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Effect of biological pretreatment of *Agropyron elongatum* 'BAMAR' on biogas production by anaerobic digestion

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HIGHLIGHTS

- *Agropyron elongatum* 'BAMAR' (Tall Wheat Grass) was treated with *Flammulina velutipes*.
- Mesophilic anaerobic digestion of fungal pretreated Tall Wheat Grass for biogas production.
- Fungal pretreatment caused as much as 35% of lignin degradation.
- Both biogas and methane production were significantly improved.

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ABSTRACT

The aim of this work was to analyze the impact of three different moisture contents (MC), at 45% MC, 65% MC, 75% MC, on the degradation of cellulose, hemicellulose, and lignin during fungi treatment by *Flammulina velutipes* of *Agropyron elongatum* 'BAMAR' and on biogas production. The analysis of chemical composition shown that *F. velutipes* had greater selectivity for lignin biodegradation with the highest hemicellulose and lignin removal at 29.1% and 35.4%, respectively, and lowest cellulose removal (20.48%) at 65% MC. *F. velutipes* cultivated at 65% MC increased biogas production of 398.07 $Ndm^3\ kg^{-1}\ VS^{-1}$, which was 120% higher than the untreated sample. These treatment conditions resulted in 134% more methane yield compared with untreated sample. The results of this study suggested that *A. elongatum* is a potential biomass for biogas production in agriculture biogas plant and white-rot fungus *F. velutipes* provides an effective methods for improve biodegradation of *A. elongatum*.

1. Introduction

Anaerobic digestion (AD) of energy crops is widely used as a promising and alternative renewable energy resource to fossil fuels (Lalak et al., 2015). However, lignocellulosic biomass like *Agropyron elongatum* 'BAMAR' is not fully biodegraded in AD-process on industrial scale due to their complex physical and chemical structure, which results in lower energy recovery in terms of methane yield. The biodegradability of lignocellulosic substrates can be increased by a pre-treatment. Effective pretreatment can break down the linkage between polysaccharides and lignin to make cellulose and hemicelluloses more accessible and more readily degradable to anaerobic bacteria (He et al., 2008).

Different pretreatment technology have been extensively studied and reported in the literature for reducing the recalcitrance of lignocellulosic material such as, physical, chemical and biological methods. Physical and chemical methods are regarded as promising pretreatment technology for industrial application, which can improve the biodegradability by increasing the interaction surfaces of biomass and change the lignin structure and solubilize lignin (Zheng et al., 2009). Nevertheless, these pretreatment may be require high-energy input, require special equipment, or produce inhibitors, i.e., furfural, hydroxymethylfurfural (HMF), and phenolic compounds, that can be determined to subsequent methane fermentation (van Kuijk et al., 2015).

Biological methods using white-rot fungi and their enzyme extracts are regarded as more economically viable and environmentally friendly alternatives (Rasmussen et al., 2010; van Kuijk et al., 2015). Among biological methods, fungal treatment by white-rot fungi (i.e., *Phanerochaete chrysosporium*, *Trametes versicolor*, *Flammulina velutipes*) is the most effective and extensively used technology for reduce the recalcitrance of lignocellulosic biomass and increase the hydrolysis of the carbohydrates (Zhao et al., 2014). Several studies have shown improved biogas production or

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glucose yield after fungal pretreatment of several feedstocks, for instance corn stover, hardwood, and rubber wood (Amirta et al., 2006; Wan and Li, 2010; Nazarpour et al., 2013; Zhao et al., 2014). Nevertheless, whether fungal pretreatment could selectively degrade lignin in *A. elongatum* 'BAMAR' and consequently increase methane production is still unclear.

During biological pretreatment, the moisture content is a key factor for fungal growth in solid-state cultivation. Water is critical in transferring nutrients, so sufficient water is needed for normal ligninolytic activity and an appropriate growth and development of fungi. On the other hand, too much water may inhibit the growth of fungi by reducing the available oxygen (Singhania et al., 2009; Zhao et al., 2014). Shi et al. (2008) found that the moisture content and culture time affected the fungal pretreatment of cotton stalk using *P. chrysosporium*. Alam et al. (2005) indicated that the moisture content and inoculum size affected ligninase production during the fungal pretreatment of oil palm biomass. For that reason, optimization of moisture content during fungi treatment is necessary for effectively degrading lignin and, in consequence, decomposing the complicated structure of lignocellulosic biomass to improve the biogas yield (Zhao et al., 2014; Kamcharoen et al., 2014).

