for 5 h as the difference from the ash content (Sluiter et al., 2008). Total solids and volatile solids measurements were performed in triplicate.

The fibre composition, including neutral detergent soluble (NDS), neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL), was determined using a Fiber Analyzer

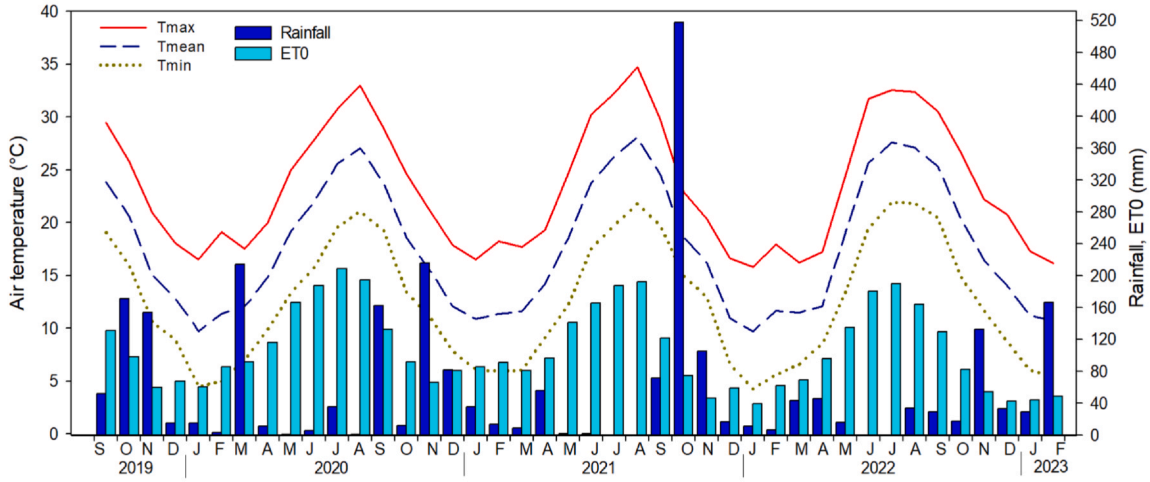


**Fig. 1.** Meteorological variables (mean air temperature and monthly cumulative rainfall) and reference evapotranspiration (ET0) during the 3 growing seasons at the experimental site (Catania, 37°24′ N, 15°03′ E, 10 m a.s.l.).

(Fibertec Velp Scientifica, model FIWE) according to the method of Van Soest et al. (1991). The amounts of hemicellulose and cellulose were determined as the difference between NDF and ADF, and ADF and ADL, respectively. The lignin content was calculated as the difference between ADL and ash content, produced by igniting the ADL residue in a muffle furnace at 550 °C for 5 h.

### 2.4. Inoculum preparation

Biomass pretreatment was carried out using two fungal strains: *Pleurotus ostreatus* (MUT00002977) and *Irpex lacteus* (MUT00005918), purchased from Mycotheca Universitatis Taurinensis (MUT) of the Department of Life Sciences and Systems Biology, University of Turin (Turin, Italy). Fungi activation was conducted using Malt Extract Agar (MEA) plates, with the cultures maintained in a thermostatically controlled incubator at 26 °C for 7 days. The experiments for fungal pretreatment were conducted using sterile giant reed biomass colonized by *P. ostreatus* and *I. lacteus* as the inoculum.

To prepare the inoculum, the biomass was prepared as follows: 30 g (dry basis) of biomass was placed in 0.5 L reactors, adding deionized water to obtain a moisture content of 70 %. Reactors were autoclaved at 121 °C for 20 min followed by cooling to room temperature. Subsequently, four agar discs (approximately 1 cm in diameter) taken from actively growing mycelium on the MEA plates were used to inoculate sterilized giant reed biomass under aseptic conditions. Reactors were incubated at 26 °C until complete colonization occurred. Full colonization typically took four weeks from the start of the incubation. The fungal-colonized giant reed biomass was thoroughly mixed and utilized as inoculum for subsequent fungal pretreatment.

### 2.5. Fungal pretreatment

The previously prepared inoculum and the sterile biomass to be pretreated were combined into 0.5 L reactors. Fungal pretreatments were carried out at a 30 % inoculum ratio (based on dry weight). Deionized water was added to reach a moisture content of 70 %. Cotton plugs were used to seal the reactors, which were then placed in an incubator at 26 °C for 30 days. Reactors with non-inoculated sterile biomass were included to serve as negative controls. At the end of the pretreatment, samples were collected, dried in a ventilated oven at 65 °C until their weight remained constant, and analysed for cellulose, hemicellulose, and lignin content. The degradation of each component was expressed as a percentage of the initial dry weight and fibre fractions before fungal pretreatment.

### 2.6. Biochemical methane potential (BMP) tests

The BMP test was carried out utilising an automatic methanogenic potential detection system (AMPTS II, Automatic Methane Potential Test System, Bioprocess Control AB, Lund, Sweden). The AMPTS II is a standardized laboratory set-up specifically developed for automatic BMP determination of any biodegradable material. It consists of 15 parallel reactors, each equipped with corresponding gas flow meters (flow cells) linked to a detection unit for online, automatic data acquisition.

