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Enhancing biogas production from perennial energy crops through bioaugmentation with perlite immobilised microbial consortia

Povećanje proizvodnje bioplina iz višegodišnjih energetskih kultura putem bioaugmentacije s mikrobnim konzorcijima imobiliziranim na perlite

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ABSTRACT

With increasing concerns over energy security and environmental sustainability, the search for sustainable alternatives to conventional biogas feedstock has become increasingly important. As part of efforts to reduce dependence on maize silage, perennial energy crops such as *Miscanthus* x *giganteus*, *Arundo donax*, and *Panicum virgatum* are emerging as promising candidates to meet the increasing demand for biogas production. In this study, these plants were subjected to anaerobic digestion in bioaugmented bioreactors, and the biogas production potential (BPP) was monitored during a 26-day batch anaerobic digestion process. The results showed that bioaugmentation significantly increased the BPP in certain cases. The addition of *M. giganteus* to bioaugmented reactors increased BPP by 36%, whereas reactors with *P. virgatum* showed a 73% increase in BPP. Results indicate that bioaugmentation of reactors fed with alternative energy crops represents a viable and sustainable solution to enhance biogas production.

Keywords: bioaugmentation, biogas, perennial energy crops, biodegradation, anaerobic digestion, sustainable bioenergy

SAŽETAK

S rastućom zabrinutošću oko energetske sigurnosti i održivosti okoliša, potraga za održivim alternativama konvencionalnoj sirovini za bioplin postala je sve važnija. Kao dio napora za smanjenje ovisnosti o kukuruznoj silaži, višegodišnje energetske kulture poput *Miscanthus* x *giganteus*, *Arundo donax* i *Panicum virgatum* pojavljuju se kao obećavajući kandidati za zadovoljavanje rastuće potražnje za proizvodnjom bioplina. U ovoj studiji, ove biljke su podvrgnute anaerobnoj digestiji u bioaugmentiranim reaktorima, a potencijal proizvodnje bioplina (PPB) praćen je tijekom 26 - dnevnog procesa anaerobne digestije u šaržnom ciklusu. Rezultati su pokazali da je bioaugmentacija značajno povećala PPB u određenim slučajevima. Dodavanje *M. giganteusa* u bioaugmentirane reaktore povećalo je PPB za 36 %,dok je *P. virgatum* pokazaopovećanje PPB-a od 73 %. Rezultati pokazuju da bioaugmentacija reaktora te korištenje alternativnih energetskih usjeva predstavlja održivo rješenje za povećanje proizvodnje bioplina.

Ključne riječi: bioaugmentacija, bioplin, višegodišnje energetske kulture, biorazgradivost, anaerobna digestija, održiva energija

INTRODUCTION

As global energy demand continues to rise, significant investments are being made in research to introduce new technologies for energy production. One promising avenue for renewable energy lies in agricultural production. While energy production in agriculture is not a new concept, recent technological advances are helping to optimise these processes to increase their efficiency. Examples of such advances include several pretreatment methods, applications of digestion strategies, and digital process monitoring, along with optimisation tools. As Johnson et al. (2007) pointed out, this approach represents a long-term renewable solution that is both carbon neutral and capable of meeting global energy needs.

According to recent estimates by the Intergovernmental Panel on Climate Change (IPCC), bioenergy production is expected to reach 100 exajoules (EJ) by 2050, contributing almost 20% to the global energy demand. The European Union's energy policy, through initiatives such as REPowerEU (European Commission, 2022), aims to increase biogas and biomethane production by 20% by 2030. In line with this policy, biogas is an important source of energy produced by the anaerobic digestion of organic material. This process is both cost-effective and relies on the activity of a diverse group of microorganisms to break down the biomass (Bhatia et al., 2014; Hans and Kumar, 2019). Currently, in some countries of the European Union, maize silage is still often used as a raw material for biogas production (Sobczak et al., 2022). This preference is primarily due to the high energy yield of maize silage (Fuksa et al., 2020). However, significant challenges emerged, especially after the COVID-19 pandemic, which caused the first price fluctuations. These problems were worsened by the outbreak of the Russian and Ukrainian conflict, which led to a significant increase in grain prices, especially maize (Aliu et al., 2023). As a result, biogas plants that rely on maize silage are faced with higher feedstock costs, which have reduced the profitability of biogas production from that material. This economic pressure emphasised the need for self-sufficiency in energy production and highlighted the importance of sustainable practices. Such circumstances also highlight the competition between food and energy agriculture, emphasising that biogas production should not compromise food security. This change is in line with the requirements of the Renewable Energy Directive (RED III), which mandates that biogas production must comply with sustainability standards to ensure a stable and sustainable energy supply which is not in competition with food production (Peni et al., 2022; Dahmen et al., 2019).

Therefore, agricultural lignocellulosic biomass, which is abundant and renewable, has emerged as a promising alternative to traditional feedstock (Dahmen et al., 2019). In this study, three types of lignocellulosic biomass were investigated: *Miscanthus* x *giganteus* (MG), *Arundo Donax* (AD) and *Panicum virgatum* (PV). These plants are well-suited for cultivation on marginal land, require low inputs and can produce high biomass yields, making them ideal candidates for lignocellulosic feedstock for biogas production (Angelini et al., 2009; Naik et al., 2010; Špelić et al., 2024). Moreover, AD is quite distributed within the Mediterranean area, and as such, provides an abundant quantity of biomass from its natural habitat. However, due to the high lignin content, lignocellulosic materials are resistant to microbial degradation during anaerobic digestion, which poses a major challenge to achieving high biogas yields (Kuijk et al., 2015; Saini et al., 2015). Although various pretreatment methods, such as chemical treatments, have been proposed to improve biodegradability, these pretreatments are often harmful to the environment (Zheng et al., 2014; Wei, 2016). Pretreatments, although effective, often require significant financial investment, time, and additional specialised equipment for implementation. So, the challenge can be overcome by applying alternative methods to increase the biodegradability rate of the raw material used in anaerobic digestion. Biological pretreatments have also been developed, offering more environmentally friendly and cost-effective alternatives.

One of the biological solutions is the process of bioaugmentation, which uses various microbial consortia to enhance the anaerobic digestion process. This treatment offers a more sustainable solution as it increases microbial activity without the use of chemicals (Herrero and Stuckey, 2015). The main concept behind the bioaugmentation is to introduce customised microorganisms that effectively degrade the substrate, ensure better anaerobic digestion efficiency and thus increase the biogas yield. Various microorganisms can be used to achieve this, including methanogens, hydrolytic, acidogenic, and acetogenic bacteria, or a combination of these (Akila and Chandra, 2010; Weiß et al., 2011; Donkor et al., 2022). To implement bioaugmentation, these targeted and isolated bacteria must be added to the anaerobic digester to maximise their impact on substrate degradation and biogas production. Although bioaugmentation can also be applied with a single microbial species, consortia are generally preferred due to their synergistic interactions and broader substrate degradation potential.