The objectives of the investigation are the following: (1) determine the influence of moisture content during fungal treatment with *F. velutipes* on the biodegradation of total solid (TS), cellulose, hemicellulose, and lignin; (2) evaluate the effects of biological pretreatment using white-rot fungi on biogas yield of *A. elongatum* 'BAMAR'.

2. Methods

2.1. Preparation of raw material

The main substrate for the process of methane fermentation was *A. elongatum* 'BAMAR' (common Tall Wheat Grass) obtained from The Plant Breeding and Acclimatization Institute (IHAR), National Research Institute, Radzików, Poland (52°12′49.7″N 20°3 8′38.9″E). It was air-dried to a moisture content of less than 10%, and ground with plant crasher to a sample size of 1.5–2.0 mm. And then, were bagged and stored at ambient conditions until used.

2.2. Inoculum for anaerobic digestion

Anaerobic digester sludge was obtained from mesophilic biogas plant in Siedliszczki, Poland (51°08′14.9″N 22°52′07.5″E). The sludge had a pH of 7.20–7.28 and contained on average 4.47% total solids and 31.57% total volatile solids. Before AD-process it was incubated at 37 °C for 5 days to decrease the background gas production.

2.3. Fungal strain and inoculation

White-rot fungus *F. velutipes* (CCBAS 365) (common 'golden needle mushroom') obtained from the Culture Collection of Basidiomycetes (CCBAS) of the Institute of Microbiology, Academy of Sciences of the Czech Republic was used in this study. This strain was preserved on potato dextrose agar (PDA) plates at 4 °C and routinely sub-cultured every month. To prepare the inoculum for the pretreatment process of Tall Wheat Grass, mycelium agar plugs with 10 mm in diameter, were cut along the edge of the actively growing colonies, which had been cultivated on PDA plates for 7 days at 28 °C.

2.4. Fungal pretreatment of Tall Wheat Grass and calculation

The fungal pretreatment was carried out in a 250 mL Erlenmeyer flask with 10 g of air-dried Tall Wheatgrass, then deionized (DI) water was added to obtain moisture contents (MC) such as 45%, 60%, and 75% (w/v) (Zhao et al., 2014). The samples at different moisture contents were sterilized in the autoclave for 20 min at 121 °C and then inoculated with inoculum. The cultures were incubated statically at 28 °C for 4 weeks. The non-inoculated samples were served as the control. All experiments were performed in triplicate. After pretreatment, all samples were used as a feedstock for anaerobic digestion process (AD-process).

The compositional variations of Tall Wheat Grass after fungal pretreatment were calculated as follow (Li et al., 2015) (Eqs. (1)–(4)):

$$\text{Solid Recovery SR} = (D_a/D_0)\ 100, \tag{1}$$

where D_a is the dry weight of Tall Wheat Grass after fungal pretreatment, D_0 is the original dry weight.

$$\text{Cellulose retention} = (C_a/C_0)\ 100, \tag{2}$$

$$\text{Hemicellulose retention} = (H_a/H_0)\ 100, \tag{3}$$

$$\text{Lignin removal} = ((L_0 - L_a)/L_0)\ 100, \tag{4}$$

where C_a, H_a, L_a are, respectively, cellulose, hemicellulose, and lignin fractions (%) of Tall Wheat Grass after fungal pretreatment, while C_0, H_0, L_0 are the corresponding fractions (%) of original Tall Wheat Grass.

2.5. Anaerobic digestion process

Mesophilic anaerobic digestion process was performed according to the DIN 38 414 protocol (1985). This process were carried out in a 2 L Biostat® B-plus stirred tank reactor (Sartorius Stedim Biotech, Gottingen, Germany). The temperature of the process was 37 °C and pH was 7.0. The initial loading of 10 g VS L^{-1} and substrate to inoculum ratio (S/I) of 1:1 (based on the VS) was established. Anaerobic conditions were created by blowing the whole volume of the reactor with nitrogen (Lalak et al., 2015). Once a day, the composition of biogas was determined by means of multigas monitor (GFM436, Gas Data, UK). The volume of biogas was determined by the method of liquid displacement. The process was conducted until the moment when the daily yield was less than 1% of the total biogas yield obtained till then. The biogas and methane yield were calculated as follow (Yuan et al., 2014; Lalak et al., 2015):

$$\text{Biogas yield} = (\text{biogas volume}_{\text{total}} - \text{biogas volume}_{\text{control}})/\ \text{VS of substrates added}, \tag{5}$$

$$\text{Methane yield} = (\text{methane volume}_{\text{total}} - \text{methane volume}_{\text{control}})/\ \text{VS of substrates added}, \tag{6}$$

where biogas yield and methane yield ($dm^3\ kg^{-1}\ VS^{-1}$).