The trial was conducted in 500 mL reactors, with substrates and inoculum combined at a 1:3 ratio based on grams of VS, under mesophilic conditions (38 ± 1 °C) and constant mixing. All tests were conducted in triplicate. The inoculum was originally obtained from an anaerobic digester located in Sicily and it was filtered through a 2 mm porosity sieve to remove large and undigested particles, followed by stabilisation in an incubator at 38 °C for 5 days. Each reactor was connected to an 80 mL trap bottle containing a 3 M sodium hydroxide solution utilized to capture $CO_2$ from the raw gas. The remaining gas after scrubbing was directed to ultra-low gas flow meters, connected to a data analytical and acquisition system. The BMP test was run for 30 days. Furthermore, blank samples, only containing the inoculum, were also incubated. The substrate methane output was calculated by subtracting the methane production of the blank (inoculum) from that of the substrate sample (substrate + inoculum). The cumulative methane production after the test was defined as the experimental BMP (NmL $g^{-1}$ VS) of the substrate.

### 2.7. Biomethane yield

The biomethane yield of giant reed ($m^3$ $CH_4$ $ha^{-1}$) was calculated by multiplying the biomethane potential (NmL $g^{-1}$ VS), the dry biomass yield (Mg $ha^{-1}$) and the volatile solid (% of dry matter) of both untreated and fungal-pretreated biomass.

### 2.8. Statistical analysis

Data were subjected to statistical analysis using analysis of variance (ANOVA). The Bartlett test was run to test homoscedasticity. The Shapiro–Wilk test was used to test residuals for normality. A three-way ANOVA was carried out for biomass yield data with harvest time (H) and nitrogen fertilization (F) as fixed factors and year (Y) as a random factor. Biomass content of hemicellulose, cellulose, ADL, ash, and NDS, was analysed by two-way ANOVA with harvest time and nitrogen fertilisation as fixed factors.

**Table 1**
Significance levels from ANOVA output for Year, Harvest time, Fertilization, and their interaction on dry biomass yield by fractions (stem and leaves) and on total dry biomass yield (Mg ha$^{-1}$) of giant reed. $p \leq 0.001$ (***), $p \leq 0.01$ (**), $p \leq 0.05$ (*), Not significant (ns).

| Source of Variance | Total yield (Mg ha$^{-1}$) | Stem yield (Mg ha$^{-1}$) | Leaves yield (Mg ha$^{-1}$) |
|---|---|---|---|
| Year | ns | ns | ns |
| Harvest | ns | * | * |
| Fertilisation | ns | ns | ns |
| Y x H | ns | ** | ns |
| Y x F | * | *** | ns |
| H x F | * | ** | ns |
| Y x H x F | ns | ns | ns |

Biomass composition after the fungal pretreatment, the degradation of cellulose, hemicellulose, and lignin, the cumulative biomethane, and the biomethane yield per hectare were analysed using a four-way ANOVA with the effect of harvest time, fertilisation, pretreatment, and pretreatment duration as fixed factors. Means were separated by the Tukey's HSD test at 95 % confidence level. The data were statistically analysed using CoHort Software (CoStat version 6.003, Monterey, CA, USA).

## 3. Results

### 3.1. Biomass yield

The analysis of variance (ANOVA) showed a significant effect of harvest time on the yield of stems and leaves ($p \leq 0.01$), but not for the total biomass yield. Significant Y x F and H x F interactions were found for both total and stem yield, while the Y x H interaction was significant only for stems. (Table 1). Tables 2 and 3 show the mean data for the significant interaction observed on stem and total biomass yield. Although there was no significant effect of fertilisation, the significant Y x F interaction in total dry biomass yield reveals the effect of nitrogen fertilisation on total yield in the 24th and 25th cropping cycles. The H x F interaction shows differences in fertilisation with a significant effect on the total biomass yield of winter harvest (Table 2). The significant Y x H interaction in stem yield reveals the significant effect of harvest time in the first and second years. During the second and third years, there was a significant effect of nitrogen fertilisation on stem yield (Y x F, $p < 0.001$). Considering the significant H x F interaction in stem yield, the yield positively responded to nitrogen fertilisation for both autumn and winter harvest, with the highest yield obtained in fertilised winter harvest, while no difference was observed between the yield of autumn and winter unfertilised biomass (Table 3).

On average, stem and leaf yields were 7.9 and 3.5 Mg ha$^{-1}$ in the autumn and 9.4 and 2.2 Mg ha$^{-1}$ in the winter harvest, respectively. The highest dry matter yield was observed in the fertilised biomass of the winter harvest, followed by the autumn fertilised biomass for each growing season. The 23rd cropping cycle showed an increase in biomass yield due to fertilisation of 11.2 % and 5.2 % for the winter and autumn harvests, respectively. Whereas the increases in biomass yield associated with nitrogen fertilisation were 40.4 % and 27.6 % in the 24th cropping cycle and 45.9 % and 28 % in the 25th, for winter and autumn harvests, respectively. The maximum yield was 13.9 Mg ha$^{-1}$ obtained for the fertilised biomass from the winter harvest during the 25th cropping cycle. On average, fertilisation induced a biomass yield increase of 31.9 % in winter and 8 % in autumn harvest.

**Table 2**
Mean values for Year x Fertilization and Harvest x Fertilization interactions and mean effect of tested factors on total dry biomass yield (Mg ha$^{-1}$). HSD ($p < 0.05$) for mean separation of interactions (2.97).

| Total yield (Mg ha$^{-1}$) | | | | |
|---|---|---|---|---|
| Year x Fertilisation | 2020/2021 | 2021/2022 | 2022/2023 | Mean |
| N0 | 10.6 | 9.8 | 10.1 | 10.2 |
| N80 | 11.5 | 13.1 | 13.8 | 12.8 |
| Mean | 11.1 | 11.4 | 11.9 | |
| Harvest x Fertilisation | Autumn | Winter | | Mean |
| N0 | 10.4 | 9.9 | | 10.2 |
| N80 | 12.4 | 13.2 | | 12.8 |
| Mean | 11.4 | 11.6 | | |

