Co-Fermentation of Microalgal Biomass and Miscanthus × giganteus Silage—Assessment of the Substrate, Biogas Production and Digestate Characteristics

Marcin Dębowski 1, Joanna Kazimierowicz 2,* , Marcin Zieliński 1 and Izabela Bartkowska 2

1 Department of Environmental Engineering, Faculty of Geoengineering, University of Warmia and Mazury in Olsztyn, 10-720 Olsztyn, Poland; marcin.debowskii@uwm.edu.pl (M.D.); marcin.zielinski@uwm.edu.pl (M.Z.)
2 Department of Water Supply and Sewage Systems, Faculty of Civil Engineering and Environmental Sciences, Białystok University of Technology, 15-351 Białystok, Poland; i.bartkowska@pb.edu.pl
* Correspondence: j.kazimierowicz@pb.edu.pl

Abstract: The development of a sustainable bioenergy market is currently largely fueled by energy crops, whose ever-increasing production competes with the global food and feed supply. Consequently, non-food crops need to be considered as alternatives for energy biomass production. Such alternatives include microalgal biomass, as well as energy crops grown on non-agricultural land. The aim of the present study was to evaluate how co-digestion of microalgal biomass with giant miscanthus silage affects feedstock properties, the biogas production process, biogas yields, methane fractions and the digestate profile. Combining giant miscanthus silage with microbial biomass was found to produce better C/N ratios than using either substrate alone. The highest biogas and methane production rates—628.00 ± 20.05 cm³/g VS and 3045.56 ± 274.06 cm³ CH₄/d—were obtained with 40% microalgae in the feedstock. In all variants, the bulk of the microbial community consisted of bacteria (EUB338) and archaea (ARC915).

Keywords: co-digestion; methane; C/N ratio; energy crops; microalgal biomass; bacterial community

1. Introduction

The development of a sustainable bioenergy market is currently largely fueled by dedicated energy crops [1]. Plant biomass is widely used in combustion or pyrolysis plants, as well as in bioenergy systems for producing biodiesel, bioethanol and gaseous fuels (including methane and hydrogen) [2]. It is widely held that biomass energy will reduce the production and consumption of conventional fuels, thus mitigating greenhouse gas emissions and the associated dangers to the climate [3].

However, this prevailing view is contested by some studies. It has been shown that energy crop production on arable land competes with the global food and feed supply [4]. In addition, devoting ever-larger swaths of land to biomass production leads to the creation of expansive monocultures harmful to ecosystems [5]. Researchers have also found that mismanagement of traditional energy crop reserves may actually exacerbate greenhouse gas emissions [6]. Consequently, there is a need to seek non-food plant species that could be used to grow biomass for energy production. These crops should be able to grow in low-input production systems and be characterized by high yield, low nutrient demand and good composition for industrial applications [7,8]. The data in the literature suggest that microalgal biomass is a viable alternative to typical energy crops [9–11]. Algal biomass offers an estimated productivity of 100–150 t/ha (10–15 times higher than conventional crops) and needs less area to grow compared with terrestrial plants [12]. For added environmental and commercial benefit, microalgal cultures can also be grown on waste feedstock, such as agri-food effluent or flue gas [13,14]. Much like traditional energy crops,
microalgal biomass can be converted into many types of biofuels—biodiesel, biohydrogen or biogas—or can be combusted as is [15–17]. Some species of microalgae accumulate large amounts of lipids, starches and proteins, while containing little to no hard-to-degrade lignin, making them prime candidates for biomethane production feedstocks [18,19]. Algae can generate 140 to 360 dm$^3$/kgVS biomethane, depending on the species [20]. The biogas produced from microalgal biomass contains 55 to 75% methane, matching or even exceeding the calorific value of biogas produced from other plant substrates [21]. Anaerobic digestion is currently widely used to produce renewable energy from organic biomass [22,23]. However, the performance of this process can be hampered by several limiting factors, one of which is an inadequate ratio of carbon (C) to nitrogen (N) in the reactor feedstock [24]. The optimal C/N ratio in the feedstock is between 20/1 and 30/1 [25]. By contrast, the C/N ratio of algal biomass is generally below 10, which may induce high levels of ammonia nitrogen and volatile fatty acids, thus impeding methane generation [26].

Most agricultural biogas plants in the EU use maize silage as the primary organic substrate for biogas generation [27]. However, the use of maize—a food and feed crop—for biogas production has raised controversy and concerns due to the crop’s rising price and the adverse impact of maize monoculture on water resources, soil properties and flora/fauna biodiversity [28]. It would therefore be reasonable to pursue biogas production from energy-efficient crops as an alternative to maize. Due to the high yield of biomass, low environmental requirements and the possibility of cultivation on low-quality and marginal soils, Miscanthus × giganteus is a fully justified choice [29]. Its suitability for producing many types of biofuels has been verified. In the case of methane fermentation, limitations related to the high content of lignocellulose complexes, low hydration or inappropriate C/N ratios were indicated [30]. One of the possibilities of reducing or eliminating the weaknesses of Miscanthus × giganteus biomass may be co-fermentation with microalgal biomass. A detailed analysis of the anaerobic digestion of such a substrate composition has not been presented so far, which has become the inspiration and the basis for conducting research to verify this hypothesis.

Co-digestion of the two substrates may be a way to solve both problems: limiting the land required for growing energy crops while optimizing the digestion of microalgal biomass [31,32]. The main benefits of co-digestion include more favorable moisture levels, optimization of the pH and C/N ratios, greater buffer capacity and the dilution of toxic compounds, thus improving biogas/methane yields [33]. The higher biogas output from the co-digestion of algal biomass and other organic feedstocks has been attributed to the synergistic effects related to optimization of the micronutrients needed for the optimal growth of anaerobic microbes [34]. It should be emphasized that the literature describes the efficiency of co-fermenting microalgal biomass with many organic substrates, including sewage sludge [35], Virginia mallow [36] and various types of waste and wastewater [37,38] as well as maize silage [39]. However, there are no reports on the possibility of anaerobic digestion of the feedstock composed of microalgal biomass and Miscanthus × giganteus silage. Research in this field is important because both substrates are perceived as prospective and promising biomass for biofuel production [40].