The obtained values of biogas volume were converted to the normal conditions (1013 hPa, 273 K) (DIN 38 414, 1985; Lalak et al., 2015) as indicated by Equation:

$$V = (273/T) \cdot (P/1013) \cdot V(CO_2 + CH_4), \quad (7)$$

where T is room temperature in K. P is the atmospheric pressure in the laboratory at room temperature in hPa. V is the CO_2 and CH_4 volume from the sample vessel under standard conditions in L.

2.6. Analytical methods

Total solids (TS), volatile solids (VS), and ash contents of investigated materials were determined by drying to constant weight at 105 °C and 550 °C (PN-EN 12880, 2004; PN-EN 12879, 2004). Total nitrogen (TN) was estimated by the Kjeldahl method. The Total Organic Carbon (TOC) and Chemical Oxygen Demand was measured according with manufacturer protocol (respectively: IL550 TOC; LCI400 COD, Hach-Lange, Germany). Ammonium nitrogen was measured by spectrophotometry. The analysis of lignin, cellulose and hemicellulose contents of raw material and feedstock were determined by the extraction unit for determination raw fiber. All samples were analyzed using the proximate standard procedures of AOAC (1997), and the fiber fraction content protocol by Van Soest et al. (1991). The Total Phenolic content (TP) were tested by the Folin–Ciocalteu method (Makkar, 2003) and calculated as gallic acid equivalent (GAE). Total reducing sugars were determined by the DNS method, using glucose as the standard (Miller, 1959). Macro- and microelements were tested by the Inductive Coupled Plasma-Optical Emission Spectrometer (ICP OES, Thermo Scientific iCAP Series 6500) (Lalak et al., 2015).

2.7. Statistical methods

Statistical analyses of collected data were performed using the statistical package STATISTICA (data analysis software system), Version 10 and $p < 0.05$ was considered significant.

3. Result and discussion

3.1. Physical and chemical properties of raw Tall Wheat Grass

In order to compare the composition difference between raw and treated Tall Wheat Grass, the composition of the raw Tall Wheat Grass was first analyzed. The raw Tall Wheat Grass used in this study was composed of 46.24% cellulose, 28.51% hemicellulose and 28.27% lignin. Cellulose and hemicellulose are the main carbon source of anaerobic microorganisms, accounting for 74.75% the total dry weight of Tall Wheat Grass. Formation of cellulose and hemicellulose content at such a high level, should be considered as a very advantageous in terms of bioenergy. However, a high lignin content might be one of the critical parameter in the generation of biofuels from lignocellulosic biomass. The raw biomass contained on average 90.62% total solids and 92.66% total volatile solids. The high content of organic matter results in the high content of organic carbon – as much as 46%. The carbon/nitrogen (C/N) ratio was high (73.27), requiring the addition of extra nitrogen to adjust the C/N when Tall Wheat Grass was used as the single feedstock for anaerobic digestion. The elemental composition can be used as one of the indicators determining usefulness of plant material for methane fermentation. The high content of potassium (26.5%) and silicon (43.8%) in raw material was probably caused by fertilization applied in the cultivation and its absorption by plants during the growing cycle (Oleszek et al., 2014; Lalak et al., 2015).

3.2. Compositional change of Tall Wheat Grass after fungal pretreatment

The main purpose of pretreatment is to change raw material properties, remove or dissolve lignin and hemicellulose, and reduce the crystallinity of cellulose. The effect of the three moisture contents (MC) (45%, 65%, and 75% (w/v)) on the biodegradation of Tall Wheat Grass during pretreatment by *F. velutipes* are shown in Fig. 1 and Table 1. The reduction of solid, cellulose, hemicellulose and lignin in the Tall Wheat Grass were increased by the *F. velutipes* treatment. But the highest degradation of four components was founded during pretreatment at 65% MC, followed by 75% MC. Although, the fungal pretreatment conducted at 45% MC had the lowest degradation of these components.