**Table 3**
Mean values for Year x Harvest, Year x Fertilization, Harvest x Fertilization interactions and mean effect of tested factors on stem yield (Mg ha$^{-1}$). HSD ($p < 0.05$) for mean separation of interactions (2.32).

| Stem yield (Mg ha$^{-1}$) | | | | |
|---|---|---|---|---|
| Year x Harvest | 2020/2021 | 2021/2022 | 2022/2023 | Mean |
| Autumn | 8.0 | 7.5 | 8.3 | 7.9 |
| Winter | 10.0 | 9.1 | 9.1 | 9.4 |
| Mean | 9.0 | 8.3 | 8.7 | |
| Year x Fertilisation | 2020/2021 | 2021/2022 | 2022/2023 | Mean |
| N0 | 8.8 | 7.1 | 7.4 | 7.8 |
| N80 | 9.3 | 9.5 | 10.0 | 9.6 |
| Mean | 9.0 | 8.3 | 8.7 | |
| Harvest x Fertilisation | Autumn | Winter | | Mean |
| N0 | 7.3 | 8.2 | | 7.8 |
| N80 | 8.5 | 10.6 | | 9.6 |
| Mean | 7.9 | 9.4 | | |

### 3.2. Biomass composition

The ANOVA did not find differences in N-fertilization levels while significant H x F interactions were observed for hemicellulose and lignin (Table 4). The biomass composition of untreated giant reed showed a significantly greater content of soluble substances (NDS) ($p \leq 0.01$) and ashes ($p \leq 0.001$) in the autumn (23.5 % and 1.3 %, respectively) rather than in winter harvest (21.7 % and 0.8 %) ($p \leq 0.01$). Although not significant, hemicellulose was higher in the autumn (28.9 %) than in the winter harvest (28.4 %). By contrast, cellulose and lignin content ($p \leq 0.01$), were higher in the winter harvest (37.4 % and 11.6 %, respectively) than in the autumn harvest (35.4 % and 10.7 %, respectively) (Fig. 2).

### 3.3. Degradation

The ANOVA revealed a significant effect of fungal pretreatment (P) and pretreatment duration (D) on all analysed components (lignin, cellulose, and hemicellulose degradation). Harvest time and nitrogen fertilisation significantly affected only lignin and hemicellulose degradation (Table 5). Significant H x F and F x P interactions were observed for lignin and cellulose degradation. Fig. 3a and b show the interactions and mean effects of hemicellulose, cellulose, and lignin degradation caused by fungal pretreatment on giant reed biomass after 30 and 50 days.

The maximum percentage of lignin degradation was obtained for fertilised winter biomass pretreated by *I. lacteus* (33.2 %) after 30 days of pretreatment and by *P. ostreatus* (34.2 %) after 50 days. Fertilised autumn biomass pretreated by *P. ostreatus* showed the minimum lignin degradation for both 30 and 50 days of pretreatment (17.2 % and 18.3 %, respectively). The highest cellulose and hemicellulose degradation was observed for winter fertilised biomass pretreated by *I. lacteus* after 30 days (28.9 % and 28.8 %, respectively) and for autumn unfertilised biomass pretreated by *P. ostreatus* after 50 days. (30.8 % and 37.7 %, respectively.) In comparison to the other treatment means, the longest pretreatment duration and *I. lacteus* pretreatment caused higher cellulose and hemicellulose losses compared to 30-day pretreatment and *P. ostreatus*.

**Table 4**
Significance levels from ANOVA output for Harvest, Fertilisation, and their interaction on lignin (%), cellulose (%), and hemicellulose (%) of giant reed under autumn (A) and winter (W) harvest and nitrogen fertilisation levels (N0 and N80 kg N $ha^{-1}$). $p \le 0.01$ (**), $p \le 0.05$ (*), Not significant (ns). HSD ($p < 0.05$) values for mean separation of interactions are reported.

| Source of Variance | Lignin (%) | Cellulose (%) | Hemicellulose (%) | NDS (%) | Ash (%) |
|---|---|---|---|---|---|
| Harvest | ** | ** | ns | ** | *** |
| Fertilisation | ns | ns | ns | ns | ns |
| H x F | ** | ns | * | ns | ns |
| | $HSD_{H \times F} = 0.69$ | | $HSD_{H \times F} = 1.00$ | | |