The bioaugmentation process can be enhanced by using suitable microbial carriers. Microbial immobilisation provides higher cell numbers and protects bacteria from operational fluctuations in the bioreactors. Natural organic carriers such as corn cobs, straw, plant fibers, and rice straw can be used, but these materials are often resistant to decomposition and can have questionable stability under various pH conditions. In contrast, natural inorganic minerals such as zeolite and perlite exhibit high chemical and physical resistance, allowing them to remain more reliably stable during anaerobic digestion (Dzionek et al., 2016; Dadić et al., 2023). According to Pavlović et al. (2024), an effective carrier should have a porous structure that is often rough and irregular and provides an optimal surface for microbial immobilisation. Among these carriers, perlite has been shown to be particularly effective due to its porous nature (Ivanković et al, 2010), which facilitates microbial adhesion and improves microbial retention during the digestion process.

In this study, the potential for biogas production using the three perennial energy crops. The experiments included the application of bioaugmentation, with microbial consortia immobilised on perlite, which served as the carrier material.

MATERIALS AND METHODS

Raw materials

Three perennial energy crops were used: *Miscanthus* x *giganteus* (MG), *Arundo Donax* (AD) and *Panicum virgatum* (PV). Samples of MG and PV were collected in March 2022 at the University of Zagreb Faculty of Agriculture experimental field Šašinovec (45°50'59.3"N 16°11'26.2" E). AD samples were collected in April 2022 on the island of Pag (Croatia, 44°29′48″ N14°57′33″ E), in its natural habitat. The physicochemical composition of the energy crops was determined after sample preparation, as presented in Tables 1-3.

Methods

After collection, biomass samples were dried in the laboratory oven (Memmert GmbH, Germany) at 60 °C for approximately 48 hours to preserve their quality. Before further laboratory analysis and processes were milled to a maximum particle size of <2mm by using the Retsch SM 100 cutting mill (Retsch GmbH, Germany). This particle size ensures the proper performance of laboratory analyses. In addition, this particle size increases the available surface area for microbial degradation and therefore improves the efficiency of anaerobic digestion.

Ultimate and proximate analysis

The variables of the proximate analysis included the moisture content as received (according to Croatian Standard (HRN), European Standard (EN) and International Organization for Standardization (ISO) methods - HRN EN ISO 18134-2:2017), ash (HRN EN ISO 18122:2015), coke and fixed carbon (HRN EN ISO 18123:2015, which were determined in the muffle furnace (Nabertherm GmbH, Nabertherm Controller B170, Germany).

Ash represents the inorganic residue remaining after complete combustion of the organic material, determined by heating the sample at 550 °C for 4 hours. Coke was the carbonaceous residue left after pyrolysis at 900 °C for 5 minutes in oxygen oxygen-free environment. Fixed carbon was calculated as the difference between the coke and ash content, representing the fraction of carbon remaining after the release of volatile substances. For ultimate analysis, total carbon, hydrogen, nitrogen and sulphur were determined using the dry combustion method. Analyses were conducted in a Vario Macro CHNS analyser (Elementar Analysensysteme GmbH, Germany) in accordance with the protocol (HRN EN ISO 16948:2015) for carbon, hydrogen and nitrogen and (HRN EN ISO 16994:2016) for sulphur. The oxygen content was calculated as residue.

Lignin content determination and chemical oxygen demand (COD) analysis

The lignin content was determined by Ankom Delta Fiber Analyzer (ANKOM Technology, USA) according to the acid detergent lignin method provided by Ankom Technology (2016). Chemical oxygen demand, as a measure of the biodegradable organic matter in the substrate, was determined using a modified ISO 6060:1989 method by Zupančič and Roš (2012).

Bioaugmentation and immobilisation process

For the bioaugmentation of the bioreactors for anaerobic digestion, a three-step process for the isolation of targeted bacteria was done. First, in Winkler bottles with 100 mL of MSM (Minimal Salt Media, composition in g/L of tap water: NaCl 2.5, K_2HPO_4 0.47, KH_2PO_4 0.56, $MgSO_4{\cdot}7H_2O$ 0.5, $CaCl_2{\cdot}2H_2O$ 0.1, NH_4NO_3 2.5, pH 7.0±0.2) a 1 g of either MG, PV or AD was added, along with 1 mL of sludge from the anaerobic digestor (the same sludge was used in the AD digestion experiment, section 2.1.6.). Bottles were incubated anaerobically by filling them all the way to the top, and then tightly sealing them with a cap, for one week at 37±1 °C. The idea was to use the substrate (MG, PV or AD) as a sole carbon source, and with prolonged incubation to select the bacteria capable of substrate enzymatic degradation. In the second step, an aliquot of 1 mL was taken from the bottles, serially diluted and spread onto MSM agar plates (2% agar) containing 1% of substrate (either MG, PV, or AD) and incubated at 37±1 °C under anaerobic conditions for one week. Presumably, since the substrate was the only carbon source in the agar plates, only bacteria capable of enzymatic degradation will be replicating and producing the biomass, in fact, a visible colony. After such conditioning, these bacteria were presumed as substrate degraders. Grown colonies were aseptically scraped from the agar surface, suspended in a sterile buffer solution (PBS) until the density was approximately 0.5 McFarland units (~3-5 × 10^8 CFU/mL), and this suspension was designated as microbial consortium MG, AD or PV. The alternating anaerobic (in liquid media) and aerobic incubation (on agar plates) were chosen to select growth of facultative anaerobic bacteria, not just the obligate anaerobes. From a biotechnological perspective, facultative anaerobes are preferable for large-scale cultivation. If the cultivation methodology were to be applied on site at a digestion plant, typically without an expert microbiologist present, it would be technically advantageous to use simple bioreactors or incubators that do not require the maintenance of strict anaerobic conditions. Moreover, biomass yield is significantly higher when the target bacteria are incubated under aerobic conditions. The drawback of this procedure is that some microbial species may have been present in the liquid media but failed to grow on solid media, highlighting an opportunity for targeted experimental investigations in future studies. In our previous experiments, we observed only bacterial species during cultivation on solid media (Dadić et al., 2024; Ivanković et al., 2022). However, since fungi are also considered potentially significant contributors to substrate degradation in anaerobic digestion, another future research could be extended to include fungal cultivation. The procedure could also be adjusted to cultivate bioparticles after enrichment in liquid media, potentially capturing a broader range of microorganisms.

The final step was immobilization of microbial consortium onto perlite, thus producing the bioparticles, which were again either bioparticles intended for usage with MG, AD or PV as a substrate for anaerobic digestion. The perlite (1-4 mm) was a commercial gardening product (Special Mix B.V., Gold Label, Netherlands), and it was dry sterilized at 105 °C for 1 hour before use. To immobilize the bacterial consortium, 4 g of perlite, 400 mL of sterile LB medium, and 4 mL of consortium suspension were combined in sterile laboratory bottles and incubated for 24 h at 37±1 °C on an orbital shaker (150 rpm). During the incubation, bacteria spontaneously form biofilm on the perlite surface. After incubation, bacteria that were not firmly attached were removed by washing the perlite twice with 0.3% saline water. Such bioparticles were aseptically transferred to anaerobic digestion reactors.

Scanning electron microscopy

To confirm the role of perlite as a microbial carrier, an additional scanning electron microscopy (SEM) was carried out. For SEM analysis, after incubation and rinsing with sterile saline water, several perlite particles with attached bacteria were transferred to a sterile vial containing 2% paraformaldehyde and kept at 4 °C for 24 h for fixation. Samples were then washed with saline water and sequentially dehydrated in ethanol (30% for 2 min, 50% for 2 min, 70% for 5 min, 96% for 5 min, and twice in 100% for 5 min each), followed by drying at 50 °C for 30 min. A single particle was coated with gold-palladium for 180 s and imaged using a TESCAN Vega3 Easy Probe SEM at 7 keV.