The aim of the present study was to evaluate how co-digestion of microalgal biomass with giant miscanthus silage affects feedstock properties, the biogas production process, biogas yields, methane fractions and the digestate profile. The experiment was carried out in continuous reactors. Different proportions of microalgal biomass were tested for their effect on the system’s performance.

2. Materials and Methods
2.1. Study Design

Microalgal biomass was co-digested with giant miscanthus silage in continuous reactors. Throughout the experiment, different ratios of dry microalgal organic matter (MA) to dry giant miscanthus organic matter (MS) were tested. A water-diluted mixture of MA
and MS biomass (90% water content) was used as a feedstock, with the following ratios of dry organic matter: Variant 1, 100:0% (MA100); Variant 2, 80:20% (MA80 + MS20); Variant 3, 60:40% (MA60 + MS40); Variant 4, 40:60% (MA40 + MS60); Variant 5, 20:80% (MA20 + MS80); Variant 6, 0:100% (MA100).

2.2. Materials

The MA biomass was self-cultivated and consisted of a mixed culture of *Chlorella* sp. (70%) and *Scenedesmus* sp. (30%). The MA biomass was grown in circulation ditch-type photobioreactors with an active volume of 2.0 m$^3$ (Figure 1). Digestate liquor was used as the growth medium. The culture and photobioreactor parameters were described in Dębowski et al. [41]. The mixotrophic culture of MA was conducted over a period of 14 days, targeting a biomass concentration of 1500 mgTS/dm$^3$. The resulting biomass was dewatered on a drum microsieve with a mesh diameter of 10.0 µm, then concentrated in a laboratory centrifuge (3000 RPM for 6 min).

Figure 1. Photobioreactor for the cultivation of microalgae biomass used as feedstock in anaerobic digestion: (a) microalgae biomass; (b) general view of the photobioreactor.

Giant miscanthus (*Miscanthus × giganteus*) silage was sourced from cropland owned by the Teaching and Research Station of the University of Warmia and Mazury in Olsztyn. The station is sited in the village of Baldy (Poland) (53.59962 N, 20.61155 E). The anaerobic reactors were inoculated with anaerobic sludge sourced from the enclosed digesters of the Municipal Water Treatment Plant in Olsztyn (53.81321 N, 20.44956 E) operating at OLR = 2.2 kgVS/m$^3$·d and HRT = 20 days. The profiles of the feedstock and anaerobic sludge are presented in Table 1.
Table 1. Profile of the feedstock digested in different variants and anaerobic sludge (inoculum).  

| Parameter                       | Unit       | 1—MA100+MS0 | 2—MA80+MS20 | 3—MA60+MS40 | 4—MA40+MS60 | 5—MA20+MS80 | 6—MA0+MS100 | Inoculum |
|---------------------------------|------------|-------------|-------------|-------------|-------------|-------------|-------------|----------|
| Dry matter (TS) %               |            | 10.4 ± 1.5  | 12.0 ± 1.4  | 14.0 ± 1.2  | 17.1 ± 1.1  | 21.8 ± 1.0  | 30.2 ± 0.9  | 3.8 ± 0.2 |
| Dry organic matter (VS) % TS    |            | 87.7 ± 1.1  | 88.9 ± 0.9  | 90.2 ± 0.7  | 91.4 ± 0.5  | 92.6 ± 0.4  | 93.8 ± 0.2  | 68.5 ± 2.5 |
| Total nitrogen (TN) mg/gTS      |            | 46.0 ± 3.9  | 39.0 ± 3.3  | 32.0 ± 2.7  | 25.1 ± 2.1  | 18.1 ± 1.5  | 11.1 ± 0.9  | 33.1 ± 3.4 |
| Total phosphorus (TP) mg/gTS    |            | 4.4 ± 0.9   | 4.0 ± 0.8   | 3.6 ± 0.7   | 3.2 ± 0.5   | 2.8 ± 0.4   | 2.4 ± 0.3   | 1.7 ± 0.2  |
| Total carbon (TC) mg/gTS        |            | 464 ± 25    | 463 ± 23    | 462 ± 20    | 462 ± 18    | 461 ± 15    | 460 ± 13    | 309 ± 28  |
| Total organic carbon (TOC) mg/gTS|          | 437 ± 20    | 438 ± 19    | 439 ± 18    | 440 ± 17    | 440 ± 16    | 441 ± 15    | 199 ± 34  |
| C:N ratio                       |            | 9.5 ± 0.4   | 11.2 ± 0.7  | 13.7 ± 0.9  | 17.5 ± 1.2  | 24.3 ± 1.4  | 39.6 ± 1.7  | 9.4 ± 0.1  |
| pH                              |            | 8.1 ± 0.8   | 7.8 ± 0.6   | 7.4 ± 0.5   | 6.9 ± 0.4   | 6.0 ± 0.2   | 4.4 ± 0.1   | 7.2 ± 0.3  |
| Protein % TS                    |            | 28.7 ± 2.5  | 20.9 ± 2.1  | 13.1 ± 1.7  | 24.8 ± 1.4  | 17.0 ± 1.0  | 9.2 ± 0.6   | 20.7 ± 2.8 |
| Lipids % TS                     |            | 20.0 ± 1.4  | 12.9 ± 1.2  | 5.8 ± 1.0   | 16.4 ± 0.9  | 9.3 ± 0.7   | 2.2 ± 0.5   | 3.1 ± 0.5  |
| Sugars % TS                     |            | 15.8 ± 2.6  | 33.7 ± 2.3  | 51.5 ± 2.0  | 24.8 ± 1.6  | 42.6 ± 1.3  | 60.4 ± 1.0  | 1.6 ± 0.4  |