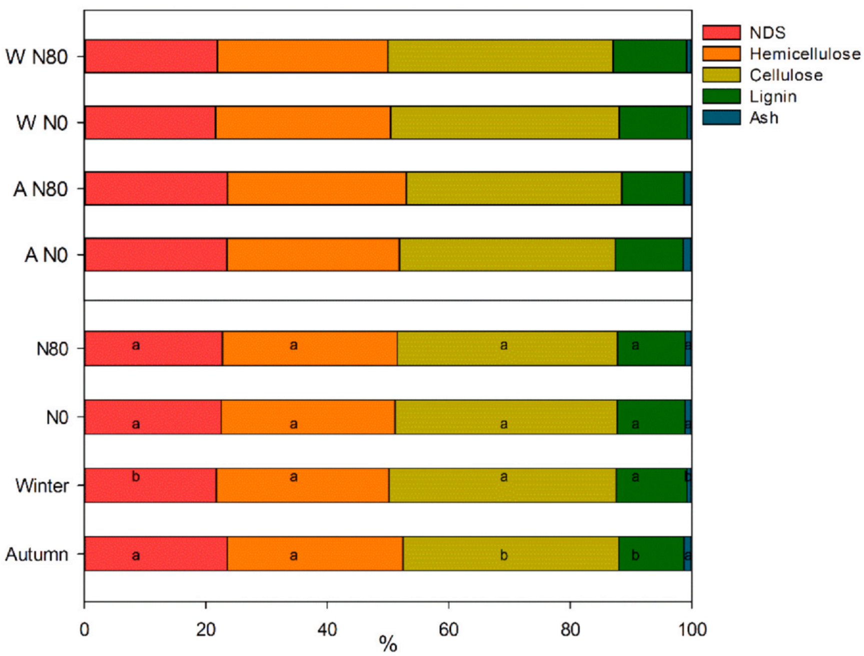


**Fig. 2.** Biomass composition (% w/w), such as hemicellulose, cellulose, lignin, neutral detergent soluble (NDS) and ash of untreated giant reed under autumn (A) and winter (W) harvest and nitrogen fertilisation levels (N0 and N80 kg N $ha^{-1}$). Different letters correspond to significantly different means within each biomass fraction, according to HSD Tukey's test ($p < 0.05$).

**Table 5**
Four-way ANOVA output for main effects of Harvest time(H), Fertilization (F), Pretreatment (P), Pretreatment duration (D), and their interactions on lignin, cellulose, and hemicellulose degradation (%). Adjusted mean square and significance level at $p \le 0.001$ (***), $p \le 0.01$ (**), $p \le 0.05$ (*), Not significant ($^{ns}$) are reported.

| Source of Variance | Lignin (%) | Cellulose (%) | Hemicellulose (%) |
|---|---|---|---|
| Harvest | $575^{***}$ | $8^{ns}$ | $32^{**}$ |
| Fertilisation | $30^{**}$ | $7^{ns}$ | 16 * |
| Pretreatment | $23^{**}$ | $107^{**}$ | $41^{***}$ |
| Duration | $40^{***}$ | $561^{***}$ | $442^{***}$ |
| H x F | $168^{***}$ | 55 * | $0.96^{ns}$ |
| H x P | $4^{ns}$ | $2^{ns}$ | $118^{***}$ |
| H x D | $0.20^{ns}$ | $0.24^{ns}$ | $97^{***}$ |
| F x P | $152^{***}$ | $146^{***}$ | $158^{***}$ |
| F x D | $0.58^{ns}$ | $116^{**}$ | $58^{***}$ |
| P x D | $0.45^{ns}$ | $79^{**}$ | $0.01^{ns}$ |
| H x F x P | $3^{ns}$ | $0.00^{ns}$ | 9 * |
| H x F x D | $2^{ns}$ | $0.02^{ns}$ | $0.10^{ns}$ |
| H x P x D | $3^{ns}$ | 54 * | $0.09^{ns}$ |
| F x P x D | $1^{ns}$ | $23^{ns}$ | $40^{***}$ |
| H x F x P x D | $1^{ns}$ | $86^{ns}$ | $60^{***}$ |

### 3.4. Biomethane production

The ANOVA showed a significant effect of fertilisation on cumulative biomethane production from untreated biomass (Table 6). The cumulative biomethane production obtained by pretreated biomass was influenced by harvest time, fertilization, fungal pretreatment, and duration. Significant H x P and P x D interactions were also observed (Table 7). The cumulative biomethane production from untreated biomass was higher in fertilised biomass (112 and 106NmL $g^{-1}$VS for winter and autumn, respectively) than in unfertilised (89 and 95 NmL $g^{-1}$VS for winter and autumn, respectively) (Table 7). Biomass pretreated by *P. ostreatus* for 30 days showed the highest BMP values for both harvest time and fertilisation levels, with the maximum production (148 NmL $g^{-1}$VS) obtained by winter fertilised biomass. The 50-day pretreatment and *I. lacteus* pretreatment induced a decrease in biomethane production compared to untreated biomass. The mean effect of the main factors on cumulative biomethane production is reported in Fig. 4.

### 3.5. Biomethane yield

The ANOVA showed that the biomethane yield of the untreated biomass was significantly affected by fertilisation ($p \le 0.001$), but no significant effect of harvest time was found (Table 6). Regarding the pretreated biomass, harvest time, fertilisation, pretreatment, and duration significantly affected biomethane yield. Additionally, significant H x F, H x P, H x D, and P x D interactions were observed (Table 8). The untreated fertilised winter biomass (WN80) showed the highest biomethane yield (1614 $m^3$ $CH_4$ $ha^{-1}$), followed by the fertilised autumn biomass (1159 $m^3$ $CH_4$ $ha^{-1}$) (Table 9).

The highest biomethane yield from pretreated biomass was obtained from fertilised winter biomass pretreated with *P. ostreatus* for 30 days (2122 $m^3$ $CH_4$ $ha^{-1}$) and 50 days (1736 $m^3$ $CH_4$ $ha^{-1}$). *Pleurotus ostreatus* 30-day pretreatment was associated with a 31.5 % increase in biomethane yield compared to the untreated biomass. The lowest biomethane yields were observed in unfertilised biomass pretreated with *I.*