A maximum dry mass loss during the fungal pretreatment process of 70.91% was reached when Tall Wheat Grass was at 65% MC. Higher dry mass loss generally resulted from higher reduction of cell wall components such as hemicellulose, cellulose, and lignin. This can also be proved by the higher lignocellulose degradation (20.48% cellulose, 29.1% hemicellulose, and 35.4% lignin) in Tall Wheat Grass treated at 65% MC as compared with untreated substrates (Fig. 1). As shown in Fig. 1, great delignification ability of *F. velutipes* is observed at different conditions of pretreatment, with higher degradation of hemicellulose and lignin. These indicated that *F. velutipes* not produced cellulases to attack the low crystallinity of cellulose (Wan and Li, 2010). But, the low reduction of cellulose content by *F. velutipes* was generally positive for the reason that cellulose is considered as the most important substrate for anaerobic digestion (Zhao et al., 2014).

Reducing the lignin content of the biomass allows to expose the ordered crystalline structure of cellulose and facilitates substrate access by enzymes (Nazarpour et al., 2013). Lignin biodegradation from the pretreated Tall Wheat Grass by *F. velutipes* was significantly higher than the untreated biomass. Significant different in lignin degradation were observed between the biomass with different moisture contents. In this study the highest level of lignin degradation was obtained when Tall Wheat Grass was at 65% MC, followed by 75% MC. Zhao et al. (2014) reported similar results, that the highest lignin degradation was founded for the yard trimmings treated at 60% MC. However, recent studies suggest that the optimal moisture content for lignin biodegradation was about 70–80% MC (Wan and Li, 2010). According to Singhania et al. (2009), the reason for the difference in moisture contents might be associated with the lower water holding capability of Tall Wheat Grass compared to other substrates such corn silage. Consequently, too much free water may reduce the inter particle spaces and therefore reduce the available oxygen for the fungal cultivation and metabolism required for delignification (Zhao et al., 2014).

Scharer and Moo-Young (1979) and Zhong et al. (2011) founded that the biodegradability of the substrate may be evaluated by ratio of lignin and cellulose. The Lig./CEL ratios of fungal pretreated Tall Wheat Grass at 65% MC were lower than the untreated biomass, reflecting the improvement of degradability with fungal pretreated biomass.

The total sugar content decreased during treatment at different moisture contents of Tall Wheat Grass. These indicated that fungi utilized reducing sugar during treatment. Muthangya et al. (2009) and Nuchdang et al. (2015) founded that white rot fungi metabolize sugar in preference to lignin and cellulose.

Presence of phenolic compounds in the hydrolysates indicates the possibility of partial degradation of lignin. However, these compounds have in many cases an inhibitory or toxic effect on bac-

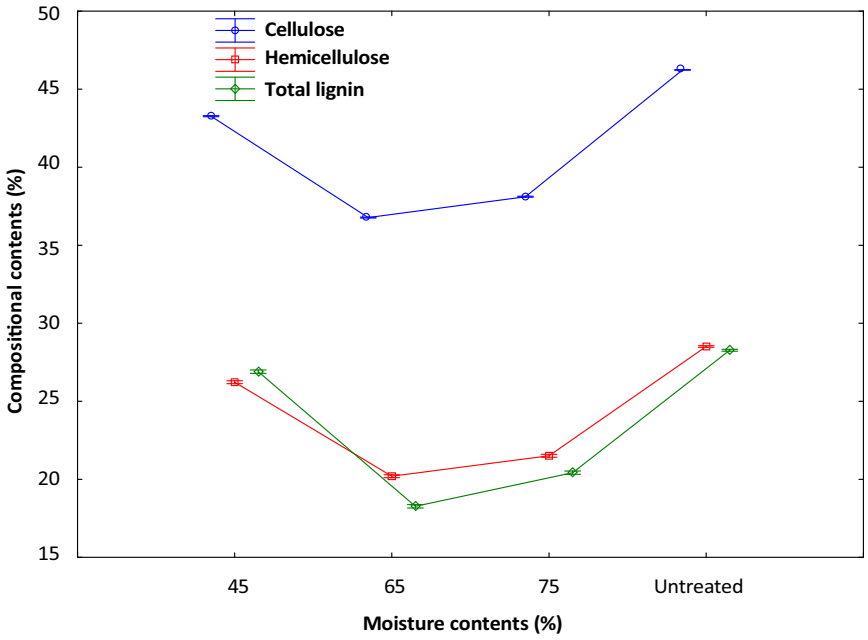

Fig. 1. Compositions of *A. elongatum* 'BAMAR' during 4-weeeks fungal pretreatment at various moisture contents.