2.3. Experimental Set-Up

The digestion process was conducted in anaerobic reactors with full mixing and an active volume of 4.0 dm³ (total volume: 5.0 dm³) (Figure 2). The reactors were kept in an insulated, heated chamber. The temperature in the reaction chambers was maintained at 38 °C. The starting concentration of anaerobic sludge in the anaerobic chambers was approximately 4.0 gTS/dm³. The start-up took 60 days, during which the reactors were filled with the inoculum, then fed with a continuous supply of the feedstock. After start-up, the organic load rate (OLR) was around 2.0 gVS/dm³·d. The hydraulic retention time (HRT) was 40 d in all of the variants. The experiment was run for 80 days, with samples taken every 4 days. The study design is presented in Table 2.

![Figure 2. The set of anaerobic reactors used in the experiments: (a) technical diagram; (b) general view; (c) thermally insulated chamber with a heating system.](image-url)
### Table 2. Study design.

| Variant     | Reactor Active Volume (dm$^3$) | OLR (gVS/dm$^3·$d) | MA Wet Mass (g/d) | MS Wet Mass (g/d) | Initial Water Content of MA + MS Mixture (%) | Volume of Water Used for Dilution (cm$^3$) | Daily Volume Supply of Feedstock (cm$^3$) | Mixture Water Content (%) | VS (% TS) | HRT (d) | Days of Experiment (d) |
|-------------|-------------------------------|---------------------|-------------------|-------------------|---------------------------------------------|-------------------------------------------|-------------------------------------------|--------------------------|------------|--------|----------------------|
| 1: MA100/MS0 | 4.0                           | 2.0                 | 87.7              | 0                 | 89.6                                        | 90.9                                      | 87.7                                      | 40                       | 80         |        |                      |
| 2: MA80/MS20 | 52.6                          | 11.3                | 88.1              | 5.6               | 85.9                                        | 91.0                                      | 87.8                                      | 40                       | 80         |        |                      |
| 3: MA60/MS40 | 35.0                          | 16.9                | 82.9              | 29.8              | 91.0                                        | 91.0                                      | 87.9                                      | 40                       | 80         |        |                      |
| 4: MA40/MS60 | 17.5                          | 22.6                | 78.2              | 41.4              | 91.0                                        | 91.2                                      | 88.1                                      | 40                       | 80         |        |                      |
| 5: MA20/MS80 | 0                             | 28.2                | 69.8              | 76.7              | 91.5                                        | 91.3                                      | 88.4                                      | 40                       | 80         |        |                      |

#### 2.4. Calculation Methods

The fermentation rate coefficient (Equation (1)) and the VS removal rate (efficiency) coefficient (Equation (2)) were determined using the following equations:

\[
\eta_{\text{MSS}} = \frac{\text{VS}_{\text{in}} \cdot \rho_{\text{in}} \cdot Q_{\text{in}} - \text{VS}_{\text{out}} \cdot \rho_{\text{out}} \cdot Q_{\text{out}}}{\text{VS}_{\text{in}} \cdot \rho_{\text{in}} \cdot Q_{\text{in}}}
\]

\[
\eta_{\text{VS}} = \frac{\text{VS}_{\text{in}} \cdot Q_{\text{in}} - \text{VS}_{\text{out}} \cdot Q_{\text{out}}}{\text{VS}_{\text{in}} \cdot Q_{\text{in}}}
\]

where $\eta_{\text{MSS}}$ is the portion digested (%), $\eta_{\text{VS}}$ is the VS removal rate (%), $\text{VS}_{\text{in}}$ is the VS in the supplied influent (g/d), $\text{VS}_{\text{out}}$ is the VS in the discharged digestate (g/d), $\rho_{\text{in}}$ is the influent’s density (g/cm$^3$), $\rho_{\text{out}}$ is the digestate’s density (g/cm$^3$), $Q_{\text{in}}$ is the daily volume of feedstock coming in (cm$^3$/d) and $Q_{\text{out}}$ is the daily volume of digestate going out (cm$^3$/d).

The yields of biogas/CH$_4$ per VS removed (Equation (3)) and the yields of biogas/CH$_4$ per VS load fed in with the influent (Equation (4)) were calculated as:

\[
Y_{\text{b/CH}_4}^{\text{VS}_{\text{removed}}} = \frac{V_{\text{b/CH}_4}}{(\text{VS}_{\text{in}} \cdot \rho_{\text{in}} \cdot Q_{\text{in}} - \text{VS}_{\text{out}} \cdot \rho_{\text{out}} \cdot Q_{\text{out}})/1000}
\]

\[
Y_{\text{b/CH}_4}^{\text{VS}_{\text{in}}} = \frac{V_{\text{b/CH}_4}}{(\text{VS}_{\text{in}} \cdot \rho_{\text{in}} \cdot Q_{\text{in}})/1000}
\]

where $Y_{\text{b/CH}_4}^{\text{VS}_{\text{removed}}}$ is the biogas/CH$_4$ yield in relation to VS$_{\text{removed}}$ (cm$^3$/gVS$_{\text{removed}}$), $Y_{\text{b/CH}_4}^{\text{VS}_{\text{in}}}$ is the biogas or CH$_4$ yield in relation to the VS in the influent (cm$^3$/gVS$_{\text{in}}$), $V_{\text{b/CH}_4}$ is the volume of biogas or CH$_4$ produced from the influent load (cm$^3$/d), $Q_{\text{in}}$ is the volume of a single load of influent fed into the reactor (cm$^3$) and $Q_{\text{out}}$ is the volume of a single discharge of AD digestate from the reactor (cm$^3$).