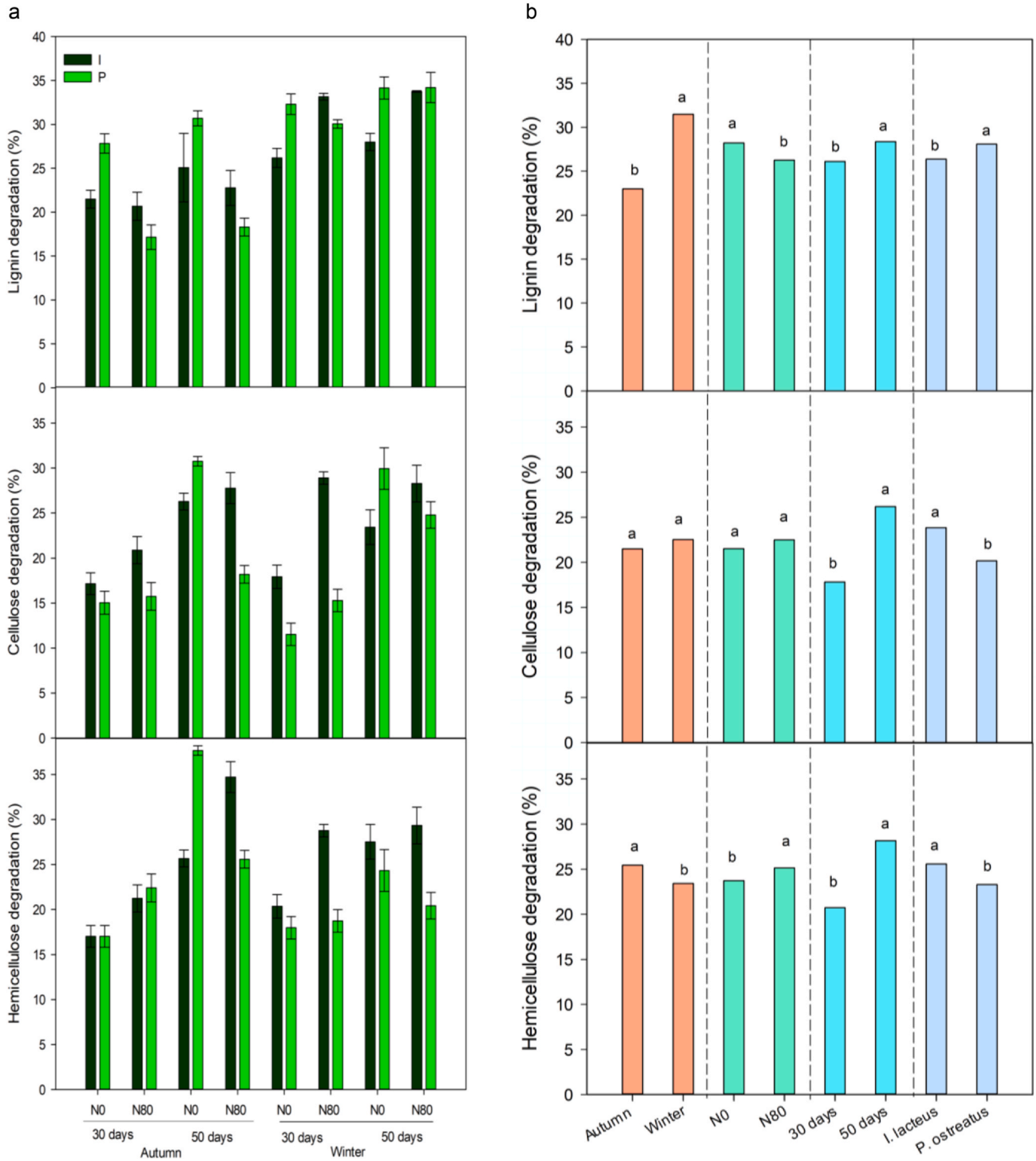


**Fig. 3.** a. Degradation (% ± SE) of giant reed components: lignin, cellulose, and hemicellulose after 30 and 50-day of fungal pretreatment in unfertilised N0 and fertilised N80 winter and autumn by *I. lacteus* (I) and *P. ostreatus* (P) pretreatments. b. Mean effect of Harvest period, Fertilization, Pretreatment duration, and Pretreatment type on degradation (%) of biomass components (lignin, cellulose, and hemicellulose). Different letters correspond to significantly different means according to HSD Tukey's test ($p < 0.05$) for each main factor.

*lacteus* for both harvest times and pretreatment durations. The mean effects of untreated and pretreated biomass are reported in Fig. 5.

## 4. Discussion

The high dry biomass yield obtained from giant reed, ranging between 9.6 (WN0) and 13.9 (WN80) Mg ha$^{-1}$ for the 25th cropping cycle, confirms the high productivity of *Arundo donax*, even when grown for more than 20 years and maintained in rainfed conditions, as reported by other long-term studies (Alexopoulou et al., 2015; Ciria et al., 2020; Monti and Zegada-Lizarazu, 2016). Harvest time did not induce significant differences in total dry biomass yield. However, there was a significant difference considering the leaf fraction yield due to the winter fall of leaves, which caused a decrease in total yield. A similar

**Table 6**
Two-way ANOVA output for main effects Harvest (H), Fertilization(F), and their interactions on biomethane production (Nml $g^{-1}$ VS) and yield per hectare ($m^3$ $ha^{-1}$) from untreated giant reed biomass. Adjusted mean square and significance level at $p \leq 0.001$ (***), $p \leq 0.01$ (**), $p \leq 0.05$ (*), not significant ($^{ns}$) are reported.

| Source of Variance | BMP (Nml $g^{-1}$ VS) | Biomethane yield ($m^3$ $ha^{-1}$) |
|---|---|---|
| Harvest | $0.04^{ns}$ | $50357^{ns}$ |
| Fertilisation | $583^{**}$ | $665390^{***}$ |
| H × F | $88^{ns}$ | 175217 * |

result was observed by Fagnano et al. (2015) who reported that postponing the harvest from October-November to January-February over 9 years of cultivation, modified the biomass composition by reducing the presence of leaves from 28.7 % to 12.9 %.

