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ORIGINAL PAPER

Effects of replacing mineral fertilizers with sewage sludge and biogas digestate on ethanol production of *Miscanthus sacchariflorus**

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Abstract

The benefits of using sewage sludge and biogas digestate for energy-efficient cultivation of *Miscanthus*, a promising source of lignocellulosic ethanol, are well documented. However, there is limited knowledge on the effects of these biofertilizers on the conversion of *Miscanthus sacchariflorus* to ethanol, which has not been studied in this context. This study investigated the effects of different fertilizers (sewage sludge, biogas digestate, and mineral fertilizers) at doses of 100 and 160 kg N ha^{-1} on ethanol production from *M. sacchariflorus*. The fertilization with digestate and mineral fertilizers significantly increased ethanol production and fermentation efficiency compared to the control treatment. Digestate (160 kg N ha^{-1}) was as productive as mineral fertilizers (100 kg N ha^{-1}), with corresponding productivities of 2295.2±181.4 and 2276.0±58.83 dm^3 A_{100} ha^{-1}. Moreover, fertilization with mineral fertilizers and digestate alone was sufficient to support yeast fermentation of *M. sacchariflorus* biomass, eliminating the need for nutrient supplementation and reducing production costs. In addition, sequential hydrolysis and fermentation proved more efficient than simultaneous saccharification and fermentation, resulting in 14% (95% confidence interval (CI): 5-23%) higher ethanol concentrations for digestate and 12% (95% CI: 4-19%) for mineral fertilizer. This study shows that bioethanol can be cost-efficiently produced using *M. sacchariflorus* fertilized with digestate as a substitute for mineral fertilizers.

Keywords: bioethanol, fertilization treatment, biogas digestate, *Miscanthus sacchariflorus*, nutrient supplementation

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INTRODUCTION

Bioethanol has gained attention as a promising alternative to fossil fuels owing to its environmental and energy benefits. Unlike fossil fuels, the combustion of bioethanol does not produce harmful pollutants, making it a preferred option (Thangavelu et al. 2016). Currently, industrial ethanol production mainly uses annual crops, such as sugarcane and corn (Devi et al. 2022). However, the high costs of fertilization, maintenance, and protection associated with using traditional annual crops for industrial ethanol production have been criticized. Moreover, this approach poses a threat to natural ecosystems and competes with food production (Robak, Balcerek 2018, David et al. 2023). Therefore, there is an urgent need to explore alternative feedstocks for bioethanol production that are both sustainable and cost-effective.

Miscanthus, a fast-growing perennial rhizomatous grass, is a promising energy crop for bioethanol production because of its desirable biomass properties (Wang et al. 2021). It produces a high biomass yield (Dubis et al. 2019, Dubis et al. 2020, Wang et al. 2021), with high energy value (Wang et al. 2023) and high cellulose and hemicellulose content (Cerazy-Waliszewska et al. 2019). In addition, growing *Miscanthus* provides environmental benefits, such as reducing greenhouse gas emissions (Weik et al. 2022) and increasing soil organic carbon content (Fu et al. 2022). With a long growing season of up to 25 years and low inputs of soil and agricultural technology, *Miscanthus* can provide extensive opportunities to meet future global energy needs (Clifton-Brown et al. 2008, Wang et al. 2021).

The genus *Miscanthus* includes several grass species, e.g. *M. sinensis*, *M.* × *giganteus*, and *M. sacchariflorus*. While *M.* × *giganteus* is preferred for its high yields (Briones et al. 2023), its large-scale cultivation can lead to ecological limitations, diseases, and pest infestations (Ouattara et al. 2022). In contrast, *M. sacchariflorus* offers several advantages as a feedstock for biofuel compared to *M.* × *giganteus*. It has higher tolerance to drought, salinity, and frost (Lewandowski et al. 2016, van der Weijde et al. 2016), making it well suited for cultivation in colder climates and poorer site conditions (Bonin et al. 2014). In addition, *M. sacchariflorus* has lower lignin content (Kim et al. 2012), which increases its suitability for conversion to biofuels.

Bioenergy crops are economically viable for commercial ethanol conversion provided they are highly productive and have low cultivation costs. One approach to increase biomass yields is to use high-input production technologies, mainly fertilization with mineral fertilizers. However, the use of mineral fertilizers is the largest cost factor in crop cultivation, primarily because of the high cost and significant energy required for their production (Jankowski et al. 2016). To address this issue, recent studies have investigated the use of biowaste, such as sewage sludge and digestate, as an alternative to mineral fertilizers. Dubis et al. (2020) found that replacing mineral

fertilizers with sewage sludge and digestate reduced energy requirements for *M.* × *giganteus* production by 34% without decreasing biomass yield. Similarly, fertilization of *M. sacchariflorus* with sewage sludge or digestate reduced energy requirements by 31-48% compared to mineral fertilizers (Dubis et al. 2022). In addition, these waste products are rich in organic matter and micro- and macroelements (Cristina et al. 2020, Lee et al. 2021) that can be easily taken up by plants, which has a positive effect on biomass yields (Lee et al. 2021, Jankowski et al. 2023) and soil quality (Holatko et al. 2023). Despite these benefits, little research has been conducted on how these fertilization methods affect *Miscanthus* ethanol production. Furthermore, previous studies examining these issues have focused on *M.* × *giganteus* (Dubis et al. 2017), while there is a lack of data on the effects of fertilization on ethanol production in *M. sacchariflorus*.

Therefore, the objective of this study was to evaluate the effects of sewage sludge, biogas digestate, and mineral fertilizers applied at two doses (100 and 160 kg N ha^{-1}) on ethanol production from *M. sacchariflorus* hydrolysates. We also investigated whether this fertilization could eliminate the need for additional nutrient supply during fermentation of the hydrolysate by comparing sequential hydrolysis and fermentation with simultaneous saccharification and fermentation methods. We hypothesized that the application of these fertilizers to *M. sacchariflorus* would significantly affect ethanol production from its hydrolysates and increase ethanol yield and productivity. In addition, we hypothesized that biowaste-based fertilizers, such as sewage sludge and digestate, could replace mineral fertilizers in ethanol production. These results would have significant implications for the commercial feasibility of lignocellulosic ethanol as a sustainable alternative to food-derived ethanol.

MATERIALS AND METHODS

Field experiment

M. sacchariflorus (10th year of cultivation) was cultivated at the Production and Experimental Station in Bałcyny (53°35′46.4″ N, 19°51′19.5″ E), which belongs to the University of Warmia and Mazury in Olsztyn (northeastern Poland). The field tests were carried out in triplicate on Haplic Luvisol developed from boulder clay (IUSS Working Group WRB 2015). The arable layer (0-30 cm) was slightly acidic (pH in 1 M KCl was 6.5) and a had high content of available phosphorus and magnesium, and moderate content of potassium. The rhizomes were planted manually in rows with a spacing of 75 × 75 cm, and the area of each plot for harvesting was 44 m^2.