Anaerobic digestion setup and biogas monitoring

Anaerobic digestion (Figure 1), following the batch biogas production (CROTEH, Croatia), was carried out according to the VDI 4630 method under mesophilic conditions at 37±1 °C in a water bath, over a period of 26 days. The laboratory scale system consisted of 15 bioreactors of 500 mL volume, each equipped with mixers operating at a rotation speed of 60 RPM and during a 15-minute per hour period. BPP was measured in triplicate by using a volumetric method, with the results automatically expressed in normal liters (NL) and then calculated to NL/kg of dry matter (DM). Each bioreactor was individually connected to a gas meter, which recorded the volume of gas produced. The system was designed so that when the gas-tight container was filled with biogas, it performed one cycle, corresponding to a known calibrated volume. The system automatically counted the number of cycles and multiplied this value by the calibrated volume to obtain the total biogas production.

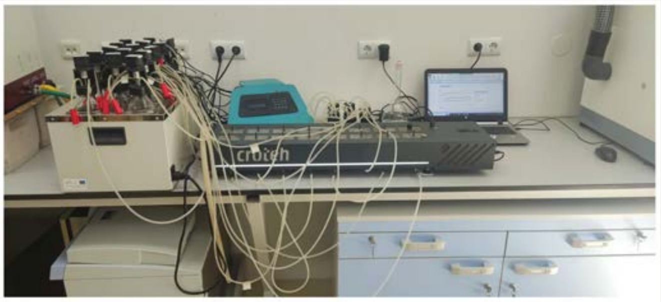


Figure 1. Anaerobic digestion setup for batch biogas production

For the anaerobic digestion setup, the digestate was taken from the nearest biogas plant in Zagreb. By using it as an inoculum, a stable and well-adapted microbial community for an anaerobic digestion setup was established. The volume of anaerobic sludge in all bioreactors was 400 ml, along with 4 g DM of dried selected crops in the tested bioreactors. Anaerobic conditions in all bioreactors were ensured by flushing with nitrogen before sealing and by keeping them airtight. Consequently, the experiment was carried out by adding the same weight percentage (1%) of each of the tested constituents in the bioreactors with anaerobic sludge (inoculum) – (i) anaerobic sludge as blank sample (negative control), (ii) a selected crop 4 g, (iii) a selected crop 4 g with perlite 4 g (carrier) (iv) selected crop with suspension of microbial consortium 4 mL (v) selected crop with bioparticles 4 g. Biogas production was monitored for all experimental setups. The value obtained from the blank sample (negative control) (i) was subsequently subtracted from each of the tested samples to accurately represent the biogas yield attributable solely to the investigated biomass. For this investigation, three batches were performed under the same conditions.

X-Ray Microtomography

The X-Ray Microtomography (micro-CT) allowed the examination of perlite particle distribution inside the opaque bottles (bioreactors) with anaerobic sludge. The perlite density is smaller than that of water, and so it floats on the water surface, which could be an interesting alternative for the AD process. Using the micro-CT, we were able to image the 3D distribution of added bioparticles inside the AD reactors, and we compared the starting condition (a few hours after the addition) and after 5 days. For comparison, we did the same with zeolite particles of 0.5 – 1 mm size (ZeoSand®, Velebit Agro, Zagreb, Croatia)

For micro-CT imaging we used the same setup as for the digestion experiment, adding 1% weight percentage of the perlite in the laboratory bottles, mixed initially and subjected to scanning on a custom made RX Solutions (Annecy, France) laboratory tomograph that is equipped with a Hamamatsu X-ray source (Hamamatsu City, Japan) and a Varian flat panel detector (Varian Medical Systems, Salt Lake City, UT, USA). A scanning setup was an X-ray beam generated with a 100 kV 100 µA electron beam on a tungsten target, and we made 1400 angular projections equally spaced over 360°. The chosen pixel size was 10 µm. The obtained 2D radiographs were converted to 3D datasets using the in-built software with a filtered back-projection algorithm. Finally, 3D datasets were further analyzed with Fiji/ImageJ software, v. 1.54j.

Statistical analysis

Statistical analysis of proximate and ultimate analysis data was performed by using TIBCO STATISTICA 13.3.0 software (StatSoft TIBCO Software Inc., Palo Alto, CA, USA). The results are expressed as mean value with standard deviation and post hoc Tukey's test. For anaerobic digestion, the results are presented in graphical form as a mean value to better illustrate the process itself. In addition, correlations are shown in a heatmap to better understand the relationships between the variables and biogas production, which was generated using the Python programming language.

RESULTS

To assess the suitability of the selected energy crops for anaerobic digestion, proximate and ultimate analyses, together with lignin content and COD, were first determined (Table 1).

Table 1. Proximate analyses of the investigated biomass samples

Sample	MC (%)	Ash (% db)	Coke (% db)	FC (% db)
MG	55.73±2.35^{b}	2.56±0.1^{a}	9.68±0.23^{a}	7.13±0.24^{a}
AD	49.94±1.29^{a}	4.06±0.06^{c}	18.26±0.91^{c}	14.2±0.91^{c}
PV	52.93±0.35^{ab}	3.22±0.25^{b}	15.12±0.84^{b}	11.68±0.72^{b}
ss	*	*	*	*

Note: Different letters in the same column represent the difference between the observed values according to the Tukey HSD post hoc test (*$P < 0.05$).

Abbreviations: MC – moisture content; FC – Fixed carbon; **db – dry basis; MG - *Miscanthus x giganteus*; AD – *Arundo donax*; PV – *Panicum virgatum*; ss – statistical significance.

Table 1 shows the proximate analyses data; it can be observed that MG showed the highest moisture content of 55.73%, while AD had the lowest of 49.94%. In terms of the ash content, AD had the highest (4.06%) while MG had the lowest (2.56%). The coke content followed a similar pattern, with AD exhibiting the highest value of 18.26%, whereas MG had the lowest of 9.68%. Lastly, the fixed carbon values reflect similar trends, with AD showing the highest value of 14.2% and MG the lowest of 7.13%. These results provide an initial insight into the variability of the proximate composition among the crops. These compositions directly influence their energy potential and biodegradability in anaerobic digestion experiments.

Table 2 shows that nitrogen content is identical for MG and AD, both having 0.33%, while PV has lower nitrogen content (0.20%). In terms of the carbon content, MG and PV have similar values, 50.60 and 50.87%, respectively, both significantly higher than AD, with

Table 2. Ultimate analyses of the biomass samples investigated

Sample	Nitrogen (% db)	Carbon (% db)	Sulphur (% db)	Hydrogen (% db)	Oxygen (% db)
MG	0.33±0.01^{a}	50.6±0.44^{a}	0.02±0.01^{a}	5.82±0.13^{a}	43.08±0.56^{a}
AD	0.33±0.01^{a}	46.62±0.03^{b}	0.1±0^{c}	5.56±0.01^{b}	47.42±0.03^{b}
PV	0.2±0.01^{b}	50.87±0.03^{a}	0.03±0.01^{b}	5.98±0.01^{a}	42.94±0.02^{a}
ss	*	*	*	*	*

Note: Different letters in the same column represent the difference between the observed values according to the Tukey HSD post hoc test (**P*<0.05)
Abbreviations: db – dry basis; MG - *Miscanthus x giganteus*; AD – *Arundo donax*; PV – *Panicum virgatum*; ss – statistical significance.

the carbon content of 46.62%. For sulfur content, AD shows the highest value of 0.1%, significantly higher than PV (0.03%) and MG (0.02%). As for hydrogen content, PV has the highest content of 5.98%, followed by MG with 5.82%. AD has a slightly lower hydrogen content of 5.56%. Lastly, the oxygen content shows the highest value for AD (47.42%), which is considerably higher than MG (43.08%) and PV (42.94%). The values highlight clear differences in elemental composition between the three crops, which directly influence their biodegradability and energy potential.