The N-fertilized biomass yield was significantly higher, demonstrating the positive effect of nitrogen on plant productivity in accordance with previous studies (Nassi o Di Nasso et al., 2010). Angelini et al. (2005) reported that fertilization increased biomass yield by approximately 15 % compared to unfertilized conditions, with yields rising from 23 to 27 dry Mg $ha^{-1}$ over six years. They also observed higher dry matter yields in winter harvests compared to autumn harvests, suggesting that delaying harvest to winter can optimize the yield under fertilized conditions.

The adaptability of giant reed to changing climate conditions, including rising temperatures and altered precipitation patterns, makes it a valuable crop for sustainable bioenergy production and ecological restoration (Sánchez et al., 2021). Its cultivation, particularly in marginal areas, can yield economic benefits and provide a profitable alternative to high-input energy crops. Therefore, growing giant reed in small-scale marginal areas of the Mediterranean region could be a wise choice both economically and environmentally (Jámbor and Török, 2019).

The results of biomass quality from our study were in line with those of Ceotto et al. (2021). These authors reported cellulose as the major component of the giant reed biomass (35–43 % of the total solids), followed by hemicellulose (23–30 %) and lignin (6–11 %). Except for hemicellulose, we found significant differences between harvest times for all components, and the interaction "harvest time x fertilisation" was observed for hemicellulose and lignin content. The quantities of soluble substances, hemicellulose, and ashes decreased from autumn to winter, whereas lignin and cellulose were higher in the winter harvest. This trend is consistent with the higher lignin deposition and mineral translocation to the rhizomes that occurs during winter, as well as the decrease in the fraction of leaves, which have lower lignin and ash content than the stems (Monti et al., 2008; Nassi O Di Nasso et al., 2011).

The qualitative analysis of giant reed biomass indicates its potential to be used as a substrate for anaerobic digestion thanks to the high percentage of cellulose and hemicellulose. Dragoni et al., 2015 carried out a study to compare ensiled giant reed with conventional annual crops such as maize and two sorghum varieties proving their suitability for biogas production and their land use and nitrogen utilisation efficiency. The results showed that giant reed can achieve higher methane yields than maize and sorghum in double harvest systems; giant reed was also more land-efficient, requiring only 0.22 ha $kWe^{-1}$ under double harvest systems and 0.27 ha $kWe^{-1}$ under single late harvest, compared to about 0.30 ha $kWe^{-1}$ for annual crops like maize and sorghum. Moreover, giant reed showed higher nitrogen utilization efficiency with

**Table 7**
Cumulative biomethane production (NmL $g^{-1}$ VS ± SE) for pretreated giant reed biomass unfertilised N0 and fertilised N80 winter and autumn harvest pretreated giant reed after 30 and 50 days of fungal pretreatment by *I. lacteus* and *P. ostreatus* pretreatment, and for untreated giant reed biomass.

| | | Autumn | | Winter | |
|---|---|---|---|---|---|
| | Days | N0 | N80 | N0 | N80 |
| *Pleurotus ostreatus* | 30 | 107 ± 3 | 124 ± 11 | 134 ± 6 | 148 ± 11 |
| | 50 | 70 ± 3 | 110 ± 4 | 85 ± 7 | 121 ± 7 |
| *Irpex lacteus* | 30 | 76 ± 2 | 95 ± 5 | 80 ± 3 | 92 ± 7 |
| | 50 | 68 ± 7 | 91 ± 1 | 71 ± 25 | 85 ± 4 |
| Untreated | | 95 ± 3 | 106 ± 3 | 89 ± 1 | 112 ± 8 |

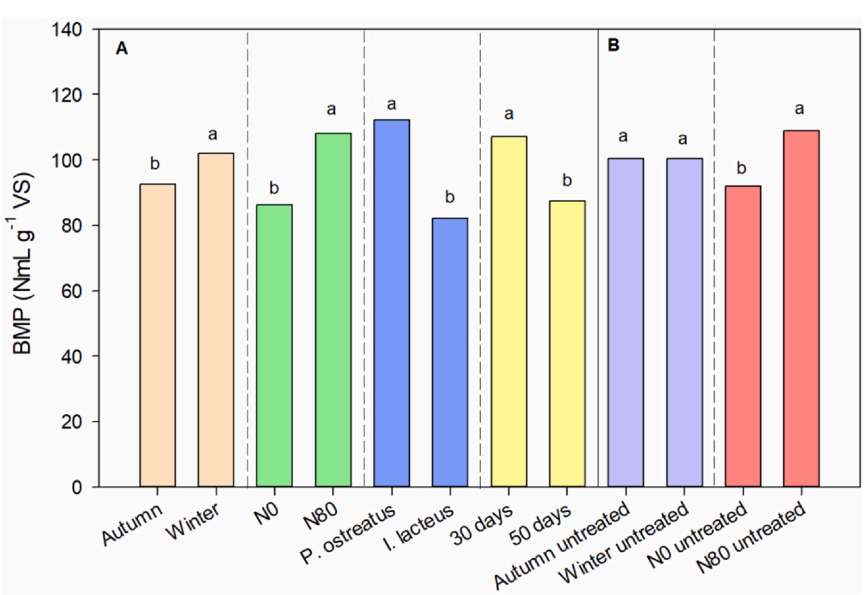


**Fig. 4.** (A) Mean effect of Harvest period, Fertilization, Pretreatment type, and Pretreatment duration on cumulative biomethane (NmL $g^{-1}$ VS) for pretreated giant reed biomass. (B) Mean effect of Harvest period and Fertilization on cumulative biomethane (NmL $g^{-1}$ VS) for untreated giant reed biomass. Different letters correspond to significantly different means according to HSD Tukey's test ($p < 0.05$) for each main factor.