Before the growing period, the crops were subjected to seven fertilizer treatments with varying doses and types of fertilizers, namely: control,

without fertilizer; S_{100} and S_{160}, sewage sludge at a dose equivalent to 100 and 160 kg N ha^{-1}, respectively; O_{100} and O_{160}, organic fertilizer Naturgal based on biogas digestate at a dose equivalent to 100 and 160 kg N ha^{-1}, respectively; M_{100} and M_{160}, mineral fertilizers at a dose equivalent to 100 and 160 kg N ha^{-1}, respectively. The sewage sludge was obtained from a municipal wastewater treatment plant in northeastern Poland. The commercial organic fertilizer sold under the brand name Naturgal was produced by Biogal (Mroczno, northeastern Poland) and was based on digestate obtained by mesophilic digestion. Specific information on the chemical composition of biowaste used as a nutrient source can be found in Table 1. Doses of sewage sludge and digestate were calculated based on their content of total nitrogen and dry mass (DM). In addition to nitrogen, crops were fertilized with 50 kg P_2O_5 and 100 kg K_2O ha^{-1} each year. Ammonium nitrate (34% N), enriched superphosphate (40% P_2O_5), and potassium salt (50% K_2O) were used for mineral fertilization.

M. sacchariflorus was harvested at the end of the flowering mature stage, in the first ten days of October 2017, using a self-propelled chopper.

Table 1

Characteristics of sewage sludge and organic fertilizer Naturgal obtained from digestate originated from agricultural biogas plant

Indicator	Unit	Sewage sludge[1]	Naturgal[2]
pH	–	8.600	7.800
Dry mass (DM)	(%)	11.60	6.980
Organic dry mass	(% DM)	59.20	86.30
Nitrogen	(% DM N)	7.470	1.580
Phosphorus	(% DM P)	4.000	–
	(% DM P_2O_5)	–	2.790
Potassium	(% DM K_2O)	n.a.[#]	1.360
Calcium	(% DM Ca)	4.200	n.a.
Magnesium	(% DM Mg)	0.500	n.a.
Cadmium	(mg Cd kg^{-1} DM)	1.400	0.140
Copper	(mg Cu kg^{-1} DM)	388.0	n.a.
Nickel	(mg Ni kg^{-1} DM)	34.10	8.500
Lead	(mg Pb kg^{-1} DM)	22.80	<5.300
Zinc	(mg Zn kg^{-1} DM)	1100.0	n.a.
Mercury	(mg Hg kg^{-1} DM)	0.420	0.009
Chromium	(mg Cr kg^{-1} DM)	61.20	10.60

[1] Analyses were performed in the Environment, Health and Safety Laboratory in Pszczyna, Poland;

[2] Biogas digestate-based organic fertilizer produced by Biogal Sp. z o.o., analyses were performed in the Institute of Soil Science and Plant Cultivation in Pulawy, Poland;

[#] Not analyzed.

The freshly collected biomass was oven dried at 65°C for 24 h and chopped into 1 to 2 mm long pieces. After drying, the estimated dry mass content of the biomass was 93%.

Pretreatment *of Miscanthus sacchariflorus*

To make the cellulosic material available for subsequent enzymatic hydrolysis, an alkaline pretreatment was performed. For this purpose, 20 g DM of the dried and chopped plants was soaked in 1% w/w sodium hydroxide at a solid-liquid ratio of 1:9 (w/v), to obtain a final total mass of 200 g. The sample was then incubated at 134°C for 1 h, cooled, and centrifuged at RCF 4240 × g for 10 min at a temp. of 10°C using Sigma 3-18K centrifuge (Germany). The resulting supernatant was decanted and distilled water was added to the remaining solid fraction to reach the final mass of 200 g, followed by centrifugation under the same conditions as before. Rinsing of the solid mass with water was repeated one more time.

After pretreatment, the samples were oven dried at 65°C for 24 h to determine the DM. Distilled water was then added to the resulting solid to obtain the final mass of 200 g, and the pH was adjusted to 5.1 using 85% orthophosphoric acid and a pH meter (Hanna HI 9024, USA). Finally, the medium was pasteurized at 90°C for 20 min.

Sequential hydrolysis and fermentation (SHF)

Enzymatic hydrolysis

After pretreatment, enzymatic hydrolysis was performed with three commercial enzymes: Cellulase from *Trichoderma longibrachiatum* (15 U g^{-1} DM of substrate), Xylanase from *T. longibrachiatum* (15 FXU g^{-1} DM of substrate), and Cellobiase from *Aspergillus niger* (30 BDU g^{-1} DM of substrate). Hydrolysis was performed in an Innova 40 incubator (New Brunswick Scientific, USA) at 42°C for 72 h with shaking at 250 rpm.

Fermentation

The fermentation was performed in two variants. In the first variant, the hydrolysates were supplemented with additional nitrogen [$(NH_4)_2SO_4$] and phosphorus (KH_2PO_4) sources, while in the second variant, the fermentation was performed without nutrient supplementation. In both variants, the hydrolysates were inoculated with *Saccharomyces cerevisiae* 7 (5% by volume) and all experiments were performed in triplicate under anaerobic conditions for 72 h at 30°C.

Simultaneous saccharification and fermentation (SSF)

Hydrolysis was first run for 24 h at 42°C and 250 rpm in an Innova 40 incubator (New Brunswick Scientific, USA). After 24 h, nitrogen and phosphorus mineral salts were added to some hydrolysates, while others were left without nutrient supplementation to compare the efficiency of fermentation

with and without nutrient supplementation. Then, the temp. was lowered to 38°C, and 5% by volume of *S. cerevisiae* AS4 inoculum was added. Each experiment was performed in triplicate for 96 h under anaerobic conditions.

Analytical methods

Neutral fiber content (NDL) was determined according to the method described by van Soest et al. (1991), while acid detergent content (ADF) and acid detergent lignin content (ADL) were determined according to the method described in PN-EN ISO13906 (2009). The FibertecTM 1020 system was used to analyze the fiber fraction. Cellulose content was calculated by subtracting ADL from ADF, while hemicellulose content was determined by subtracting ADF from NDF. The efficiency of hydrolysis was evaluated by determining the concentration of enzymatically released reducing sugars using the 3,5-dinitrosalicylic acid method (Miller 1959). Alcohol concentration was determined by the distillation method described by AOAC (1990).

Calculations

The fermentation efficiency was expressed as a percentage of the theoretical yield of ethanol and calculated according to the following formula:

Fermentation efficiency (% of theoretical ethanol yield) =
ethanol concentration (% v/v) × 100/reducing sugar concentration (%) × 0.65,

where 0.65 is a theoretical yield of ethanol from glucose ($dm^3\ A_{100}\ kg^{-1}$).

The ethanol yield indicates the volume of ethanol produced per kg DM of *M. saccharifl orus* that has been subjected to the entire process (pretreatment plus SHF or SSF), and it is expressed as follows:

Ethanol yield (dm3 A100 kg-1 DM) =
volume of ethanol after fermentation (dm3 A100)/mass of biomass (kg DM)

The ethanol productivity was expressed as ethanol obtained from each hectare of *M. saccharifl orus* cultivation, and calculated according to the following formula:

Ethanol productivity (dm^3 A100 ha^{-1}) = ethanol yield (dm^3 A100 kg^{-1} DM) × biomass yield (t DM ha^{-1}) ×1000.

Statistical analysis

The Tukey's HSD (honestly significant difference) test was used to determine the effects of fertilizer treatment on lignocellulose composition and ethanol concentration, yield, and productivity. Differences were considered significant at $P \leq 0.05$.