Table 1
Changes in chemical components of Tall Wheat Grass after different pretreatment.

Experimental factors	SR[a] (%)	Compositional of treated sample					
MC (%)		Lignin removal[a] (%)	Reducing sugar[a] (%)	TP[a] (mg GAE g TS^{-1})	HCEL retention[a] (%)	CEL retention[a] (%)	Lig./CEL ratio[a]
Untreated	100	–	59.72 (0.42)	0.0325 (0.000)	–	–	0.61 (0.00)
45	88.85 (0.1)	4.86 (0.75)	59.42 (0.40)	0.0383 (0.003)	91.97 (0.23)	93.58 (0.03)	0.62 (0.01)
65	74.76 (0.21)	35.38 (0.21)	48.29 (0.07)	0.0553 (0.001)	70.84 (0.58)	79.48 (0.04)	0.50 (0.07)
75	83.35 (0.07)	27.75 (0.06)	59.00 (0.30)	0.0483 (0.000)	75.44 (0.13)	82.42 (0.05)	0.54 (0.00)

MC – moisture content; SR – Solid Recovery; CEL – cellulose, HCEL – hemicellulose; Lig./CEL ratio – lignin/cellulose ratio; TP – total phenol; mg GAE gTS^{-1} – milligram gallic acid per gram total solids.

[a] Data are the means of three measurements and number in parentheses are standard deviations.

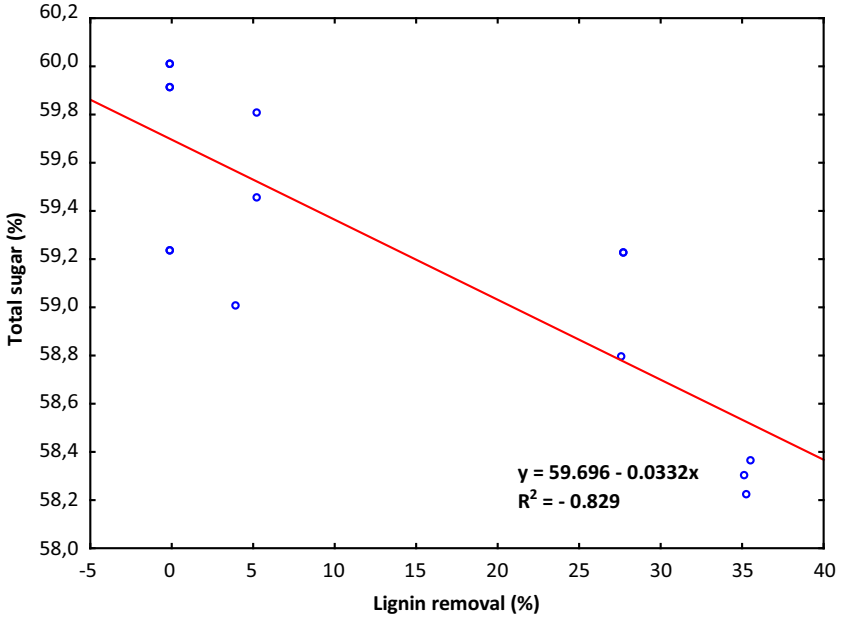

Fig. 2. Effect of lignin removal on enzymatic hydrolysis of Tall Wheat Grass pretreated with *F. velutipes*.

Table 2
Biogas and methane yield in anaerobic digestion process of *Agropyron elongatum* 'BAMAR' after various pretreatment.

Moisture contents %	Biogas yield[a]			Methane yield[a]		
	$Ndm^3\ kg^{-1}$ f.m.$^{-1}$	$Ndm^3\ kg^{-1}\ TS^{-1}$	$Ndm^3\ kg^{-1}\ VS^{-1}$	$Ndm^3\ CH_4\ kg^{-1}$ f.m.$^{-1}$	$Ndm^3\ CH_4\ kg^{1}\ TS^{-1}$	$Ndm^3\ CH_4\ kg^{-1}\ VS^{-1}$
Untreated	277.23 (4.02)	305.93 (4.43)	330.16 (4.78)	105.59 (1.46)	116.52 (1.61)	125.75 (1.74)
45	288.89 (3.09)	318.79 (3.41)	333.16 (15.46)	116.85 (0.06)	128.95 (0.07)	134.80 (7.5)
65	334.26 (2.74)	368.86 (3.02)	398.07 (3.26)	142.11 (1.58)	156.82 (1.74)	169.24 (1.88)
75	305.56 (2.42)	337.18 (2.67)	363.89 (2.88)	124.30 (2.17)	137.16 (2.40)	148.03 (2.59)

f.m. – fresh matter; TS – total solids, VS – volatile solids.
[a] Data are the means of three measurements and number in parentheses are standard deviation.