To complement these findings, Table 3 highlights differences in lignin content and COD values, parameters that are more directly related to the anaerobic digestion process. MG had the highest lignin content at 37.54%, while AD contained 25.65%, and PV had the lowest at 20.41%. A similar pattern was observed in COD values, where MG reached 927.09 mgO/g, followed by PV with 719.34 mgO/g, while AD had the lowest at 650.42 mgO/g. The observed variation in lignin and COD among the crops underlines the challenges of their direct anaerobic digestion, thereby justifying the application of bioaugmentation as a strategy to enhance biodegradability.

To further investigate how bioaugmentation influences the anaerobic digestion process, bacterial suspension and perlite bioparticles were applied as carriers for microbial enhancement. To bioaugmented anaerobic digestors, we added either bacterial suspension or perlite bioparticles. Figure 2, an SEM image, revealed a substantial number of bacterial cells attached to the perlite surface.

Table 3. Lignin content and COD analysis for selected energy crops

	MG	AD	PV
Lignin (%)	37.54±1.71^{c}	25.65±1.08^{b}	20.41±0.63^{a}
COD (mgO/g)	927.09±11.46^{c}	650.42±16.51^{a}	719.34±35.56^{b}
ss	*	*	*

Note: Different letters in the same column represent the difference between the observed values according to the Tukey HSD post hoc test (**P*<0.05).

Abbreviations: MG - *Miscanthus x giganteus*; AD – *Arundo donax*; PV – *Panicum virgatum*; COD – *chemical oxygen demand*; ss – statistical significance.

Perlite has previously been demonstrated to accommodate high concentrations of immobilised cells due to its highly porous structure, characterised by irregular cavities and fractures, which provide an ideal surface for microbial attachment (Ivanković et al., 2010). Furthermore, the protocol described here encompasses bacterial isolation, cultivation, and immobilisation previously resulted in perlite particles with immobilised bacteria that could potentially enhance substrate biodegradation and improve biogas production under laboratory experimental conditions (Dadić et al., 2024; Ivanković et al., 2022).

After confirming the colonisation of carriers, the focus was shifted to the anaerobic digestion performance, which is graphically presented in the following (Figures 3-5). The results are shown separately for each investigated crop to illustrate how different bioaugmentation strategies affected biogas yield. According to Figure 3, which displays the bio-gas yield for MG and different combinations: selected crop (MG), with bioparticles (MG/BP), perlite (MG/P), and suspension (MG/S).

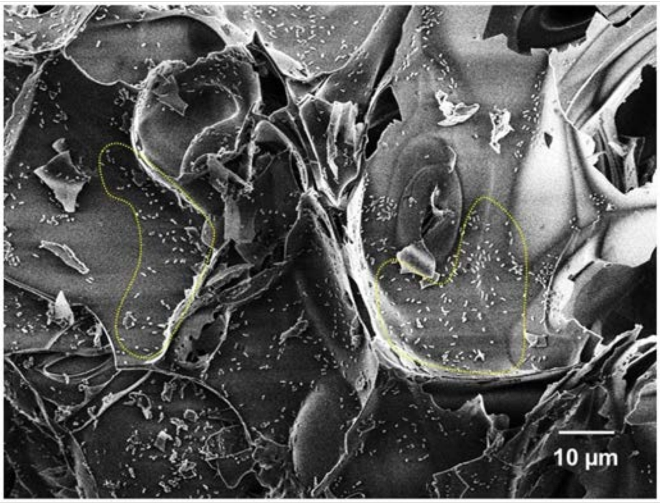


Figure 2. SEM image of the perlite particle with bacterial cells visible as bright structures all over the perlite's surface and additionally accented by a yellow line, magnification 500×

It is evident that the combination with bioparticles (MG/BP) achieves the highest biogas yield, reaching a maximum of 159.43 NL/kg DM after 26 days, while the MG itself results in the lowest yield, peaking at 117.71 NL/kg DM. The perlite (MG/P) and suspension (MG/S) combination reaches intermediate values, with final yields of around 124.89 NL/kg DM and 143.54 NL/kg DM, respectively. All combinations exhibit a similar growth trend during the first 10 days, after which production stabilises. Differences between the combinations become most pronounced after day 15 of anaerobic digestion.

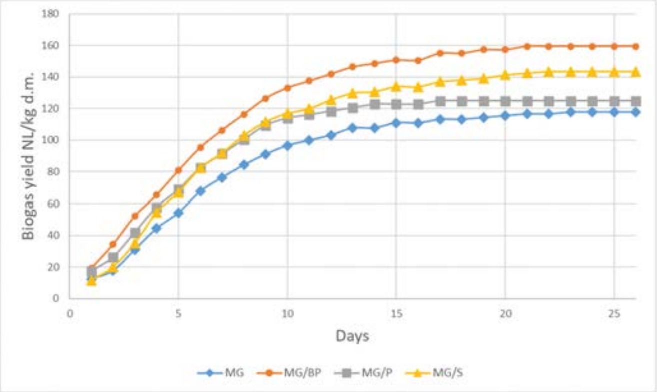


Figure 3. Cumulative biogas production during anaerobic digestion for MG with different combinations

Figure 4 illustrates the biogas production for plant AD. Like MG, the bioparticles combination (AD/BP) shows the highest yield, reaching 99.81 NL/kg DM by day 26, while the AD is the least effective, with a maximum yield of 80.20 NL/kg DM. The perlite (AD/P) and suspension (AD/S) combinations produce intermediate values, reaching 84.19 NL/kg DM and 86.03 NL/kg DM, respectively. However, for this plant, the difference between combinations is not as pronounced as they are for MG, suggesting that AD is less prone to bioaugmentation.

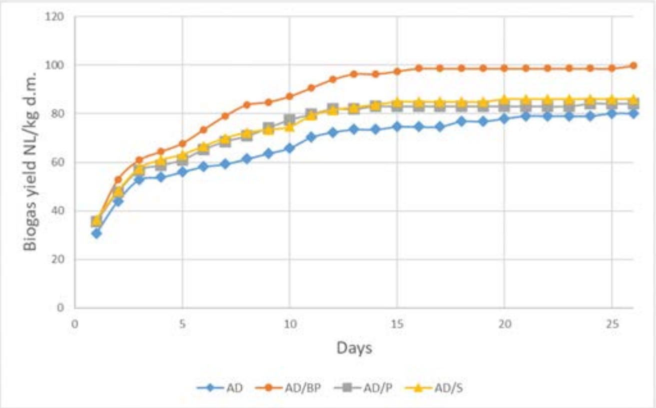


Figure 4. Cumulative biogas production during anaerobic digestion for AD with different combinations

Figure 5 presents the biogas yield for PV, where the bioparticles combination (PV/BP) results in the highest yield, reaching 115.05 NL/kg DM by day 26. The perlite combination (PV/P) achieves lower values, with a final yield of around 85.53 NL/kg DM, while the PV shows the lowest yield, peaking at around 66.41 NL/kg DM. The suspension combination (PV/S) reached a final yield of 103.94 NL/kg DM. The production trend for PV indicates faster growth during the initial days, followed by stabilization, with the differences between combinations becoming more apparent after day 10.