To evaluate the effect of nutrient supplementation on ethanol concentration, a supplementation ratio was calculated by dividing the mean ethanol

concentration in the non-supplemented variant by the mean ethanol concentration in the corresponding supplemented variant [SHF(–)/(SHF(+) or SSF(–)/SSF(+)]. The ethanol concentration was chosen as a criterion due to its importance in offsetting processing costs, especially the energy-consuming steps such as dehydration or distillation. The statistical significance of nutrient supplementation was determined by calculating 95% confidence intervals (CIs) for the supplementation ratio. CIs were calculated using Student's independent *t*-tests. A CI containing only positive values indicates that the mean ethanol concentration is higher without nutrient supplementation, and vice versa. The range covered by each CI indicates where the true difference would likely be found if the experiment were repeated an infinite number of times.

To quantify the effect of the fermentation mode on ethanol production, the differences between the mean ethanol concentrations of each variant were calculated. To determine the statistical significance of differences between all SHF and SSF pairs, 95% confidence intervals (CIs) were calculated using Student's independent *t*-test. All statistical analyses were performed using Statistica 13.1 software (StatSoft Inc., USA).

RESULTS AND DISCUSSION

Composition of the lignocellulose fraction in *Miscanthus sacchariflorus* fertilized with different treatments

Analysis of the lignocellulosic composition of *M. sacchariflorus* revealed different effects of fertilization. Fertilization with sewage sludge and digestate significantly increased cellulose content. The highest cellulose content (54.9±2.126% DM), which was 20% higher than that of the control plants, was observed in the biomass treated with S_{100}. Fertilization with digestate increased cellulose content by 7% (O_{100}) and 13% (O_{160}) – Table 2. Similar positive effects of sewage sludge fertilization on cellulose content were also observed in other crops, such as rice, with increases ranging from 8% to 21% (Zahoor et al. 2017). However, the addition of mineral fertilizers had a minimal effect as the cellulose content increased by less than 4% in M_{100} treatment (46.4±0.571% DM) and was even lower in M_{160} treatment (39.7±0.491% DM) than in the control sample. In contrast, the hemicellulose content in *Miscanthus* biomass showed an inverse relationship. The biomass treated with sewage sludge and digestate had lower hemicellulose content compared to the control sample (25.1±0.308% DM). Conversely, the plants fertilized with mineral fertilizers had higher hemicellulose content, with values of 28.6±0.719% DM for M_{100} and 31.1±0.437% DM for M_{160}. As a results, the M_{160} biomass had the highest Hem/Cel ratio of 0.783, while the S_{100} biomass had the lowest Hem/Cel ratio of 0.312.

Table 2

Composition of lignocellulose of *Miscanthus sacchariflorus* fertilized by various treatments. Means followed by different letters within the same row for a given biomass component are statistically different according to the Tukey's HDS test at $P \leq 0.05$. The standard deviation of the mean is given after the mean (N=3)

Biomass component	Unit	Fertilization treatment						
		Control	S_{100}	S_{160}	O_{100}	O_{160}	M_{100}	M_{160}
Cellulose (Cel)	(% DM)	44.78 ± 0.758^{a}	54.90 ± 2.126^{b}	45.38 ± 0.585^{ac}	48.19 ± 1.046^{cd}	51.07 ± 1.150^{dc}	46.42 ± 0.571^{ac}	39.73 ± 0.491^{e}
Hemicelluloses (Hem)	(% DM)	25.09 ± 0.308^{a}	17.09 ± 1.711^{b}	23.73 ± 1.255^{a}	24.45 ± 1.318^{a}	24.11 ± 1.015^{a}	28.61 ± 0.719^{c}	31.12 ± 0.437^{c}
Holocellulose	(% DM)	69.87 ± 0.924^{a}	71.99 ± 0.416^{b}	69.11 ± 1.313^{a}	72.64 ± 0.306^{b}	75.18 ± 0.154^{c}	75.03 ± 0.637^{c}	70.85 ± 0.750^{ab}
Lignin (ADL)	(% DM)	11.29 ± 0.331^{a}	10.86 ± 0.599^{a}	12.86 ± 0.478^{b}	10.63 ± 0.275^{a}	11.37 ± 0.445^{a}	10.69 ± 0.293^{a}	13.36 ± 0.662^{b}
Hem to Cel ratio	–	0.560 ± 0.009^{a}	0.312 ± 0.044^{b}	0.523 ± 0.029^{ac}	0.508 ± 0.039^{ac}	0.473 ± 0.031^{c}	0.616 ± 0.021^{ad}	0.783 ± 0.012^{e}

Abbreviations: DM dry mass, M_{100} and M_{160} – mineral fertilizer at a dose equivalent to 100 and 160 kg N ha^{-1}, respectively, O_{100} and O_{160} – digestate-based organic fertilizer Naturgal at a dose equivalent to 100 and 160 kg N ha^{-1}, respectively, S_{100} and S_{160} – sewage sludge at a dose equivalent to 100 and 160 kg N ha^{-1}, respectively, holocellulose – cellulose and hemicelluloses combined, ADL – acid detergent lignin

As for the holocellulose content, all fertilized *Miscanthus* biomasses showed a slight increase, except for the S_{160} treatment, where both polysaccharide contents slightly decreased compared to the unfertilized sample (Table 2). The highest holocellulose content of 75% DM was observed in *Miscanthus* treated with O_{160} and M_{100}. These results show the potential of *M. sacchariflorus* fertilized with digestate as a cost-effective feedstock for the production of ethanol from lignocellulose.

As with the hemicellulose content, plants fertilized with S_{160} and M_{160} had significantly higher lignin content than unfertilized plants and plants fertilized with S_{100} and M_{100}, indicating that the lignin content in the *Miscanthus* biomass is affected by the fertilizer dose. It is worth noting that these lignin contents (Table 2) were lower than those reported for *M.* × *giganteus* by Lee and Kuan (2015) and Turner et al. (2021), which ranged from 12.7% to 22.4% DM and 21.4% to 24.9% DM, respectively. In the context of bioethanol production, high lignin content poses a challenge because its derivatives, such as phenolic compounds, hinder hydrolysis and fermentation processes. Conversely, significant cellulose and hemicellulose content in *Miscanthus* biomass is crucial for increasing the process efficiency, as these polysaccharides serve as a source of fermentable sugars for ethanol conversion (Rosales-Calderon, Arantes 2019). Therefore, it is desirable to obtain biomass with minimal lignin content while maximizing cellulose and hemicellulose content to ensure optimal bioethanol production.