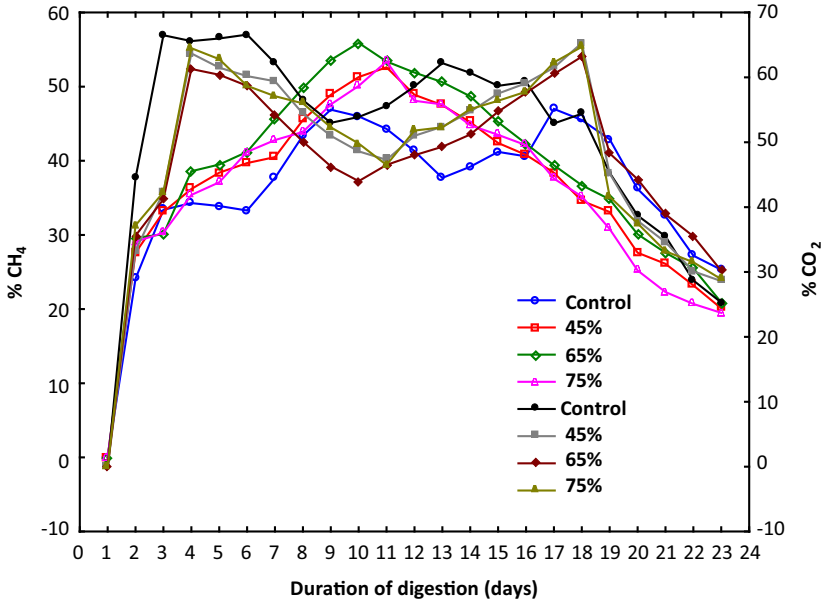

Fig. 3. Methane and carbon dioxide contents in percentage during anaerobic digestion of *A. elongatum* 'BAMAR'.

teria, yeast and methanogens/archa. (Hendriks and Zeeman, 2009). In this study was found slightly less total phenolics amounts as compared to the untreated substrates, but the difference was not significant ($p > 0.05$). This reduction in phenolics may be attributed to the fungal degradation of phenolic compounds.

3.3. Relationship between lignin removal and reducing sugar yield

Lignin content has an impact on the enzymatic hydrolysis. The degradation of lignin after fungal pretreatment can increase pore size in the biomass substrate and provide a more available surface area to cellulose (Nazarpour et al., 2013). Fig. 2 shows the relationship between lignin removal and reducing sugar yield during fungal pretreatment. There is a linear correlation between lignin removal and reducing sugar yield for Tall Wheat Grass by *F. velutipes* ($R^2 = 0.891$). Nazarpour et al. (2013) who conducted fungal pretreatment of rubberwood with *Ceriporiopsis subvermispora*, achieve the higher regression ($R^2 = 0.969$), which might be attributed to simultaneous holocellulose and lignin degradation during fungal pretreatment. Wan and Li (2010) founded that enzymatic hydrolysis yield (i.e., reducing sugar yield) was highly related to the lignin removal during microbial pretreatment. The authors suggested that the cellulose digestibility may be potentially enhanced by preferential degradation of lignin.

3.4. Biogas production

Fungal pretreatment at different moisture contents could significantly increase biogas and methane yield. The biogas production potential is presented in terms of biogas yield, methane content and methane yield (Table 2). The biogas and methane yield of the untreated and fungal treated Tall Wheat Grass yielded more biogas and methane then the untreated sample. The highest productivity of biogas per kilogram of VS was obtained in the case of sample pretreated at 65% MC – 398.07 $Ndm^3\ kg^{-1}\ VS^{-1}$, which was 120% higher than the untreated sample. Methane yields per kilogram of VS of the substrates studied were in the range of 134.8–169.24 $Ndm^3\ CH_4\ kg^{-1}\ VS^{-1}$. These results indicates that the fungal pretreatment at different moisture contents is capable of enhancing biogas and methane yield. This effect is because that the water molecule plays an important role in the fungal treatment at different moisture contents; i.e., bound water also can form hydrogen bonds with cellulose, which causes swelling of crystalline cellulose structure and increases the accessibility to enzymes (Chen, 2014; Zhao et al., 2014).