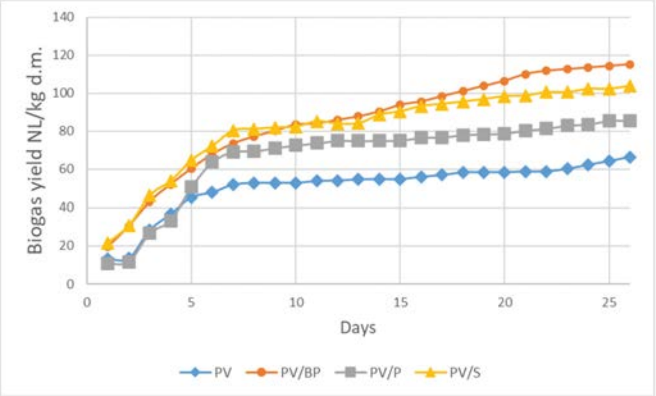


Figure 5. Cumulative biogas production during anaerobic digestion for PV with different combinations

Overall, to represent data for the total yield of biogas using all groups tested in Table 4 that were previously depicted in the graphical representations (Figures 2-4). In this way, the table complements the graphical data and enhances the clarity of the statistical interpretation of biogas production results. It can be observed that the anaerobic digestion process is highly volatile, which highlights the complexity and dynamic nature of the biogas production process.

Figure 6 shows the percentage increase in biogas yield for each combination relative to the selected crop for all three plants. Bioparticles emerged as the most effective additive for all plants, with the highest increase observed for PV. Perlite and suspension also show significant increases, but to a lesser extent compared to bioparticles. These differences underline that the effectiveness of bioaugmentation is crop dependent. Therefore, to gain a clearer understanding of how biomass composition influences biogas yield, a correlation analysis was conducted. The results are presented in the following figure.

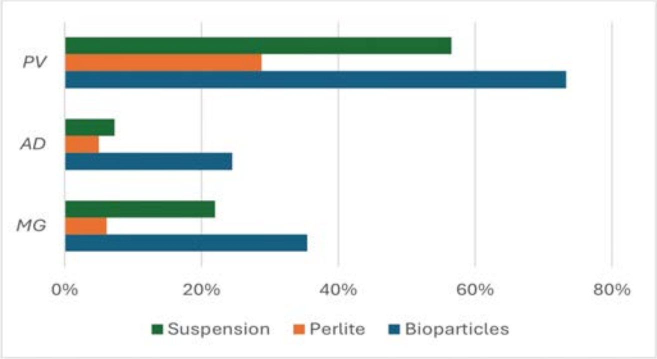


Figure 6. Percentage increase in biogas yields relative to the selected culture

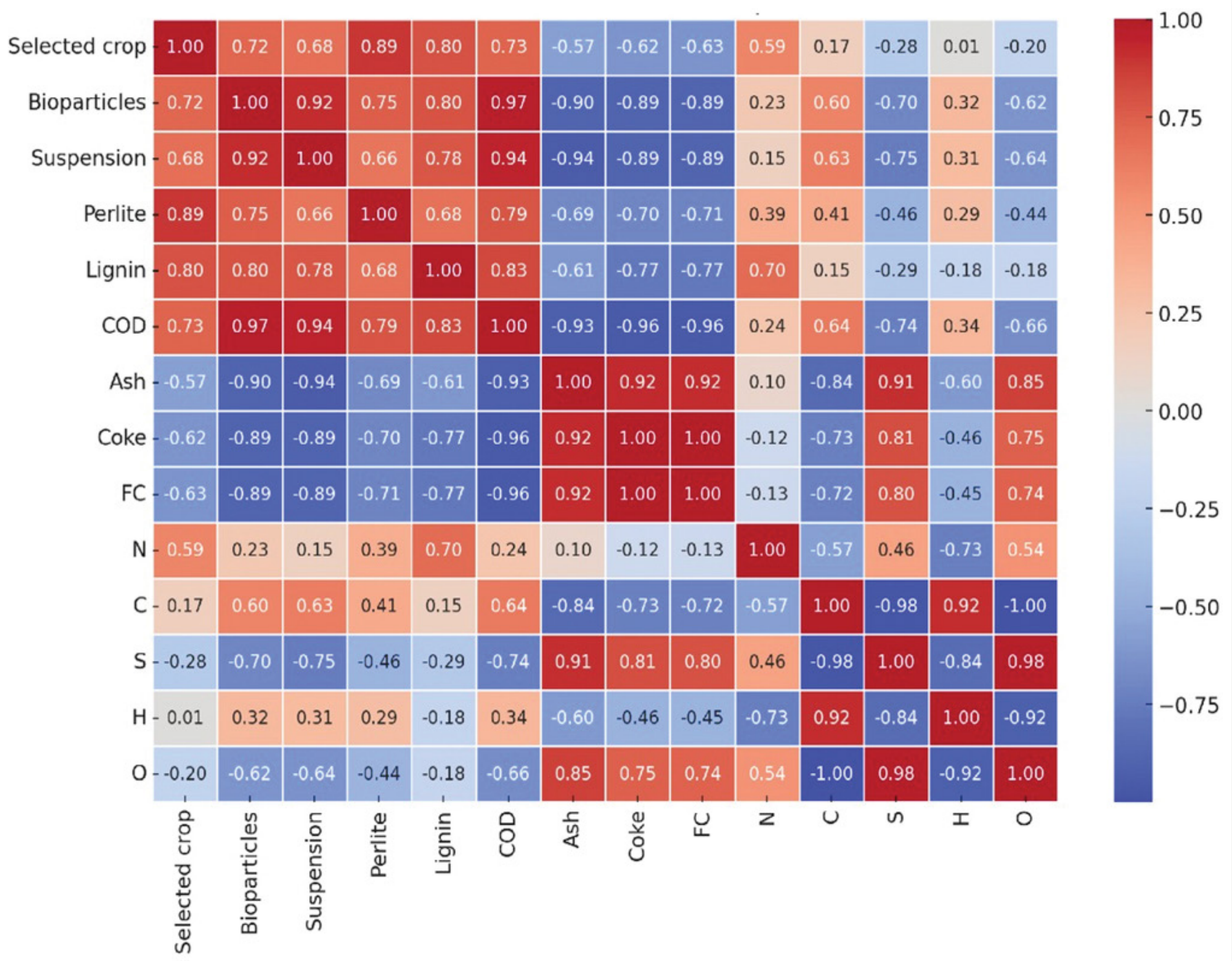
According to the correlation matrix heatmap in Figure 7, it can be observed that ash, coke and fixed carbon show a negative correlation with biogas production under all experimental conditions. This indicates that substrates with a higher content of these proximal components have a lower biodegradability. Furthermore, the parameters of the ultimate analysis show no significant correlation with biogas production. This indicates that the elemental composition of the substrate alone does not have a major influence on the anaerobic digestion process. It is evident that COD and lignin show the highest correlation with biogas production under all conditions, especially for substrates containing bioparticles. The strong correlation between COD and biogas production ($r = 0.97$, $P < 0.01$) confirms that the availability of biodegradable organic material is a key factor for biogas production. Lignin content also correlated with COD ($r = 0.83$, $P < 0.05$), which reflects its association with the overall chemical structure of biomass. However, it is well recognized that lignin itself is poorly degradable, and its high content usually limits biogas production by restricting access to cellulose and hemicellulose. This highlights that COD is the most reliable predictor of biogas yield, whereas lignin content provides additional context regarding biomass recalcitrance.