**Table 8**
Four-way ANOVA output for main effects Harvest (H), Fertilization (F), Pretreatment (P), Pretreatment duration (D), and their interactions on biomethane production (N mL $g^{-1}$ VS) and yield ($m^3$ $CH_4$ $ha^{-1}$) from pretreated giant reed biomass. Adjusted mean square and significance level at $p \leq 0.001$ (***), $p \leq 0.01$ (**), $p \leq 0.05$ (*), not significant ($^{ns}$) are reported.

| Source of Variance | BMP (Nml $g^{-1}$ VS) | Biomethane yield ($m^3$ $ha^{-1}$) |
|---|---|---|
| Harvest | 703** | 305750** |
| Fertilisation | 3856*** | 3051438*** |
| Pretreatment | 7241*** | 1025519*** |
| Duration | 3084*** | 274440** |
| H x F | 77ns | 150557 * |
| H x P | 766** | 134345 * |
| H x D | 88ns | 144305 * |
| F x P | 191ns | 112489ns |
| F x D | 311ns | 117447ns |
| P x D | 1234*** | 136735 * |
| H x F x P | 6ns | 23024ns |
| H x F x D | 1ns | 17253ns |
| H x P x D | 62ns | 17126ns |
| F x P x D | 198ns | 17342ns |
| H x F x P x D | 0.17ns | 908ns |

single harvests reaching up to 64 $Nm^3$ $CH_4$ $kgN^{-1}$ , compared to 28–40 $Nm^3$ $CH_4$ $kgN^{-1}$ for maize and sorghum.

Although there is a high concentration of cellulose and hemicellulose, the conversion process is complicated due to the significant lignin content. Therefore, it becomes necessary to adopt a pretreatment method to reduce the lignin concentration, facilitate hydrolysis, and improve the cellulose and hemicellulose digestibility. In this study, a biological pretreatment using white-rot fungi (*P. ostreatus* and *I. lacteus*) was performed on giant reed biomass for 30 and 50 days. The longer pretreatment leads to higher lignin degradation but also results in an excessive consumption of cellulose and hemicellulose resulting in lower biomethane production. During the 30-day pretreatment, *I. lacteus* achieved the highest lignin degradation for fertilised winter biomass, while also causing the highest cellulose degradation involving a decrease in biomethane production compared to untreated biomass. During biological pretreatment, microorganisms produce oxidative hydrolytic enzymes to break down the phenyl bonds in lignin, but to also obtain from biomass the carbon source for their growth resulting in cellulose and hemicellulose degradation. The synergic mechanisms of these enzymes are complex and not fully understood (Manyi-Loh and Lues, 2023). Previous studies reported that *P. ostreatus* has a remarkable ability to adapt and respond to various substrates by secreting enzymes more suited for woody materials, with a particular preference for lignin degradation, although several factors affect the enzyme production by *Pleurotus* spp. during the biological pretreatment. For example, the enzyme adsorption capacity of a substrate could be influenced by roughness, biomass composition, and interactions between substrate and enzymes (Baig, 2020; Durán-Aranguren et al., 2021). These factors related to raw materials, together with fungal strains, inoculum preparation methods, and cultivation conditions, influence fungal growth, resulting in conflicting results regarding the incubation time and the ability of white-rot fungi to selectively degrade lignin and maintain cellulose and hemicellulose. An increase of 120% in biomethane production was observed by Mustafa et al. (2016) when rice straw was pretreated with *P. ostreatus* for 20 days compared to untreated straw, while Wyman et al. (2018) observed a negative impact on methane production after a *P. ostreatus* pretreatment of corn stover for 30 days. Taniguchi et al. (2005) found a similar negative effect, reporting

**Table 9**
Biomethane yield ($m^3$ $CH_4$ $ha^{-1}$ ± SE) for untreated and pretreated giant reed after 30 and 50 days of fungal pretreatment in unfertilised N0 and fertilised N80 winter and autumn harvest by *I. lacteus* and *P. ostreatus*.

| | | Autumn | | Winter | |
|---|---|---|---|---|---|
| | Days | N0 | N80 | N0 | N80 |
| *Pleurotus ostreatus* | 30 | 1048 ± 64 | 1369 ± 191 | 1306 ± 191 | 2122 ± 147 |
| | 50 | 688 ± 54 | 1458 ± 274 | 699 ± 139 | 1736 ± 340 |
| *Irpex lacteus* | 30 | 743 ± 45 | 1048 ± 117 | 778 ± 116 | 1321 ± 92 |
| | 50 | 666 ± 92 | 1192 ± 167 | 596 ± 273 | 1218 ± 119 |
| Untreated | | 879 ± 52 | 1159 ± 96 | 741 ± 92 | 1614 ± 112 |