Ethanol production from *Miscanthus sacchariflorus* hydrolysates in sequential hydrolysis and fermentation (SHF)

To select the experimental *M. sacchariflorus* biomasses most suitable for conversion to bioethanol, the hydrolysates of all seven fertilizer treatments were supplemented with mineral nitrogen and phosphorus salts and then fermented in a sequential system (SHF). The results showed that the use of digestate and mineral fertilizers significantly increased ethanol production and fermentation efficiency, regardless of the dose applied (Figure 1). Among the treatments, O_{160} and M_{100} produced the highest ethanol concentrations of 2.29±0.040% v/v and 2.36±0.081% v/v, corresponding to 56.3±1.615% and 58.2±0.577% of the theoretical ethanol yield, respectively. The plants subjected to these fertilization methods had the highest content of holocellulose (Table 2), the main fraction of lignocellulose useful for ethanol fermentation. Our results are in agreement with the study of Kang et al. (2013) on *M. sacchariflorus*, in which ethanol concentrations of 1.2-3.3% w/v (35.4–64.3% of theoretical ethanol yield) were obtained after pretreatment by alkali extrusion with a twin-screw. Furthermore, our results also exceeded the ethanol concentrations of *M.* × *giganteus* pretreated with NaOH determined by Kordala et al. (2023), which ranged from 1.76-2.04% w/v (44.1-51.7% of the theoretical ethanol yield). These results demonstrate the feasibility of digestate as fertilizer in the cultivation of *M. sacchariflorus* for bioethanol production.

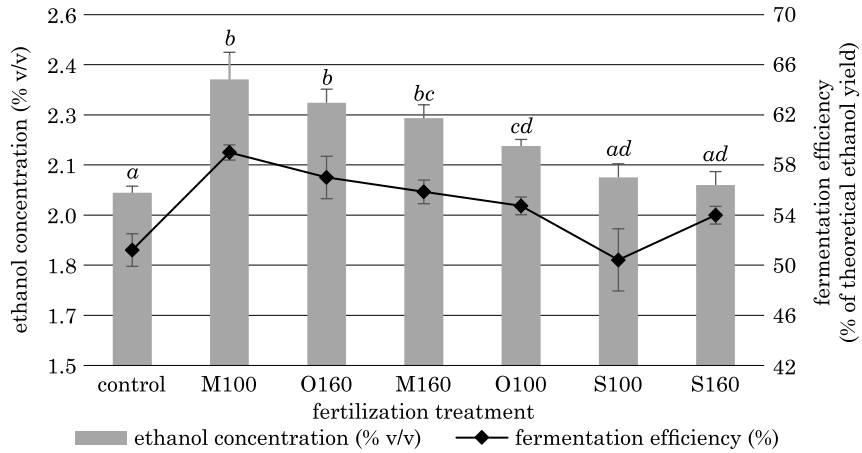

Fig. 1. The effect of different fertilization treatment on the fermentation of *Miscanthus sacchariflorus* hydrolysates in the SHF system with the addition of KH_2PO_4 and $(NH_4)_2SO_4$. Vertical whiskers represent the standard deviation of the mean (N=3). Means followed by different letters are statistically different according to the Tukey's HDS test at $P \leq 0.05$

On the other hand, unfertilized *Miscanthus* and *Miscanthus* fertilized with sewage sludge were found to be less suitable for ethanol production. This could be due to their lower holocellulose content, especially the lower hemicellulose to cellulose ratio (Table 2), as shown in previous studies by Xu et al. (2012) and van der Weijde et al. (2017). These authors reported that *Miscanthus* biomasses with higher hemicellulose content and lower cellulose content were more digestible and therefore produced more ethanol.

Ethanol production from *Miscanthus sacchariflorus* hydrolysates without nutrient supplementation in sequential hydrolysis and fermentation (SHF)

To achieve efficient fermentation of lignocellulosic feedstocks, additional sources of macro- and micronutrients such as peptone, yeast extract, or salt are usually added to the hydrolysates in laboratory experiments. Unfortunately, the use of these additives in large-scale processes is prohibitive due to cost and practicality (Pereira et al. 2010). Therefore, it is important to investigate whether *Miscanthus* fertilization can eliminate the need for additional hydrolysate addition during fermentation, which would be a cost-effective and sustainable approach for large-scale ethanol production from *M. sacchariflorus*.

To evaluate the effect of the lack of nutrient supplementation on ethanol production, this experiment focused on *M. sacchariflorus* plants treated with O_{160} or M_{100}, which had high ethanol production in the earlier phase of the study. To maintain consistency, fermentation was performed with the same yeast strain (*S. cerevisiae* 7) and under the same conditions as in the first phase, except that the addition of mineral salts to the hydrolysate was omit-

ted. The results of the study show that an additional supply of nutrients is not required for ethanol production from selected *M. sacchariflorus* biomasses (Figure 2). This indicates that digestate and mineral fertilizers already provide sufficient nitrogen and phosphorus for yeast fermentation. Moreover, ethanol concentration even increased when no additional nutrients

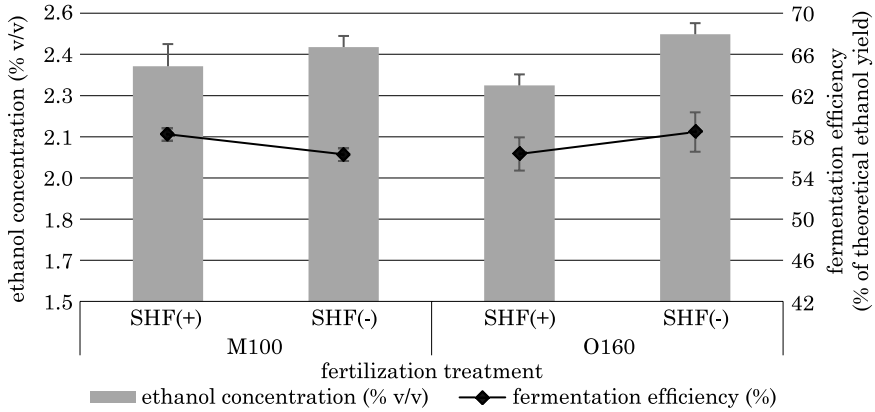

Fig. 2. The effect of the absence of hydrolysate supplementation on the fermentation of *Miscanthus sacchariflorus* hydrolysates in the SHF system. Vertical whiskers represent the standard deviation of the mean (N=3); (+)/(–), fermentation supplemented/ /non-supplemented with KH_2PO_4 and $(NH_4)_2SO_4$. 95% CIs for unpair t-Student express the differences between fermentation variants (see Table 3)

were added to the hydrolyses of O_{160}-treated biomass. The unsupplemented O_{160} variant provided the highest ethanol concentration of 2.47±0.040% v/v, corresponding to 58.5±1.916% of the theoretical ethanol yield (Figure 2), which was 8% higher than the variant with added nutrients (supplementation ratio, 1.082; 95% CI, 1.036 to 1.127) – Table 3. Similarly, the absence of nitrogen and phosphorus supplementation in the M_{100} variant did not significantly affect ethanol concentration. M_{100}-treated plants fermented without supplemental nutrients showed only a slight, insignificant increase in ethanol concentration of 3% (supplementation ratio, 1.031; 95% CI, 0.903 to 1.158). These results indicate that fertilization with mineral fertilizers and especially digestate allows ethanol production from *M. sacchariflorus* without the additional cost of nutrient supplementation.