Table 4. Total biogas production for each crop and treatment

Crop/treatment	Selected crop	Bioparticles	Suspension	Perlite
MG	117.71 ± 35.2^{a}	159.43 ± 4.1^{b}	143.54 ± 14.7^{b}	124.90 ± 25.3^{ab}
AD	80.20 ± 1.7^{c}	99.81 ± 5.8^{bc}	86.03 ± 0.7^{c}	84.20 ± 2.4^{c}
PV	66.41 ± 16.9^{c}	115.05 ± 19.6^{b}	103.94 ± 20.1^{b}	85.53 ± 12.6^{c}
ss	*	*	*	*

Note: Different letters in the same column represent the difference between the observed values according to the Tukey HSD post hoc test (*$P<0.05$).

Abbreviations: MG - *Miscanthus x giganteus*; AD – *Arundo donax*; PV – *Panicum virgatum*.



	Selected crop	Bioparticles	Suspension	Perlite	Lignin	COD	Ash	Coke	FC	N	C	S	H	O
Selected crop	1.00	0.72	0.68	0.89	0.80	0.73	-0.57	-0.62	-0.63	0.59	0.17	-0.28	0.01	-0.20
Bioparticles	0.72	1.00	0.92	0.75	0.80	0.97	-0.90	-0.89	-0.89	0.23	0.60	-0.70	0.32	-0.62
Suspension	0.68	0.92	1.00	0.66	0.78	0.94	-0.94	-0.89	-0.89	0.15	0.63	-0.75	0.31	-0.64
Perlite	0.89	0.75	0.66	1.00	0.68	0.79	-0.69	-0.70	-0.71	0.39	0.41	-0.46	0.29	-0.44
Lignin	0.80	0.80	0.78	0.68	1.00	0.83	-0.61	-0.77	-0.77	0.70	0.15	-0.29	-0.18	-0.18
COD	0.73	0.97	0.94	0.79	0.83	1.00	-0.93	-0.96	-0.96	0.24	0.64	-0.74	0.34	-0.66
Ash	-0.57	-0.90	-0.94	-0.69	-0.61	-0.93	1.00	0.92	0.92	0.10	-0.84	0.91	-0.60	0.85
Coke	-0.62	-0.89	-0.89	-0.70	-0.77	-0.96	0.92	1.00	1.00	-0.12	-0.73	0.81	-0.46	0.75
FC	-0.63	-0.89	-0.89	-0.71	-0.77	-0.96	0.92	1.00	1.00	-0.13	-0.72	0.80	-0.45	0.74
N	0.59	0.23	0.15	0.39	0.70	0.24	0.10	-0.12	-0.13	1.00	-0.57	0.46	-0.73	0.54
C	0.17	0.60	0.63	0.41	0.15	0.64	-0.84	-0.73	-0.72	-0.57	1.00	-0.98	0.92	-1.00
S	-0.28	-0.70	-0.75	-0.46	-0.29	-0.74	0.91	0.81	0.80	0.46	-0.98	1.00	-0.84	0.98
H	0.01	0.32	0.31	0.29	-0.18	0.34	-0.60	-0.46	-0.45	-0.73	0.92	-0.84	1.00	-0.92
O	-0.20	-0.62	-0.64	-0.44	-0.18	-0.66	0.85	0.75	0.74	0.54	-1.00	0.98	-0.92	1.00

Figure 7. Correlation matrix for all observed variables and combinations

The micro-CT scans revealed that, just after the addition, almost all perlite particles accumulated at the sludge surface, entirely covering the sludge/air interface (Figure 8), with no appreciable difference between the start and day 5. It is important to emphasize that in our experimental setup, the bioreactors were mixed just initially, after the addition of perlite and zeolite, and not between days 1 and 5, either internally or externally. The two time points were chosen to test whether perlite particles might gradually absorb water over five days and subsequently sink into the sludge. However, this was not observed. In contrast, zeolite behaved oppositely: particles were located at the bottom of the sludge both at the beginning and after five days.

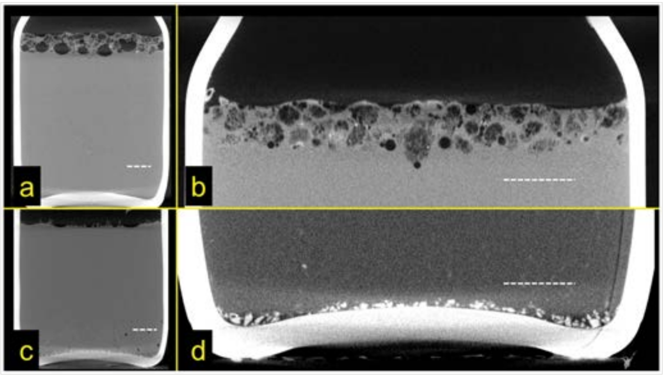
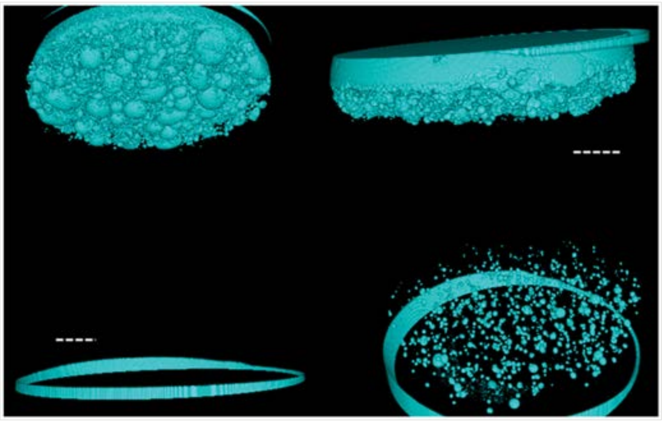


Figure 8. The radiographs of bottles filled with anaerobic sludge, lateral view in the middle of the 400 mL laboratory bottle; perlite particles can be seen at the sludge/air interface just after the addition (a) and after 5 days (b). Zeolite particles were located at the bottom of the bottle, both at the start (c) and after 5 days (d). The scale bar is 10 mm

During image analysis, we further observed that, in addition to perlite and zeolite particles, the scans distinctly captured gas bubbles, which appeared as completely black regions. This clear contrast enabled image segmentation and visualization of the spatial distribution of gas bubbles throughout the entire reactor volume. In the case of perlite, bubbles were initially concentrated at the surface (Figure 9), likely trapped between perlite particles, whereas after five days, additional bubbles appeared in the lower regions of the reactor, while others remained trapped at the surface.