#### 2.5. Analytical Methods

The identification of microalgae species was carried out in non-solid or semi-solid preparations [29]. TS and VS in the MA biomass, MS biomass and the anaerobic sludge were determined gravimetrically. TC, TOC and TN were quantified using a Flash 2000 analyzer (Thermo Scientific, Waltham, MA, USA). The content of TP and sugars (saccharides) was determined with a DR 2800 spectrophotometer (HACH Lange, Loveland, CO, USA). The lipid content was assayed using the Soxhlet method with an extractor (B-811 Büchi, Flawil, Switzerland). Total protein content was estimated by multiplying total nitrogen by a
conversion factor of 6.25. The pH of aqueous homogenized solutions was measured with a pH meter (1000 L, VWR, Radnor, PA, USA). Potassium (K) was determined by using an air acetylene flame atomic absorption spectrometer (Perkin Elmer Analyst 700 Model, Louisville, KY, USA).

The current and total biogas flow rate were measured continuously using XFM17S digital gas flow meters (Aalborg Instruments and Controls, Inc., Orangeburg, NY, USA). The composition of the biogas was measured every 24 h using a GMF 430 analyzer (gas data) and a gas chromatograph (GC 7890 A Agilent, Santa Clara, CA, USA). The gas chromatograph was equipped with two Haysep Q columns (80/100), two molecular sieve columns (60/80) and a Porapak Q column (80/100) working at 70 °C. The temperature at the injection port and the detector port was 150 °C and 250 °C, respectively. Helium and argon were used as the carrier gases (flow rate: 15 mL/min).

Anaerobic microbe consortia were identified by FISH. Four molecular probes were used for hybridization: a bacteria-universal probe EUB338 [30], an archaea-universal probe ARC915 [31], a Methanosarcinaceae-specific probe MSMX860 and a Methanosaeta-specific probe MX825 [32]. The samples were examined in an epifluorescent microscope (100× lens, 1000× total magnification) (Nikon Eclipse, Nikon, Tokyo, Japan). Microbial counts for the tested species were calculated from DAPI-stained cells using Image-J (http://rsbweb.nih.gov/ij/, accessed on 18 May 2022).

2.6. Statistical Analysis

Each experimental variant was conducted in triplicate. Statistical analysis of the results was performed using Statistica 13.1 PL. The hypothesis assuming the normality of distribution of each analyzed variable was verified using the Shapiro–Wilk W-test. One-way analysis of variance (ANOVA) was used to determine significant differences between variables. Homogeneity of variance in the groups was determined using the Levene test. Significant differences between the variables were determined via Tukey’s HSD test. Differences were considered significant at \( \alpha = 0.05 \).

The progressive stepwise method with multiple regression was used to develop empirical equations for estimating biogas and methane yields. Significant predictors of changes in the estimated parameter values were identified in the model systems. The model’s goodness-of-fit to the empirical data was verified using the coefficient of determination.

3. Results

3.1. Feedstock Properties and Parameters

It is widely acknowledged that the composition of anaerobic reactor feedstock is a major determinant of the growth rate of anaerobic bacteria and the resulting biogas yields. According to Aboudi et al. [42], insufficient trace elements and alkalinity in methane-digested plant biomass can disrupt the methane production process. Data in the literature indicate that co-digestion of various substrates leads to more balanced physicochemical parameters of the fermented mixture and thus to improved methane output [34,35]. Many authors have also noted that co-digestion has additional technological, commercial and environmental benefits [43]. Research to date has indicated that there are several factors which limit the efficiency of methane digestion and can effectively limit or stop biogas production in microalgae-mediated processes [44,45]. Such limiting factors include cellulose or hemicellulose in the cell walls (which are resistant to anaerobic digestion), formation of compounds that are toxic to anaerobic bacteria by certain algae strains and an insufficient C/N ratio in the digested feedstock [46].

The tested microalgal biomass had an organic load rate of 87.7 ± 1.1% (Table 1). The TC and TOC contents were 464 ± 25 mg/gTS and 437 ± 20 mg/gTS, respectively. TN content averaged 46.0 ± 3.9 mg/gTS, while TP content approximated 4.4 ± 0.9 mg/gTS. The biomass C/N ratio, a key parameter for methane fermentation, was very poor at 9.5 ± 0.4. The fractions of total protein, lipids and carbohydrates in the mixed microalgal culture were 28.7 ± 2.5% TS, 20.0 ± 1.4% TS and 15.8 ± 2.6% TS, respectively.
The giant miscanthus silage had higher rates of organic matter, with the carbon in dry matter at 93.8 ± 0.2% TS, an average TC of 460 ± 13 mg/gTS and TOC of around 441 ± 15 mg/gTS (Table 1). The nitrogen and phosphorus contents in the silage were found to be significantly lower than in the microalgal biomass, with values of 11.1 ± 0.9 mg/gTS and 2.4 ± 0.3 mg/gTS, respectively. The C/N ratio averaged 39.6 ± 1.7. The respective contents of the total protein fraction, the lipid fraction and reducing sugars were 9.2 ± 0.6% TS, 2.2 ± 0.5% TS, and 60.4 ± 1.0% TS.

Mixing giant miscanthus silage with microalgal biomass in different proportions had a direct effect on the key feedstock parameters for methane digestion, particularly the C/N ratio (Table 1), which ranged from 9.5 ± 0.4 to 39.6 ± 1.7, depending on the experimental variant. The best C/N ratios were obtained when the proportion of microalgal biomass was between 20% and 40% dry organic matter: 24.3 ± 1.4 and 17.5 ± 1.2, respectively (Table 1). Different feedstock combinations also affected the other tested parameters, including dry organic matter, and total phosphorus, protein, lipids and sugars (Table 1). However, these variations were less significant than in the case of the C/N ratios.