Our results are consistent with those of Dubis et al. (2017), who also found no clear evidence of the value of supplementing *M.* × *giganteus* hydrolysates with nutrients. However, studies with different types of biomasses have shown that supplementation can enhance ethanol production. For example, Ünal et al. (2020) showed an increase in ethanol yield of 13.5% and 17.9% from rejected watermelon and sugar melon, respectively, when these substrates were supplemented with peptone (initial concentration of total fermentable sugars of 90-100 g dm^{-3}). Xiros and Olsson (2014) demonstrated that the addition of yeast extract to a fermentation medium containing spruce manure as a substrate increased ethanol production in a sequential

Table 3

95% CIs for unpair *t*-Student for ethanol concentration after fermentation of *Miscanthus sacchariflorus* hydrolyzates supplemented or not supplemented with mineral salts in SHF and SSF systems. The standard deviation of the mean is given after the mean

Fertilization treatment	Fermentation method	Mean (% vol. N=3)	Supplementation ratio (95% CI)	Mean difference (95% CI)
O_{160}	SHF(+)	2.287±0.040	1.082 (1.036–1.127)	
	SHF(–)	2.473±0.040		
	SSF(+)	2.333±0.040	1.000 (0.926–1.074)	
	SSF(–)	2.333±0.040		
	SHF(+) vs. SSF(+)			–0.047 (–0.138–0.045)
	SHF(+) vs. SSF(–)			–0.047 (–0.138–0.045)
	SHF(–) vs. SSF(+)			0.140 (0.048–0.232)
	SHF(–) vs. SSF(–)			0.140 (0.048–0.232)
M_{100}	SHF(+)	2.357±0.081	1.031 (0.903–1.158)	
	SHF(–)	2.427±0.040		
	SSF(+)	2.357±0.040	0.980 (0.919–1.042)	
	SSF(–)	2.310±0.020		
	SHF(+) vs. SSF(+)			0.000 (–0.145–0.145)
	SHF(+) vs. SSF(–)			0.047 (–0.087–0.180)
	SHF(–) vs. SSF(+)			0.07 (–0.022–0.162)
	SHF(–) vs. SSF(–)			0.117 (0.044–0.189)

Abbreviations: SHF – sequential hydrolysis and fermentation, SSF – simultaneous saccharification and fermentation, (+)/(–) – fermentation supplemented/non-supplemented with KH_2PO_4 and $(NH_4)_2SO_4$, CI – confidence interval

high-gravity hydrolysis and fermentation process (200 g kg^{-1} water-insoluble solids). Similarly, Phukoetphim et al. (2019) reported a positive effect of yeast extract addition on high-gravity fermentation of sweet sorghum juice. Thus, the different results between this study and previous studies may be due to the use of different substrates, lower sugar concentration

in the hydrolysates (about 60 g dm^{-3}), and the use of a different nitrogen source (mineral ammonium sulfate vs. organic nitrogen compounds).

Ethanol production from *Miscanthus sacchariflorus* hydrolysates without nutrient supplementation in simultaneous saccharification and fermentation (SSF)

The next phase of the study involved the production of ethanol by simultaneous saccharification and fermentation. Based on the results obtained in the previous phase, *M. sacchariflorus* biomass (O_{160} and M_{100} treatments) was fermented with and without the addition of mineral salts.

The effect of the absence of mineral salts on the alcohol concentration in the distillate was found to be negligible (Figure 3). SSF fermentation results for biomass treated with M_{100} showed minimal and insignificant dif-

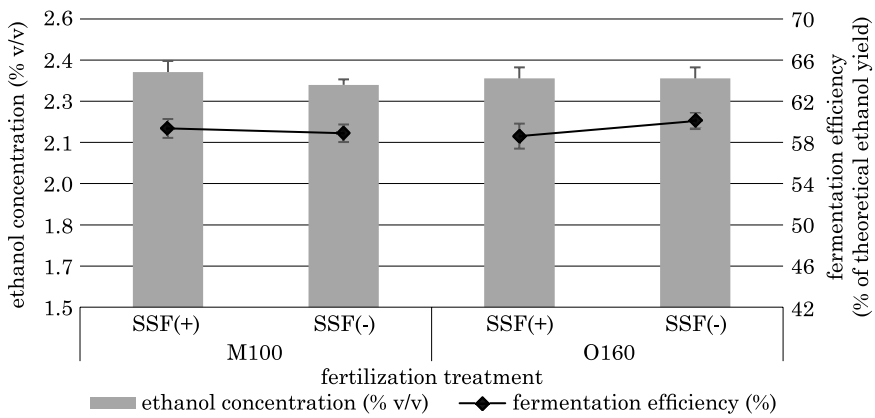

Fig. 3. The effect of the absence of hydrolysate supplementation on the fermentation of *Miscanthus sacchariflorus* hydrolysates in the SSF system. Vertical whiskers represent the standard deviation of the mean (N=3); (+)/(–) – fermentation supplemented/non-supplemented with KH_2PO_4 and $(NH_4)_2SO_4$. 95% CIs for unpair t-Student express the differences between fermentation variants (see Table 3)

ferences (supplementation ratio, 0.980; 95% CI, 0.919 to 1.042). The same trend was observed for O_{160}-treated *Miscanthus* with and without mineral salt supplementation, where there was no significant difference in alcohol concentration (supplementation ratio, 1.000; 95% CI, 0.926 to 1.074) – Figure 3 and Table 3.

Building on the previous results, we compared the efficiency of the SHF and SSF methods in producing bioethanol from *M. sacchariflorus* biomass. The results showed that without nutrient supplementation, the SHF method was more efficient than the SSF method in producing bioethanol. Specifically, *M. sacchariflorus* treated with O_{160} had a 14% higher mean ethanol concentration in SHF than in SSF, with a mean difference of 0.140 and a 95% CI of 0.048 to 0.232 (Table 3). Similarly, ethanol concentrations of M_{100}-treated plants were 12% higher in SHF than in SSF, with 95% CIs ranging from 4%

to 19%. However, the ethanol production of the SHF method with nutrient supplementation was lower and the alcohol concentration was lower than that of the SSF method regardless of supplementation. However, none of the differences between SHF with supplementation and SSF with or without supplementation were significant (Table 3).

Several previous studies have reported on the comparison of SHF and SSF methods for ethanol production, with varying results. López-Linares et al. (2014) found that the SHF produced 15.7% more ethanol than the SSF in the bioconversion of rapeseed straw treated with sulfuric acid. Similarly, Dubis et al. (2017) observed that after experiments with *M.* × *giganteus* treated with sewage sludge and mineral fertilizers, respectively, the SHF produced 11.1% and 19.5% more ethanol than the SSF under similar experimental conditions as in our study. On the other hand, Cotana et al. (2015) reported slightly better performance of the SSF system in bioconversion of Cardoon pretreated by a steam explosion (7.44 vs. 7.01% v/v ethanol concentration). Nguyen et al. (2017) found that SSF produced more than twice the concentration of ethanol than SHF for popping-pretreated mixed agricultural biomass residues (2.6% v/v and 1.2% v/v, respectively). Similarly, Szambelan et al. (2018*a*, *b*), found that ethanol production from sorghum and corn (*Z. mays* L.) grains was twice as effective with SSF as with SHF. Nevertheless, it is important to note that much better results with SSF in some studies could be due to higher biomass loading than SHF (10 vs. 5% w/v DM (Nguyen et al. 2017); 25 vs. 14.3% w/w (Szambelan et al. 2018*a*, *b*).