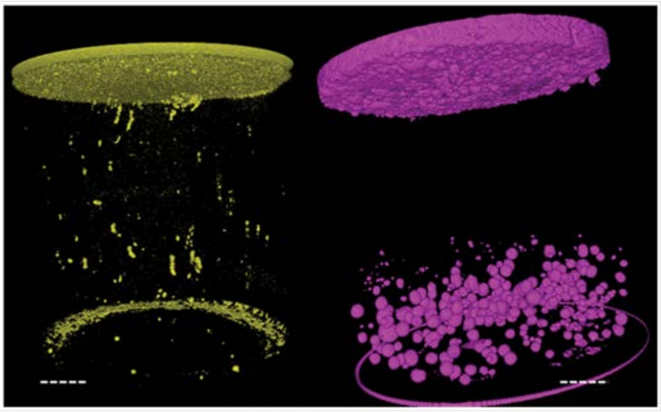


Note – in this image analysis, the radiograph data were segmented so that exclusively gas bubbles are shown. The rings at the bottom are artefacts from image analysis and reconstruction, the same as the disc at the top of the bottle, which is air. The scale bar is 10 mm.

Figure 9. 3D reconstruction of gas bubbles in bottles with anaerobic sludge and perlite particles at the start (left) and after 5 days (right)

In the case of zeolite, almost no bubbles were present initially, but after five days, a considerable number were detected at the bottom of the sludge (Figure 10), though not at the surface.

The bubbles observed at the starting point most likely resulted from mixing during the initial addition of perlite and zeolite, while those detected after five days undoubtedly originated from microbial digestion. Overall, the data support the conclusion that the surface layer of perlite impedes the release of a fraction of gas bubbles from the sludge, potentially altering gas–liquid mass transfer dynamics.



Note – in this image analysis, the radiograph data were segmented so that exclusively gas bubbles are shown. The rings at the bottom are artefacts from image analysis and reconstruction, the same as the disc at the top of the bottle, which is air. The scale bar is 10 mm.

Figure 10. 3D reconstruction of gas bubbles in bottles with anaerobic sludge and zeolite particles at the start (left) and after 5 days (right)

DISCUSSION

This study aimed to evaluate the biogas potential of *Miscanthus* x *giganteus*, *Arundo Donax*, and *Panicum virgatum* when bioaugmentation treatment was employed. The results indicate that bioaugmentation significantly enhances biogas yields, with bioparticles emerging as the most effective combination for all three crops.

Among the three energy crops, MG consistently showed the highest biogas yields in our research, with an average yield of 117.71 NL/kg DM. However, other studies have found significantly higher biogas yields for this crop. Von Cossel et al. (2019) found that the methane yield in a three-year trial was between 230 and 270 NL/kg volatile solids. In their study, the methane content in the biogas averaged 55.1%, which leads to an approximate biogas yield of 190 to 230 NL/kg DM based on the data presented. It should be noted here that methane content was not directly measured in our study. Although this represents a shortcoming and a limitation of the research, many studies have demonstrated a strong and consistent correlation between cumulative biogas and methane yields under comparable anaerobic digestion conditions (Dandikas et al., 2014; Dandikas et al., 2015; Pilarska et al., 2021; Gong et al., 2011). Perhaps mostly applicable to our setup, Dandikas et al. (2014) reported a robust positive correlation across different energy crops and confirmed this relationship with a correlation coefficient of r = 0.96 for grassland plant species (Dandikas et al., 2014). These findings indicate that cumulative biogas production can serve as a reliable proxy for methane potential, thereby supporting the validity of our results despite the absence of direct methane quantification.

Other research has shown similar results for the biogas yield of MG, including studies by Kiesel and Lewandowski (2014), Mangold et al. (2019), and Kulichkova et al. (2020). However, these studies used MG samples harvested in the fall (green harvest), a time when the lignin content is much lower (Huyen et al., 2010; Kiesel and Lewandowski, 2017). This lower lignin content makes the MG more biodegradable and explains the higher biogas yields compared to our results, where the harvest took place in winter, a time when the lignin content is higher and reduces biodegradability. In our case, the one point of investigation was to determine the effect of bioaugmentation when crops have higher lignin content. In that case, the purpose of bioaugmentation is easier to detect in those harsh conditions.

The AD and PV had lower total biogas yields when compared to MG, with AD achieving a biogas yield of 80.02 NL/kg DM. According to research by Špelić et al. (2024), the biogas yield of AD in continuous anaerobic digestion was on average 25.90 NL/day with daily feeding. As shown in Figure 3, the biogas yield was 35.11 NL/kg DM on the first day, after which it decreased during the anaerobic digestion process, indicating that our results are consistent with the mentioned work. In terms of total biogas yield, AD harvested in September performed better with a yield of 229.05 NL/kg DM (Ragaglini et al., 2014). Although our research showed significantly lower biogas yields, the main advantage of using AD in biogas production is its high biomass yield. Previous studies reported biomass yields of up to 30 tons per hectare DM Angelini et al., 2005; Riffaldi et al., 2010), while MG yields up to 25 t per hectare DM (El Bassam, 2010; Whittaker et al., 2016).

The biogas yield of PV was the lowest, reaching a total of only 66.40 NL/kg DM. As already mentioned, the harvest was carried out in March, when the lignin content is higher. This obviously had a major impact on the biogas potential, especially for PV. Other studies show higher biogas yields for this energy crop (Baute et al., 2018; Kupryś-Caruk et al., 2019). However, as shown in Figure 5, PV exhibited the highest percentage increase in biogas production when bioparticles were applied. This suggests that despite its initially low biogas yield, the structural characteristics of PV allowed for a more pronounced effect of bioaugmentation, likely due to improved microbial accessibility and enhanced degradation of its organic components. This observation aligns with the data presented in Table 3, where PV had the lowest lignin content (20.41%), compared to AD (25.65%) and MG (37.54%). A closer look at this relationship is given in Figure 6, which shows that lignin has the strongest correlation with biogas production. For instance, Nakhate et al. (2021) reported that the lowest biogas production (411 mL/g of volatile solids (VS)) was observed for cotton waste, which had the highest lignin content (23.97%). In contrast, the highest biogas yield (622 mL/g VS) was recorded for rice straw, which contained the lowest lignin content (16.9%). This supports the hypothesis that a lower lignin content facilitates enzymatic and microbial degradation, leading to a more efficient conversion of organic material into biogas when bioaugmentation is introduced.

Furthermore, the results presented in Figure 6 suggest that COD could be a reliable predictor of biogas yield (Syaichurrozi and Sumardiono, 2013), emphasising the importance of substrate treatment strategies aimed at increasing the biodegradability of organic material. By improving COD availability, the overall potential of biogas production can be increased, and the performance of anaerobic digestion can be further optimised. Kumar et al. (2020) reported that the reduction of COD due to microbial activity directly correlates with increased biogas production, achieving a maximum biogas yield of 3571.14 mL with a 52.03% COD reduction under optimal mesophilic conditions. These findings align with our study, where substrates showing greater COD reduction demonstrated higher biogas yields, confirming that efficient COD removal enhances biogas production potential.