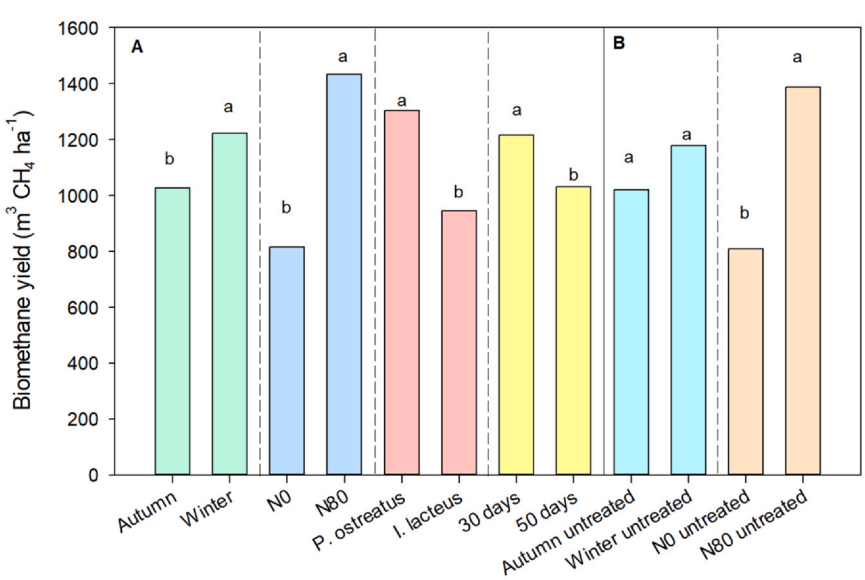


**Fig. 5.** (A) Mean effect of Harvest period, Fertilization, Pretreatment type, and Pretreatment duration on biomethane yield ($m^3$ $CH_4$ $ha^{-1}$) for pretreated giant reed biomass. (B) Mean effect of Harvest period and Fertilization on biomethane yield ($m^3$ $CH_4$ $ha^{-1}$) for untreated giant reed biomass. Different letters correspond to significantly different means according to HSD Tukey's test($p < 0.05$) for each main factor.

reduced cellulose and hemicellulose content and lower methane production in rice straw after a long pretreatment of 60 days with *P. ostreatus*. Basinas et al. (2022) reported *I. lacteus* strain as being a non-selective white-rot fungus that reduced the biomethane yield from corn silage by 20.6% due to the consumption of holocellulose. On the other hand, Testa et al. (2023) observed a greater selective ability of *I. lacteus* on castor husks with lignin degradation of 25% compared to *P. ostreatus* that achieved a 20% of lignin degradation after 30 days of pretreatment.

The rate of degradation of biomass components reflects the final biomethane production. This results in the highest BMP from winter fertilised and unfertilised biomass pretreated by *P. ostreatus*, with an increase of 31% and 51%, respectively, compared to untreated biomass. In contrast, *I. lacteus* pretreatment induced a lower BMP than untreated biomass for both harvest times and fertilisation levels. Similar results were previously reported by Piccitto et al. (2022), who found a negative effect of *I. lacteus* pretreatment on giant reed biomass that decreased the methane yield by −27%, while *P. ostreatus* showed a + 34% methane yield compared to untreated biomass.

Considering the most common pretreatment methods to improve biomethane production from giant reed biomass, chemical pretreatments, particularly alkaline methods using NaOH or $Ca(OH)_2$, have been effective in improving enzymatic digestibility and methane production. NaOH pretreatment increased methane yields up to 1.6-fold, although it was not cost-effective due to high chemical costs (Jiang et al., 2017). In contrast, $Ca(OH)_2$ pretreatment provided a net economic benefit, making it a more feasible option. Similarly, KOH pretreatment enhanced methane yield by 21% and improved the overall efficiency of the anaerobic digestion process (Vasmara et al., 2021). Mechanical pretreatment, such as dry milling, effectively increases methane yields by reducing particle size and enhancing the surface area for microbial action. Dell'Omo et al. (2018) reported a 137% increase in biogas production from mechanically pretreated giant reed compared to untreated material. This method also reduces the energy required for pretreatment, making it economically viable for large-scale applications (Dell'Omo and Spena, 2020).

Biological pretreatment is a safe and environmentally friendly technology. It uses no chemicals and does not involve acids, alkalis, or any reactive substances, which helps reduce waste output and lower downstream processing costs. Additionally, it results in minimal inhibitor formation and produces by-products that do not interfere with the hydrolysis process. However, despite these benefits, biological pretreatment faces several challenges. These include long processing times, the need for large spaces, and the necessity for continuous monitoring of microorganism growth. When comparing different pretreatment methods, alkaline pretreatments generally outperform others in terms of methane yield improvement. However, the economic and environmental impacts of chemical usage must be considered. Mechanical pretreatment offers a balance between efficiency and cost, while biological pretreatment, although promising, requires further optimization to enhance its selectivity and effectiveness (Vasmara et al., 2023). The biomethane yield was mainly influenced by the dry biomass yield, so the highest biomethane yield was found in fertilised biomass pretreated by *P. ostreatus* for 30 days followed by the biomass pretreated for 50 days.

## 5. Conclusion

This study highlighted the potential of giant reed to grow with no or low agronomic inputs under rainfed conditions. This trait is of considerable interest in terms of the production of bioenergy and non-food crops, especially if cultivation is conducted in the Mediterranean where water availability is a limiting factor for plant growth. Additionally, giant reed, as a long-term plantation, provided satisfactory biomass yield even after more than 20 years of cultivation, demonstrating its potential as a sustainable crop for environmentally friendly biomass production and bioenergy conversion. The biomethane production from giant reed was influenced by agronomic practices, which contributed to modification of the qualitative composition of biomass. Strategic management of harvest time and nitrogen fertilisation supply contribute to maximizing biomass yield, increasing the digestibility of the biomass and enhancing biomethane production. Moreover, the application of fungal pretreatment emerged as a promising strategy to further enhance the biodegradability of giant reed biomass and improve the overall efficiency of the bioconversion process. In this study, the fungal pretreatment using *P. ostreatus* showed the best results in terms of biomass degradation and biomethane yield. Overall, the integration of sustainable agricultural management with innovative pretreatment methods represents a viable pathway for maximizing giant reed potential.

## CRediT authorship contribution statement

**Testa Giorgio:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Cosentino Salvatore Luciano:** Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization. **Ciaramella Barbara Rachele:** Writing – original draft, Investigation, Formal analysis. **Iurato Antonella:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Piccitto Alessandra:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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