Ethanol yield and productivity of *Miscanthus sacchariflorus* fertilized with different treatments

Ethanol yield and productivity are crucial factors for plant breeders and ethanol producers because they determine the amount of ethanol that can be produced per kilogram of feedstock or per hectare of cropland. Fertilization was found to have a significant effect on both of these parameters, with values generally higher for all fertilized biomasses than for unfertilized biomass (Table 4). Among the fertilizers used, biomass treated with O_{160} showed the highest ethanol productivity and yield (2295.2±181.4 dm^3 A_{100} ha^{-1} and 0.231±0.038 dm^3 A_{100} kg^{-1} DM), which were 36% and 20% higher than unfertilized *Miscanthus*, respectively. Biomass treated with M_{100} showed similar productivity and yield to O_{160}, with a mean of 2276.0±58.83 dm^3 A_{100} ha^{-1} and 0.226±0.038 dm^3 A_{100} kg^{-1} DM, respectively (*P* values of 0.999 and 0.634). These results show that biowaste-based fertilizers, such as digestate, can replace mineral fertilizers in the cultivation of *M. sacchariflorus* for bioethanol production. Fertilization with biogas digestate has also been shown to increase potential ethanol production in other energy crops, as reported by Głąb et al. (2019) for sweet sorghum. They showed that ethanol productivity increased by up to 48-55% after fertilization with digestate, depending on the hybrid variety used.

Table 4

Ethanol yield and productivity of *Miscanthus sacchariflorus* fertilized with various treatment. Means followed by different letters within the same column for each parameter are statistically different according to the Tukey's HDS test at $P \leq 0.05$.
The standard deviation of the mean is given after the mean (N=3)

Fertilization treatment	Biomass yield (t DM ha^{-1})	Ethanol yield (dm^3 A_{100} kg^{-1} DM)	Ethanol productivity (dm^3 A_{100} ha^{-1})
Control	8.493±0.323^{a}	0.189 ±0.004^{a}	1602.1±88.13^{a}
O_{160}	9.940±0.632^{b}	0.231 ±0.038^{b}	2295.2±181.4^{d}
M_{100}	10.07±0.377^{b}	0.226 ±0.038^{b}	2276.0±58.83^{cd}
O_{100}	10.12±0.121^{b}	0.199 ±0.004^{ac}	2019.7±56.77^{bcd}
S_{100}	10.37±0.311^{b}	0.194 ±0.004^{a}	2006.8±89.35^{bc}
M_{160}	9.557±0.297^{b}	0.209 ±0.002^{bc}	2001.8±76.28^{bc}
S_{160}	9.757±0.395^{b}	0.190 ±0.002^{a}	1849.5±92.98^{ab}

The highest ethanol productivity values obtained for *M. sacchariflorus* in the present study were comparable to those obtained by Cerazy-Waliszewska et al. (2019) using the SSF method with alkaline-pretreated *M. sacchariflorus*. They reported ethanol productivity values of 2200-2400 dm^3 A_{100} ha^{-1}. However, other *Miscanthus* species such as *M.* × *giganteus* and *M. sinensis* were found to be more productive with ethanol productivity values of 4500-5600 dm^3 A_{100} ha^{-1} and 4400-5200 dm^3 A_{100} ha^{-1}, respectively (Cerazy-Waliszewska et al. 2019). Similarly, Dubis et al. (2017) reported higher ethanol productivity values for *M.* × *giganteus*, which may be due to its higher biomass yield compared to *M. sacchariflorus* (Cerazy-Waliszewska et al. 2019, Dubis et al. 2020).

CONCLUSIONS

In this study, it was found that the use of biogas digestate as fertilizer is a viable option to reduce the use of mineral fertilizer in the cultivation of *M. sacchariflorus* for bioethanol production. Ethanol yield (0.231±0.038 vs. 0.226±0.038 dm^3 A_{100} kg^{-1} dried feedstock) and ethanol productivity (2295±181 vs. 2276±159 dm^3 A_{100} ha^{-1}) obtained with digestate were comparable to those obtained with mineral fertilizer. Optimal doses of 160 kg N ha^{-1} for digestate and 100 kg N ha^{-1} for mineral fertilizer were determined for growing *M. sacchariflorus* for bioethanol production. The study also compared the efficiency of sequential hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF), with SHF showing higher ethanol production. However, further studies are needed to evaluate performance

at higher dry mass loading rates. Nutrient supplementation had minimal effect on ethanol concentration. This study highlights the potential of using digestate as fertilizer to improve the cultivation of *M. sacchariflorus* for bioethanol production.

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Credit authorship contribution statement

Bogdan Dubis: Conceptualization, Methodology, Investigation, Writing – Review & Editing; **Tomasz Pokój**: Formal analysis, Visualization, Writing – Original Draft, Writing – Review & Editing; **Małgorzata Lewandowska**: Conceptualization, Methodology, Resources, Validation, Writing – Review & Editing; **Bożena Bogucka**: Methodology, Investigation, Writing – Review & Editing; **Aneta Zajkowska**: Investigation, Writing – Review & Editing; **Artur Szatkowski**: Investigation, Writing – Review & Editing.

Declaration of competing interest

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

REFERENCES

AOAC (1990) *Official Methods of Analysis of AOAC International*, Arlington, VA: JAOAC International, USA.

Bonin, C.L., Heaton, E.A., and Barb, J. (2014) 'Miscanthus sacchariflorus – biofuel parent or new weed?', *GCB Bioenergy*, 6(6), 629-636, available: https://doi.org/10.1111/GCBB.12098

Briones, M.J.I., Massey, A., Elias, D.M.O., McCalmont, J.P., Farrar, K., Donnison, I., and McNamara, N.P. (2023) 'Species selection determines carbon allocation and turnover

in Miscanthus crops: Implications for biomass production and C sequestration', *Science of the Total Environment*, 887, 164003, available: https://doi.org/10.1016/J.SCITOTENV.2023.164003

Cerazy-Waliszewska, J., Jeżowski, S., Łysakowski, P., Waliszewska, B., Zborowska, M., Sobańska, K., Ślusarkiewicz-Jarzina, A., Białas, W., and Pniewski, T. (2019) 'Potential of bioethanol production from biomass of various Miscanthus genotypes cultivated in three -year plantations in west-central Poland', *Industrial Crops and Products*, 141, 111790, available: https://doi.org/10.1016/J.INDCROP.2019.111790

Clifton-Brown, J., Chiang, Y.-C., and Hodkinson, T.R. (2008) 'Miscanthus: Genetic Resources and Breeding Potential to Enhance Bioenergy Production', in Vermerris, W., ed., *Genetic Improvement of Bioenergy Crops*, Gainesville: Springer, New York, NY, 295-308, available: https://doi.org/10.1007/978-0-387-70805-8_10

Cotana, F., Cavalaglio, G., Gelosia, M., Coccia, V., Petrozzi, A., Ingles, D., and Pompili, E. (2015) 'A comparison between SHF and SSSF processes from cardoon for ethanol production', *Industrial Crops and Products*, 69, 424-432, available: https://doi.org/10.1016/J.INDCROP.2015.02.064

Cristina, G., Camelin, E., Tommasi, T., Fino, D., and Pugliese, M. (2020) 'Anaerobic digestates from sewage sludge used as fertilizer on a poor alkaline sandy soil and on a peat substrate: Effects on tomato plants growth and on soil properties', *Journal of Environmental Management*, 269, 110767, available: https://doi.org/10.1016/J.JENVMAN.2020.110767