The C/N ratio in the digester feed should be within the 20/1–30/1 range, whereas the ratio of most algae-based feedstocks is below 10, inhibiting methane generation [47,48]. This limiting effect of low C/N may stem from high concentrations of ammonia nitrogen and volatile fatty acids in the digesters, which are detrimental to methane production [49]. One way to counteract this hindrance to anaerobic processing of algal biomass is to improve the C/N ratio by adding biomass that is rich in organic carbon to the feedstock mix [50].

### 3.2. Biogas Yield and Composition

Studies from around the world have tested the potential of co-digesting low-C/N microalgal biomass with carbon-rich energy crops. Peng et al. [51] co-digested algal biomass with corn straw, achieving a biogas yield of around 687.3 cm$^3$ /gVS with 63.3% methane. Zhong et al. [52] observed that the co-digestion of algae and corn straw at a C/N ratio of 20/1 increased methane yield by 62% compared with the digestion of microalgae alone, reaching 325 cm$^3$ /gVS. Researchers have reported that 20/1 is the optimal C/N ratio for the feedstock mix. A study by Schwede et al. [53] has shown that even small admixtures of microalgal biomass in corn silage (1 to 6 ratio) can produce a 9% increase in the biogas yield, reaching 660 m$^3$/kgTS. The superior biogas output from the co-digestion of algal biomass and other organic feedstocks has been attributed to the synergistic effects occurring during anaerobic degradation of mixed feedstocks. Microalgal biomass can serve as a source of the nitrogen and micronutrients needed for the optimal growth of anaerobic microbes. Mata-Alvarez et al. [54] have demonstrated that using a combination of carefully selected feedstocks can directly and significantly improve the efficiency of anaerobic degradation. Matsui and Koike [55] operated a pilot-scale system where *Laminaria* sp. and *Ulva* sp. macroalgae were mixed with organic waste. Their experiment indicated that the stable operation of anaerobic reactors can be maintained if aquatic plants are mixed with the co-feedstock at appropriate ratios.

In Variant 1 (MA100/MS0), the biogas yields averaged 384.72 ± 22.11 cm$^3$ /gTS and 438.73 ± 25.21 cm$^3$ /gVS, and the biogas contained 65.12 ± 1.94% methane (Table 3). The total biogas output was around 3509.82 ± 201.71 cm$^3$/d, of which 2285.59 ± 199.46 cm$^3$/d was methane (Table 3). Variants MA80/MS20, MA60/MS40 and MA40/MS60 showed markedly improved digestion efficiency. We noted a steady improvement in biogas yields, ranging from 453.06 ± 26.05 cm$^3$/gTS in Variant 2 to 573.83 ± 12.54 cm$^3$/gTS in Variant 4 (Table 3). The methane content in the biogas ranged from 63.92 ± 3.02% in Variant MA80/MS20 to 60.62 ± 4.13% in Variant MA40/MS60 (Table 3). The total daily production of methane was: 2605.51 ± 272.94 cm$^3$/d (MA80/MS20), 2817.10 ± 256.62 cm$^3$/d (MA60/MS40) and 3045.56 ± 274.06 cm$^3$/d (MA40/MS60) (Table 3). Variant MA20/MS80 produced 612.42 ± 19.07 cm$^3$/gVS and 567.11 ± 17.66 cm$^3$/gTS biogas (Table 3). The methane fraction in the biogas averaged 57.38 ± 3.69%, which translated to a methane production rate of 351.40 ± 33.54 cm$^3$/gVS and 325.41 ± 31.06 cm$^3$/gTS (Table 3).
Biogas and methane output was 4899.33 ± 152.57 cm³/d and 2811.24 ± 268.35 cm³/d, respectively (Table 3).

### Table 3. Performance of microalgal biomass co-digestion with giant miscanthus silage.

| Variant       | Biogas production | Methane production |
|---------------|-------------------|--------------------|
|               | cm³/gTS           | cm³/gVS            |
| 1—MA100+MS0   | 384.72 ± 22.11    | 435.06 ± 26.05     |
| 2—MA80+MS20   | 438.73 ± 25.21    | 509.53 ± 11.71     |
| 3—MA60+MS40   | 3509.82 ± 201.71  | 4076.21 ± 114.24   |
| 4—MA40+MS60   | 2290.00 ± 99.73   | 2117.10 ± 149.14   |
| 5—MA20+MS80   | 250.53 ± 18.15    | 289.60 ± 20.01     |
| 6—MA0+MS100   | 285.70 ± 18.69    | 325.69 ± 25.88     |
|               | cm³/gVS_removed   | cm³/gTS            |
| 1—MA100+MS0   | 2285.59 ± 199.46  | 2655.51 ± 272.94   |
| 2—MA80+MS20   | 1491.25 ± 54.62   | 1335.25 ± 40.62    |
| 3—MA60+MS40   | 199.46 ± 26.04    | 229.12 ± 32.96     |
| 4—MA40+MS60   | 65.12 ± 1.94      | 63.92 ± 3.02       |
| 5—MA20+MS80   | 37.20 ± 1.41      | 37.38 ± 2.69       |
| 6—MA0+MS100   | 20.64 ± 1.44      | 20.64 ± 1.44       |
|               | cm³/d             | %                  |
| 1—MA100+MS0   | 152.57 ± 6.53     | 99.73 ± 2117.10    |
| 2—MA80+MS20   | 215.22 ± 7.51     | 149.12 ± 2122.07   |
| 3—MA60+MS40   | 268.35 ± 8.88     | 281.10 ± 2811.24   |
| 4—MA40+MS60   | 20.64 ± 1.44      | 20.64 ± 1.44       |
| 5—MA20+MS80   | 37.20 ± 1.41      | 37.38 ± 2.69       |
| 6—MA0+MS100   | 20.64 ± 1.44      | 20.64 ± 1.44       |

The MS-only variant performed significantly worse, with biogas yields of only 569.42 ± 8.25 cm³/gVS and 534.29 ± 7.74 cm³/gTS (Table 3). The methane content in the biogas was around 51.35 ± 2.88% (Table 3). The low methane concentration was offset by high biogas yields, meaning that the nominal biogas production was 292.40 ± 20.64 cm³/gVS and 274.36 ± 19.36 cm³/gTS (Table 3). Nevertheless, these values were still much lower than those noted for Variants MA60/MS40 and MA20/MS80. The daily biogas and methane yields averaged 4555.39 ± 65.99 cm³/d and 2339.19 ± 165.09 cm³/d, respectively (Table 3).