Bioaugmentation, either with microbial consortium suspension or bioparticles, has been shown to increase the biogas yield of all three crops tested in the study. This supports existing research highlighting the benefits of bioaugmentation in overcoming the limitations of lignocellulosic materials that are otherwise difficult to degrade. The results presented here are consistent with previous reports (Mulat et al., 2018; Ivanković et al., 2022) that have demonstrated the ability of bioaugmentation to enhance microbial activity and substrate degradation, especially for biogas production. These results are also consistent with those of Öner et al. (2018), who used bioaugmentation on wheat straw and established a 39% higher methane yield. Nkemka et al. (2015) also succeeded in increasing the methane content of maize silage in the leachate reactor by 3-10%, but they pointed out that the biogas yield decreased slightly. Interestingly, bioaugmentation with pretreatment of lignocellulosic materials resulted in high degradation of cellulose, hemicellulose and lignin up to 68.8–78.2%, 77.4–89% and 15.4–33.7%, respectively, according to a study by Hu et al. (2016). This led to an increase in methane production of 210 to 246% compared to the yield without pretreatment and bioaugmentation. Nevertheless, harsh pretreatments such as alkali, acid, thermal and ultrasound were used in these studies, which can often be considered unsustainable.

In our study, bioaugmentation also led to a clear increase in biogas yield across all tested crops, with the strongest relative improvement observed in PV, confirming the effectiveness of this approach under challenging conditions of high lignin content.

Perlite as a microbial carrier

Judging by the SEM images, perlite has proven to be an effective bacterial carrier (Figure 2), with high capacity for microbial immobilisation. In both its raw form and as bioparticles, perlite enhanced biogas production, largely due to its highly porous structure (Ivanković et al., 2010; Arnaiz et al., 2006), which provides extensive surface area for microbial adhesion and facilitates the retention of both previously immobilised and digestate-derived microorganisms. However, SEM imaging of perlite after biogas production was not performed in the current study, which would have further substantiated the evidence of bacterial attachment and reinforced the discussion on carrier reusability. Future work will therefore include post-process SEM analyses combined with comparative evaluation of carrier reusability, in order to better assess its implications for process efficiency and long-term sustainability.

The benefit of using bacterial carriers in the anaerobic digestion (AD) process is well established, primarily due to the advantages provided by bacterial biofilms formed on carrier surfaces. Microorganisms embedded within biofilms exhibit notable adaptability and resilience to adverse environmental conditions, and at the same time increase microbial retention time. Generally, the introduction of bacterial carriers to the AD process enhances overall process efficiency (Abera et al., 2024), which is described in many published studies (Pilarska et al., 2021; Gong et al., 2011; Abera et al., 2024; Liu et al., 2017; Ding et al., 2024; Pilarska et al., 2022).

Consequently, most research has focused on identifying optimal carrier materials that combine a high capacity for bacterial immobilisation with non-toxicity, environmental sustainability, and economic feasibility. Several materials tested in the literature are briefly outlined below. Gong et al. (2011) incorporated activated carbon, polyvinyl alcohol, and glass fibers into laboratory-scale AD reactors (2.5 L) and demonstrated that reactors with carriers, particularly activated carbon fibers achieved higher biogas and methane production than controls over a 50-day run. Similarly, Liu et al. (2017) tested polypropylene, polyester, polyamide, and polyurethane fiber materials, reporting that polypropylene fibers increased biogas and methane yields by 45% and 50%, respectively. Pilarska et al. (2021) used silica–lignin particles (0.5–1 mm diameter), which enhanced biogas and methane production by approximately 17–19% during a 21-day run. Ding et al. (2024) observed that the addition of polyurethane carriers increased methane yield by 6–15%.

Our systems employing perlite as a microbial carrier produced results comparable to those reported in the literature, with biogas yields increasing with the addition of just the perlite by approximately 5–29%, depending on the substrate. Perlite was selected due to several notable advantages. From both implementation and technological perspectives, perlite represents a promising carrier for bioaugmentation: unlike most carriers described in the literature, it is a natural aluminosilicate material that can be disposed of without significant environmental impact (Dadić et al., 2024; Ivanković et al., 2010). Moreover, it is inexpensive and widely available; in Croatia, the retail price ranges between 5–10 EUR per kg (approximately 1 EUR/L). Literature consistently confirms its high capacity for bacterial immobilisation.

However, the greatest novelty of the here presented perlite system, when compared to literature data, is the usage of pre-colonised bioparticles. The addition of the latter significantly increased biogas yield when compared to solely the perlite, which presents another level of improvement compared to existing research. The role of perlite as a carrier of precolonized microbial consortium has already been indicated in previous studies (Dadić et al., 2024; Ivanković et al., 2022), and it showed to significantly increase the biogas yield when olive cake was used as a substrate (Ivanković et al., 2022), but not if the substrate was pruned olive tree biomass. Such observation indicates that a beneficial synergy between the carrier and the immobilised consortium is essential for a successful result.

In addition, our findings suggest that the spatial distribution of bacterial carriers within anaerobic digesters may represent a previously underestimated factor in process optimisation. As an example, Zhang et al. (2025) highlighted that the heterogeneity of biochar carrier distribution can substantially influence digestion performance, indicating that the spatial organisation of carriers is not merely a structural detail but a determinant of reactor efficiency and stability. At the same time, Qiu et al. (2024) demonstrated that micro-computed tomography provides a powerful, non-invasive tool for three-dimensional visualisation of internal structures in porous materials and biomass, offering unique insights without compromising sample integrity.

Taking these insights into account, our study demonstrated that micro-CT can be effectively applied to anaerobic digestion systems to resolve the spatial arrangement of carriers and associated microbial communities in situ. This approach not only confirms the feasibility of imaging-based carrier distribution analysis but also sets the stage for quantitatively linking spatial heterogeneity with microbial activity and overall process performance. Beyond carrier distribution, micro-CT also resolved the in situ spatiotemporal patterns of entrained gas (bubble fields) that cogovern mixing, local shear, and mass transfer. Building on Zhang et al. (2025), who showed that carrier spatial uniformity modulates AD performance (Qui et al., 2024), who demonstrated non-destructive, three-dimensional micro-CT of porous biomatrices, we posit that combining carrier maps with bubble metrics (e.g., slice-wise gas holdup, bubble size distributions, and colocalization indices) can mechanistically link spatial heterogeneity to process stability and biogas yield. In our scans, the concurrent visualisation of carrier placement and bubble fields highlights potential structure–hydrodynamics couplings (e.g., bubble trapping at carrier clusters at the sludge/air interface) that are invisible to bulk measurements yet actionable for reactor design and mixing strategies.

CONCLUSION

This study underlines the potential of bioaugmentation as a promising strategy for increasing biogas yield from lignocellulosic energy crops. Among the investigated plants, *Miscanthus* x *giganteus* consistently produced the highest biogas yields, achieving 117.71 NL/kg DM. *Arundo Donax* had a lower biogas yield, but is still a valuable plant for biogas production due to its potential for higher biomass yields. Bioparticles proved to be the most effective bioaugmentation option that stimulated microbial activity and substrate degradation, especially in the case of *Panicum virgatum*, where a 73% increase was observed. Further research should focus on assessing the feasibility of bioaugmentation in continuous biogas production processes and its economic viability on an industrial scale. Investigating the commercial potential of these treatments, as well as their long-term stability, will be the key to determining the scalability of bioaugmentation in improving renewable energy production.

From a methodological standpoint, integration of micro-CT into anaerobic digestion research enables direct links between carrier placement, microbial activity, and process performance. Understanding how spatial architecture governs stability and biogas yield provides a mechanistic basis for designing next-generation digesters with optimised carrier distribution.

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