As shown Fig. 3 the methane contents in the biogas produced from untreated and fungal pretreated Tall Wheat Grass decreased systematically after 10 days of digestion process, reaching approximately 55%. Methane contents in the biogas production during fungal pretreatment under all the conditions was significantly ($p < 0.05$) different from that of the untreated biomass. The enhancement of methane contents in the biogas production by the effective fungal pretreatments could be explained by the breakdown of cell wall structure. The lignin degradation increased the surface area of exposed cellulose to increase its susceptibility to microbes and their enzymes (Komilis and Ham, 2003).

The data for the anaerobic digestion may be compared with earlier works. Amirta et al. (2006) reported that pretreatment of Japanese cedar wood in the presence of wheat bran by *C. subvermispora*, increased the methane yield. Similar results were reported by Zhao

et al. (2014) who conducted 4–8 weeks fungal pretreatment of yard trimming by *C. subvermispora* and obtained the following amounts of methane – 44.6 L kg^{-1} TS^{-1}, 40.3 L kg^{-1} TS^{-1} and 32.6 L kg^{-1} TS^{-1}, respectively, for yard trimming pretreated at 60% MC, 45% MC, and 75 MC%. Tuyen et al. (2013) achieved the highest results of biogas yield for oil palm fronds incubated with white-rot fungi: *C. subvermispora*, *Lentinula edodes*, and *Pleurotus eryngii* by 68–132%. Muthangya et al. (2009) found that the methane production of sisal leaf decortication residues (SLDR) could be improved by pretreatment by *Trichoderma reesei* at 25% wet weight inoculum per feedstock. On the other hand, unpromising results have also been described. For instance, Feng et al. (2013) who conducted fungal pretreatment by white-rot fungi: *Pleurotus ostreatus* and *P. eryngii* of straw and reported that the methane yield of the fungal pretreated straw was not significantly higher compere with the untreated straw. The authors suggested that some carbon such as those in reducing sugar in the straw was lost as a result of the fungal pretreatment under aerobic conditions. These results indicates that increasing of methane yield by fungal pretreatment depends on many factors such as the chemical compositions of biomass substrates, moisture contents of fungal cultivation, types of the fungal strains, and nutrition supplementation (Nuchdang et al., 2015).

3.5. The relationship between lignin removal and biogas yield

The correlation between lignin removal and biogas yield suggest that the biogas yield increased linearly with the lignin removal by fungi pretreatment ($y = 265.94 + 1.32\times$). As lignin removal increased from 0% for untreated biomass to 35%, the biogas yield was increased by 121% in the case of sample pretreated at 65% MC. The results may be compared with earlier reports that suggested that the higher lignin removal (i.e., lower the lignin content), the higher the biogas, methane, ethanol, and enzymatic hydrolysis sugar yield (Wan and Li, 2010; Zhao et al., 2014). The results could be possibly explained by the fact that the barrier of lignin was reduced and the availability of feedstock for anaerobically microbes was increased. A positive linear correlation between lignin degradation by *C. subvermispora* and methane yield was reported by Zhao et al. (2014). Whereas, Take et al. (2006) observed a negative correlation between lignin content and methane yield.

4. Conclusion

Moisture contents had a significant impact on fungal pretreatment of *A. elongatum* 'BAMAR' by *F. velutipes* and biogas yield. The pretreated biomass at 65% yielded 120% more biogas production and 134% more methane yield, compared with the control. The enhanced biogas yield was attributed to the improved biodegradability of biomass, as indicated by increase lignin and hemicellulose reduction. *F. velutipes* treatment caused significant biodegradation of hemicellulose (29.1%) and lignin (35.41%), but had slightly effect on cellulose removal (20.48%) at 65% MC. Biological pretreatment could be an efficient methods for improving the highly efficient bioconversion of *A. elongatum* into biogas.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

J.L. and A.K. performed the experiment. J.L. wrote the manuscript. A.K., D.M. and J.T. participated in the preparation of the manuscript. J.T. supervised the experiment and preparation of the manuscript. All authors read and approved the final manuscript.

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