In our study, we noted that the digestion of “pure” substrates (microalgal biomass or giant miscanthus silage) performed the worst, with biomethane production rates of 2285 cm³/d and 2339 cm³/d, respectively. Co-digestion of microalgal biomass and giant miscanthus silage at different ratios led to considerable improvements in methane yields across all of the experimental variants. The observed biogas output increased from the 384 cm³/gTS observed in the microalgae-only variant to 573 cm³/gTS in the 40/60 MA/MS variant. Variant MA40/MS60 performed the best in terms of biomethane production, yielding 3045 cm³ CH₄/d at a C/N ratio of 17.53. Lower proportions of microalgal biomass in the feedstock mix led to decreased daily yields of biogas and methane.

### 3.3. Forecast of Biogas and Methane Production

Empirical equations were developed using multiple regression in order to model biogas and methane yields from microalgal biomass + giant miscanthus silage digestion. Biogas and methane production was found to correlate significantly with factors such as the share of microalgae and the C/N ratio. The biogas production model (Equation (5)) had an estimation error of ±13.756 and accounted for approximately 96.15% of the biogas yield variation (R² coefficient of determination = 0.9615). The methane estimation model (Equation (6)) accounted for approximately 92.95% of the methane yield variation (R² coefficient of determination = 0.9295) with an estimation error of ±9.867.

\[
\text{BIOGAS} = -4.1212 \text{ Microalgae} - 9.6191 \text{ C/N} + 945.9262
\]  
(5)

\[
\text{METHANE} = -2.4233 \text{ Microalgae} - 8.0430 \text{ C/N} + 607.7298
\]  
(6)

For the volume of biogas produced under mesophilic conditions [cm³/gVS], the volume of methane produced under mesophilic conditions [cm³/gVS], and the ratio of microalgae [% C/N], the C/N ratio is expressed in terms of percentage.

### 3.4. Bacterial Community

Across all variants, bacteria (EUB338) accounted for the largest share of microbes, which fell within the narrow range of 69 ± 4 in Variant 4 (MA40 + MS60) to 72 ± 2 in Variant 1 (MA100+MS0) (Table 4). Archaea (ARC915) were also quite populous, accounting for...
23 ± 3 (Variant 6: MA0 + MS100) to 27 ± 3 (Variant 4: MA40+MS60) of all microbes (Table 4). The highest share of methanogenic bacteria from the *Methanosarcinaceae* and *Methanosaeta* groups in the anaerobic bacterial community structure was found for the substrates with the share of microalgae at the level of 20% and 40%. These were, respectively, 15 ± 2% and 10 ± 1% for *Methanosarcinaceae*, and 14 ± 2% and 11 ± 1% for *Methanosaeta* (Table 4).

Table 4. Microbial taxonomy in AS across experimental variants.

| Consortium                        | Variant       | 1: MA100 + MS0 | 2: MA80 + MS20 | 3: MA60 + MS40 | 4: MA40 + MS60 | 5: MA20 + MS80 | 6: MA0 + MS100 |
|-----------------------------------|---------------|----------------|---------------|----------------|---------------|--------------|---------------|
| Bacteria (EUB338)                 |               | 72 ± 2         | 68 ± 3        | 70 ± 3         | 69 ± 4        | 69 ± 3       | 71 ± 4        |
| Archaea (ARC915)                  |               | 24 ± 1         | 26 ± 2        | 25 ± 2         | 27 ± 3        | 26 ± 3       | 23 ± 3        |
| *Methanosarcinaceae* (MSMX860)    |               | 11 ± 2         | 12 ± 2        | 11 ± 1         | 14 ± 2        | 15 ± 2       | 12 ± 1        |
| *Methanosaeta* (MX825)            |               | 8 ± 1          | 9 ± 1         | 9 ± 1          | 11 ± 1        | 10 ± 1       | 9 ± 1         |

Other researchers also determined the impact of the substrate used and the technological parameters of anaerobic digestion on the anaerobic bacterial community. Zhao et al. [56] proved that *Methanosaeta* dominated in the silage and dry cornstalks co-fermentation process. In the study of Zhou et al. [57], it was observed that the type of substrate had a significant impact on the formation of the bacterial community. In the study of Zhou et al., pig manure and rice straw were co-fermented in various proportions (95:5; 78:22 and 65:35 w/w), which corresponded to C/N ratios of 10:1, 20:1 and 30:1, respectively. The hydrotrophic methanogenic species *Methanothermobacter* was predominant, but at higher C/N ratios, sequences related to the genus *Methanosarcina* were also detected. In contrast, Venkiteswaran et al. [58], in a co-fermentation of polyhydroxybutyrate (PHB) with primary sludge, found that the bacterial communities were changed, but no changes were observed in the archaea community in different variants of the process. This research proved that the C/N ratio can be a factor significantly affecting the taxonomic structure of anaerobic bacteria in fermentation reactors, which directly translates into the methane fermentation efficiency achieved [59]. According to Mata-Alvarez et al. [54], fueling anaerobic reactors with mixed feedstock leads to better biogas production performance compared with using separate substrates. Combined substrates can provide the microelements and nutrients necessary for anaerobic microflora growth [46]. Mussgnug et al. [60] found that digesting maize silage and microalgae in one medium can improve the methane production of *Chlamydomonas reinhardtii* by 11.0 %.