David, A.J., Abinandan, S., Vaidyanathan, V.K., Xu, C.C., and Krishnamurthi, T. (2023) 'A critical review on current status and environmental sustainability of pre-treatment methods for bioethanol production from lignocellulose feedstocks', *3 Biotech 2023*, 13(7), 233, available: https://doi.org/10.1007/S13205-023-03657-1

Devi, A., Bajar, S., Kour, H., Kothari, R., Pant, D., and Singh, A. (2022) 'Lignocellulosic biomass valorization for bioethanol production: a circular bioeconomy approach', *BioEnergy Research*, 15(4), 1820-1841, available: https://doi.org/10.1007/S12155-022-10401-9

Dubis, B., Bułkowska, K., Lewandowska, M., Szempliński, W., Jankowski, K.J., Idźkowski, J., Kordala, N., and Szymańska, K. (2017) 'Effect of different nitrogen fertilizer treatments on the conversion of Miscanthus × giganteus to ethanol', *Bioresource Technology*, 243, 731-737, available: https://doi.org/10.1016/J.BIORTECH.2017.07.005

Dubis, B., Jankowski, K.J., Załuski, D., Bórawski, P., and Szempliński, W. (2019) 'Biomass production and energy balance of Miscanthus over a period of 11 years: A case study in a large -scale farm in Poland', *GCB Bioenergy*, 11(10), 1187-1201, available: https://doi.org/10.1111/GCBB.12625

Dubis, B., Jankowski, K.J., Załuski, D., and Sokólski, M. (2020) 'The effect of sewage sludge fertilization on the biomass yield of giant miscanthus and the energy balance of the production process', *Energy*, 206, 118189, available: https://doi.org/10.1016/J.ENERGY.2020.118189

Dubis, B., Szatkowski, A., and Jankowski, K.J. (2022) 'Sewage sludge, digestate, and mineral fertilizer application affects the yield and energy balance of Amur silvergrass', *Industrial Crops and Products*, 175, 114235, available: https://doi.org/10.1016/J.INDCROP.2021.114235

Fu, T., Xu, Y., Hou, W., Yi, Z., and Xue, S. (2022) 'Long-term cultivation of Miscanthus and switchgrass accelerates soil organic carbon accumulation by decreasing carbon mineralization in infertile red soil', *GCB Bioenergy*, 14(9), 1065-1077, available: https://doi.org/10.1111/GCBB.12987

Głąb, L., Sowiński, J., Chmielewska, J., Prask, H., Fugol, M., and Szlachta, J. (2019) 'Comparison of the energy efficiency of methane and ethanol production from sweet sorghum (Sorghum bicolor (L.) Moench) with a variety of feedstock management technologies', *Biomass and Bioenergy*, 129, 105332, available: https://doi.org/10.1016/J.BIOMBIOE.2019.105332

Holatko, J., Hammerschmiedt, T., Kucerik, J., Kintl, A., Baltazar, T., Malicek, O., Latal, O., and Brtnicky, M. (2023) 'Fertilisation of permanent grasslands with digestate and its effect on soil properties and sustainable biomass production', *European Journal of Agronomy*, 149, 126914, available: https://doi.org/10.1016/J.EJA.2023.126914

IUSS Working Group WRB (2015) 'World reference base for soil resources. International soil classification system for naming soils and creating legends for soil maps.', *World Soil Resources Reports*, No. 106.

Jankowski, K.J., Dubis, B., Budzyński, W.S., Bórawski, P., and Bułkowska, K. (2016) 'Energy efficiency of crops grown for biogas production in a large-scale farm in Poland', *Energy*, 109, 277-286, available: https://doi.org/10.1016/J.ENERGY.2016.04.087

Jankowski, K.J., Kołodziej, B., Dubis, B., Sugier, D., Antonkiewicz, J., and Szatkowski, A. (2023) 'The effect of sewage sludge on the energy balance of cup plant biomass production. A six-year field experiment in Poland', *Energy*, 276, 127478, available: https://doi.org/10.1016/J.ENERGY.2023.127478

Kang, K.E., Han, M., Moon, S.K., Kang, H.W., Kim, Y., Cha, Y.L., and Choi, G.W. (2013) 'Optimization of alkali-extrusion pretreatment with twin-screw for bioethanol production from Miscanthus', *Fuel*, 109, 520-526, available: https://doi.org/10.1016/J.FUEL.2013.03.026

Kim, C., Zhang, D., Auckland, S.A., Rainville, L.K., Jakob, K., Kronmiller, B., Sacks, E.J., Deuter, M., and Paterson, A.H. (2012) 'SSR-based genetic maps of Miscanthus sinensis and M. sacchariflorus, and their comparison to sorghum', *Theoretical and Applied Genetics*, 124(7), 1325-1338, available: https://doi.org/10.1007/S00122-012-1790-1/FIGURES/3

Kordala, N., Lewandowska, M., and Bednarski, W. (2023) 'Effect of the method for the elimination of inhibitors present in Miscanthus giganteus hydrolysates on ethanol production effectiveness', *Biomass Conversion and Biorefinery*, 13(3), 2089-2097, available: https://doi.org/10.1007/S13399-020-01255-2/FIGURES/2

Lee, M.S., Urgun-Demirtas, M., Shen, Y., Zumpf, C., Anderson, E.K., Rayburn, A.L., and Lee, D.K. (2021) 'Effect of digestate and digestate supplemented with biochar on switchgrass growth and chemical composition', *Biomass and Bioenergy*, 144, 105928, available: https://doi.org/10.1016/J.BIOMBIOE.2020.105928

Lee, W.C. and Kuan, W.C. (2015) 'Miscanthus as cellulosic biomass for bioethanol production', *Biotechnology Journal*, 10(6), 840-854, available: https://doi.org/10.1002/BIOT.201400704

Lewandowski, I., Clifton-Brown, J., Trindade, L.M., Van Der Linden, G.C., Schwarz, K.U., Müller-Sämann, K., Anisimov, A., Chen, C.L., Dolstra, O., Donnison, I.S., Farrar, K., Fonteyne, S., Harding, G., Hastings, A., Huxley, L.M., Iqbal, Y., Khokhlov, N., Kiesel, A., Lootens, P., Meyer, H., Mos, M., Muylle, H., Nunn, C., Özgüven, M., Roldán-Ruiz, I., Schüle, H., Tarakanov, I., Der Weijde, T., Wagner, M., Xi, Q., and Kalinina, O. (2016) 'Progress on optimizing miscanthus biomass production for the European bioeconomy: Results of the EU FP7 project OPTIMISC', *Frontiers in Plant Science*, 7 (November 2016), 1620, available: https://doi.org/10.3389/FPLS.2016.01620/BIBTEX

López-Linares, J.C., Romero, I., Cara, C., Ruiz, E., Moya, M., and Castro, E. (2014) 'Bioethanol production from rapeseed straw at high solids loading with different process configurations', *Fuel*, 122, 112-118, available: https://doi.org/10.1016/J.FUEL.2014.01.024

Miller, G.L. (1959) 'Use of dinitrosalicylic acid reagent for determination of reducing sugar', *Analytical Chemistry*, 31(3), 426-428, available: https://doi.org/10.1021/AC60147A030/ASSET/AC60147A030.FP.PNG_V03