3.5. Correlations

The analysis showed strong positive correlations (R² > 0.93) between biogas or methane production and the C/N ratio in Variants 1 (MA100+MS0) to 4 (MA40+MS60) (Figure 3). Very strong negative correlations (R² > 0.99) between these factors were also noted in Variants 4 (MA40+MS60) to 6 (MA0 + MS100) (Figure 3). Similarly, in the same variants, bacteria (EUB338), archaea (ARC915) and *Methanosaeta* (MX825) were strongly negatively correlated (R² > 0.90) with the C/N ratio (Figure 4). The only strong positive correlation (R² = 0.9116) was found between *Methanosaeta* (MX825) and C/N in Variants 1 (MA100+MS0) to 4 (MA40+MS60). Figure 5 shows the forecasted impact of the C/N ratio and the share of microalgae on biogas yield. Other authors also confirmed the existence of possible correlations between the C/N ratio in the feedstock used and the course of methane fermentation.
Zhong et al. [52] used algae collected from Meiliang Bay, Taihu Lake (China), together with corn straw. *Microcystis* sp. was the dominant species in the mixture (99%). The experiment was conducted in reactors with a total volume of 150 cm$^3$, fed with 2.0 gVS maize and microalgal biomass. These two primary substrates were supplied in various proportions, leading to different C/N ratios across the different variants: 71:1, 25:1, 20:1, 16:1 and 6:1. Variants with a C/N ratio between 16/1 and 25/1 performed the best in terms of total biogas yield after 30 days of incubation, with values ranging from 922 cm$^3$ to 1184 cm$^3$. The 20/1 C/N variant produced the most methane-rich biogas, with the methane content reaching 54.90%. In the other variants, the methane content was closer to 51.00% [52].

An earlier study by Dębowski et al. [61,62] demonstrated that adding macroalgal biomass from Puck Bay had a positive effect on the anaerobic digestion of haylage and maize silage. The best-performing variants yielded 373.1 m$^3$ CH$_4$/MgVS methane under static conditions and 386.8 m$^3$ CH$_4$/MgVS in flow reactors, with maize silage and microalgae used as feedstocks [62]. Lower yields were observed for water plant biomass combined with...
haylage. A respirometric assay showed methane production to be at 354.7 m$^3$ CH$_4$/MgVS, compared with 359.0 m$^3$ CH$_4$/MgVS determined in flow digesters [61].

Figure 5. Forecasted plane-plotted correlation between C/N ratio or microbial share and biogas production.

3.6. Digestate Characteristics

TS removed by the anaerobic digestion process ranged from 54.8±2.1% in Variant 1 (MA100+MS0) (with the final TS at 4.7±1.3%) to 87.1±4.2% in Variant 6 (MA0+MS100) (final TS: 3.9±0.9%) (Table 5). Decreases in VS were also noted in these variants, ranging between 19.2±2.3% and 32.0±2.6% (final values: 70.9±2.5% TS to 63.8±3.2% TS) (Table 5). The ɳ$_{MSS}$ ranged from 60.27±1.82% in Variant 4 (MA40+MS60) to 71.84±2.12% in Variant 6 (MA0+MS100) (Table 5). The same variants produced the lowest and highest values of ɳ$_{VS}$ at 55.24±1.95% in Variant 4 and 67.11±1.45% in Variant 6, respectively (Table 5). TN peaked (45.3±3.1 mg/gTS) in Variant 1 (MA100+MS0), whereas the lowest TN levels were recorded for Variant 6 (MA0+MS100) (13.1±2.0 mg/gTS). For TP, the observed levels ranged from 4.0±1.0 mg/gTS to 2.0±0.8 mg/gTS (Table 5). The C/N ratio was between 6.9±0.2 in Variant MA100+MS0 to 24.0±0.6 in Variant MA0+MS100. The pH was near-neutral at 6.7±0.2 in Variant 1 (MA100+MS0) to 7.5±0.2 in Variant 6 (MA0+MS100) (Table 5).

The digestate characteristics depend primarily on the feedstock used in the fermentation process [63]. The content of the main fertilization index, N:P:K, is directly related to the concentrations of these compounds in the biomass and falls within a very wide range [64]. The highest amount of nitrogen is in digestate from the fermentation of slaughterhouse waste and animal manure [65]. The highest concentrations of phosphorus and potassium are observed in plant substrates [66]. The degree of digestion, the level of organic compounds removed and their final concentration in the digestate depend mainly on the technological parameters of the process [67]. The organic load rate of the anaerobic chamber, the hydraulic retention time and the temperature are among the factors that determine the stabilization level of post-fermentation sludge [68]. Other important aspects are the pretreatment techniques used and the support of the hydrolysis process [69]. A high degree of digestion is important to reduce the negative impact of sludge on the environment, including reducing the susceptibility to rotting and limiting the spread of odors [70].
Table 5. Digestate parameters across the experimental variants.