Nguyen, Q.A., Yang, J., and Bae, H.J. (2017) 'Bioethanol production from individual and mixed agricultural biomass residues', *Industrial Crops and Products*, 95, 718-725, available: https://doi.org/10.1016/J.INDCROP.2016.11.040

Ouattara, M.S., Laurent, A., Berthou, M., Borujerdi, E., Butier, A., Malvoisin, P., Romelot, D., and Loyce, C. (2022) 'Identifying factors explaining yield variability of miscanthus x giganteus and miscanthus sinensis across contrasting environments: Use of an agronomic dia-

gnosis approach', *Bioenergy Research*, 15(2), 672-685, available: https://doi.org/10.1007/S12155-021-10332-X/FIGURES/7

Pereira, F.B., Guimarães, P.M.R., Teixeira, J.A., and Domingues, L. (2010) 'Optimization of low-cost medium for very high gravity ethanol fermentations by Saccharomyces cerevisiae using statistical experimental designs', *Bioresource Technology*, 101(20), 7856-7863, available: https://doi.org/10.1016/J.BIORTECH.2010.04.082

Phukoetphim, N., Chan-U-Tit, P., Laopaiboon, P., and Laopaiboon, L. (2019) 'Improvement of bioethanol production from sweet sorghum juice under very high gravity fermentation: Effect of nitrogen, osmoprotectant, and aeration', *Energies 2019*, 12(19), 3620, available: https://doi.org/10.3390/EN12193620

PN-EN ISO13906 (2009) 'Feeds. Determination of the content of acid detergent fiber (ADF) and acid detergent lignin (ADL)'.

Robak, K. and Balcerek, M. (2018) 'Review of second generation bioethanol production from residual biomass', *Food Technology and Biotechnology*, 56(2), 174, available: https://doi.org/10.17113/FTB.56.02.18.5428

Rosales-Calderon, O. and Arantes, V. (2019) 'A review on commercial-scale high-value products that can be produced alongside cellulosic ethanol', *Biotechnology for Biofuels*, 12(1), 1-58, available: https://doi.org/10.1186/S13068-019-1529-1/FIGURES/6

van Soest, P.J., Robertson, J.B., and Lewis, B.A. (1991) 'Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition', *Journal of Dairy Science*, 74(10), 3583-3597, available: https://doi.org/10.3168/JDS.S0022-0302(91)78551-2

Szambelan, K., Nowak, J., Frankowski, J., Szwengiel, A., Jeleń, H., and Burczyk, H. (2018*a*) 'The comprehensive analysis of sorghum cultivated in Poland for energy purposes: Separate hydrolysis and fermentation and simultaneous saccharification and fermentation methods and their impact on bioethanol effectiveness and volatile by-products from the grain and the energy potential of sorghum straw', *Bioresource Technology*, 250, 750-757, available: https://doi.org/10.1016/J.BIORTECH.2017.11.096

Szambelan, K., Nowak, J., Szwengiel, A., Jeleń, H., and Łukaszewski, G. (2018*b*) 'Separate hydrolysis and fermentation and simultaneous saccharification and fermentation methods in bioethanol production and formation of volatile by-products from selected corn cultivars', *Industrial Crops and Products*, 118, 355-361, available: https://doi.org/10.1016/J.INDCROP.2018.03.059

Thangavelu, S.K., Ahmed, A.S., and Ani, F.N. (2016) 'Review on bioethanol as alternative fuel for spark ignition engines', *Renewable and Sustainable Energy Reviews*, 56, 820-835, available: https://doi.org/10.1016/J.RSER.2015.11.089

Turner, W., Greetham, D., Mos, M., Squance, M., Kam, J., and Du, C. (2021) 'Exploring the bioethanol production potential of miscanthus cultivars', *Applied Sciences*, 11(21), 9949, available: https://doi.org/10.3390/APP11219949

Ünal, M.Ü., Chowdhury, G., Şener, A. (2020) 'Effect of temperature and nitrogen supplementation on bioethanol production from waste bread, watermelon and muskmelon by Saccharomyces cerevisiae', *Biofuels*, 13(4), 395-399, available: https://doi.org/10.1080/17597269.2020.1724440

Wang, C., Kong, Y., Hu, R., and Zhou, G. (2021) 'Miscanthus: A fast-growing crop for environmental remediation and biofuel production', *GCB Bioenergy*, 13(1), 58-69, available: https://doi.org/10.1111/GCBB.12761

Wang, N., Torralbo, F., Naumann, H.D., and Fritschi, F.B. (2023) 'Tissue composition and higher heating values differ among six bioenergy grasses', *Industrial Crops and Products*, 205, 117437, available: https://doi.org/10.1016/J.INDCROP.2023.117437

van der Weijde, T., Kiesel, A., Iqbal, Y., Muylle, H., Dolstra, O., Visser, R.G.F., Lewandowski, I., and Trindade, L.M. (2017) 'Evaluation of Miscanthus sinensis biomass quality as feedstock

for conversion into different bioenergy products', *GCB Bioenergy*, 9(1), 176-190, available: https://doi.org/10.1111/GCBB.12355

van der Weijde, T., Torres, A.F., Dolstra, O., Dechesne, A., Visser, R.G.F., and Trindade, L.M. (2016) 'Impact of different lignin fractions on saccharification efficiency in diverse species of the bioenergy crop Miscanthus', *Bioenergy Research*, 9(1), 146-156, available: https://doi.org/10.1007/S12155-015-9669-Z/FIGURES/3

Weik, J., Lask, J., Petig, E., Seeger, S., Marting Vidaurre, N., Wagner, M., Weiler, M., Bahrs, E., Lewandowski, I., and Angenendt, E. (2022) 'Implications of large-scale miscanthus cultivation in water protection areas: A life cycle assessment with model coupling for improved policy support', *GCB Bioenergy*, 14(11), 1162-1182, available: https://doi.org/10.1111/GCBB.12994

Xiros, C. and Olsson, L. (2014) 'Comparison of strategies to overcome the inhibitory effects in high-gravity fermentation of lignocellulosic hydrolysates', *Biomass and Bioenergy*, 65, 79-90, available: https://doi.org/10.1016/J.BIOMBIOE.2014.03.060.

Xu, N., Zhang, W., Ren, S., Liu, F., Zhao, C., Liao, H., Xu, Z., Huang, J., Li, Q., Tu, Y., Yu, B., Wang, Y., Jiang, J., Qin, J., and Peng, L. (2012) 'Hemicelluloses negatively affect lignocellulose crystallinity for high biomass digestibility under NaOH and H_2SO_4 pretreatments in Miscanthus', *Biotechnology for Biofuels*, 5(1), 1-12, available: https://doi.org/10.1186/1754-6834-5-58/FIGURES/7

Zahoor, Sun, D., Li, Y., Wang, J., Tu, Y., Wang, Y., Hu, Z., Zhou, S., Wang, L., Xie, G., Huang, J., Alam, A., and Peng, L. (2017) 'Biomass saccharification is largely enhanced by altering wall polymer features and reducing silicon accumulation in rice cultivars harvested from nitrogen fertilizer supply', *Bioresource Technology*, 243, 957-965, available: https://doi.org/10.1016/J.BIORTECH.2017.07.057