| Parameter   | Unit | Variant          |
|-------------|------|------------------|
|             |      | 1: MA100 + MS0   |
|             |      | 2: MA80 + MS20   |
|             |      | 3: MA60 + MS40   |
|             |      | 4: MA40 + MS60   |
|             |      | 5: MA20 + MS80   |
|             |      | 6: MA0 + MS100   |
| TS %        | -    | 4.7 ± 1.3        |
| VS %TS      |      | 4.1 ± 1.4        |
|             |      | 4.0 ± 1.0        |
|             |      | 5.0 ± 0.9        |
|             |      | 4.3 ± 0.8        |
|             |      | 3.9 ± 0.9        |
| TN mg/gTS   | -    | 70.9 ± 2.5       |
|             |      | 67.5 ± 3.6       |
|             |      | 66.1 ± 2.8       |
|             |      | 69.4 ± 1.3       |
|             |      | 66.9 ± 1.9       |
|             |      | 63.8 ± 3.2       |
| TP mg/gTS   | -    | 45.3 ± 3.1       |
|             |      | 40.0 ± 1.2       |
|             |      | 29.9 ± 3.6       |
|             |      | 21.5 ± 1.7       |
|             |      | 18.1 ± 3.1       |
|             |      | 13.1 ± 2.0       |
| TK mg/gTS   | -    | 4.0 ± 1.0        |
|             |      | 3.6 ± 0.7        |
|             |      | 3.1 ± 1.1        |
|             |      | 2.8 ± 0.2        |
|             |      | 2.0 ± 0.8        |
|             |      | 2.3 ± 1.4        |
| TC mg/gTS   | -    | 9.1 ± 2.4        |
|             |      | 8.7 ± 1.2        |
|             |      | 8.3 ± 1.9        |
|             |      | 7.9 ± 0.7        |
|             |      | 7.3 ± 1.6        |
|             |      | 7.5 ± 1.2        |
| TOC mg/gTS  | -    | 384 ± 29         |
|             |      | 378 ± 35         |
|             |      | 336 ± 18         |
|             |      | 381 ± 18         |
|             |      | 372 ± 30         |
|             |      | 328 ± 42         |
| C:N ratio   | %    | 316 ± 30         |
|             |      | 315 ± 30         |
|             |      | 300 ± 22         |
|             |      | 333 ± 22         |
|             |      | 330 ± 31         |
|             |      | 315 ± 30         |
| pH          | %TS  | 6.9 ± 0.2        |
|             |      | 7.8 ± 0.5        |
|             |      | 10.3 ± 0.1       |
|             |      | 15.4 ± 0.1       |
|             |      | 18.2 ± 0.1       |
|             |      | 24.0 ± 0.6       |
| Protein %TS |      | 6.7 ± 0.2        |
|             |      | 6.9 ± 0.4        |
|             |      | 7.3 ± 0.1        |
|             |      | 6.8 ± 0.4        |
|             |      | 7.0 ± 0.3        |
|             |      | 7.5 ± 0.2        |
| Lipids %TS  |      | 28.3 ± 1.9       |
|             |      | 25.0 ± 0.8       |
|             |      | 18.1 ± 2.2       |
|             |      | 13.1 ± 1.1       |
|             |      | 11.2 ± 2.0       |
|             |      | 8.1 ± 1.3        |
| Sugars %TS  |      | 6.1 ± 0.8        |
|             |      | 4.2 ± 1.1        |
|             |      | 2.7 ± 0.8        |
|             |      | 3.0 ± 0.3        |
|             |      | 3.7 ± 0.7        |
|             |      | 3.0 ± 0.5        |
| I_{MSS} %   |      | 1.8 ± 0.5        |
|             |      | 2.0 ± 0.8        |
|             |      | 1.8 ± 0.4        |
|             |      | 2.0 ± 0.3        |
|             |      | 1.3 ± 0.1        |
|             |      | 2.5 ± 0.5        |
| I_{VS} %    |      | 60.43 ± 1.68     |
|             |      | 68.92 ± 1.55     |
|             |      | 70.35 ± 2.31     |
|             |      | 70.27 ± 1.82     |
|             |      | 67.38 ± 1.52     |
|             |      | 71.84 ± 2.12     |
|             |      | 58.25 ± 1.22     |
|             |      | 64.98 ± 2.01     |
|             |      | 66.58 ± 1.89     |
|             |      | 65.24 ± 1.95     |
|             |      | 62.60 ± 2.23     |
|             |      | 67.11 ± 1.45     |

4. Conclusions

The C:N ratio (a significant factor for methane fermentation) in the tested monosubstrates was 9.5 ± 0.4 in the microalgae biomass and 39.6 ± 1.7 in the Miscanthus × giganteus silage. A reasonable technological step was therefore to use appropriate proportions of these substrates to achieve the optimal C/N ratio. Appropriate values of the C/N ratio from the technological point of view were obtained when the microalgal biomass was between 20% and 40% dry organic matter of the substrate: 24.3 ± 1.4 and 17.5 ± 1.2, respectively.

The highest unit productivity of biogas (628.00 ± 20.05 cm³/gVS) and methane (380.69 ± 28.55 cm³ CH₄/gVS) was recorded when microalgae biomass accounted for 40% of the feedstock. The highest concentration of methane in biogas at the level of 65.12 ± 1.94% was observed during fermentation of microalgae biomass only. Successive increases in the proportion of Miscanthus × giganteus silage in the substrate decreased the methane content.

The highest digestion level and removal of organic compounds, amounting to 71.84 ± 2.12% and 67.11 ± 1.45%, respectively, were recorded in the variant in which only the giant miscanthus silage was introduced into the fermentation chamber. Similar values were found for the substrate containing 40% to 60% of microalgae biomass. Bacteria (EU338) dominated in the microbial environment in all variants. The highest share of methanogenic bacteria from the Methanosarcinaceae and Methanosaeta groups in the anaerobic bacterial community structure was found for the substrates with the share of microalgae at the level of 20% and 40%. These were, respectively, 15 ± 2% and 10 ± 1% for Methanosarcinaceae, and 14 ± 2% and 11 ± 1% for Methanosaeta.

In order to enable the estimation of the produced biogas and methane as a result of co-fermentation of microalgal biomass with Miscanthus × giganteus silage, empirical equations were developed by the multiple regression method. It was found that the amount of biogas and methane was significantly influenced by variables such as the share of microalgae in the substrate and the feedstock C/N ratio.

Research has shown that co-fermentation of microalgae biomass and Miscanthus × giganteus silage allows for high technological effects. An important factor determining the possibility of implementing this solution is the development of efficient, economically justified and environmentally friendly methods of feedstock production, mainly microalgae biomass. The main issue is the implementation of solutions consistent with the assumptions of a circular economy and that are conducive to reducing greenhouse gas emissions to the atmosphere.
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