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Boosting Methane Production by Fungal Pretreatment of Poplar in Anaerobic Digestion

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Today's increasing demand for sustainable technologies and circular production systems promotes the development of R&D and innovations for green, renewable, and economically viable solutions. Biomass utilization has a great opportunity in this respect, because of its flexible applicability. This study aimed to investigate the effects of fungal pretreatment on lignocellulose substrate for semi-continuous anaerobic digestion. The applied substrate was an I-214 poplar clone, and biological pretreatment was carried out with *Pleurotus* strain HK35. An intensive organic loading rate was applied in order to compare the digestion parameters between the treated and control fermenters. Results showed significant benefits in the case of daily methane yield and specific methane yield, as well (205 $mLd^{-1}L^{-1}$, 44 $mLgVS^{-1}L^{-1}$ in case of fungal pretreated and 98 $mLd^{-1}L^{-1}$, 26 $mLgVS^{-1}L^{-1}$ in case of control fermenters). Titrated volatile fatty acid content indicated stable run, cellulose and reduced lignin content demonstrated the efficiency of degradation, supporting the accelerated methane production.

1. Introduction

Considering the world's energy supply and energy security problems, solutions that have received less emphasis in the past are becoming increasingly prominent (Rouches et al., 2016). One such solution could be the expansion of biomass utilization by incorporating previously unused or research-phase substrates on an industrial scale (Brémond et al., 2018). This could be relevant for countries that rely on natural gas imports, aiming to have their sources available in the energy mix for greater security of supply (Surenda et al., 2014).
The goal of anaerobic digestion is the secondary utilization of waste materials (Alexandropoulou et al., 2017), through which we can obtain electrical energy, thermal energy, methane as an industrial raw material, and the depleted digestion residue can also be used as a nutrient supplement in agricultural and forestry fields (Hultberg et al., 2023).
Besides the most used substrates, the application of lignocellulosic systems in degradation is a challenging area (Kainthola et al., 2021). Lignocellulosic systems are typically composed of three main polymers: cellulose, hemicellulose, and lignin (Higuchi, 1997). They also contain smaller amounts of proteins, water, pectins, ash constituents, and minor components (Tomme et al., 1995). The interaction of these main polymers forms a heterogeneous matrix, mainly influenced by the specific plant species (Kratkỳ et al., 2012).
Lignocellulose is among the more recalcitrant substrates; hence, pretreatment is recommended to achieve higher efficiency in methane production (Xu et al., 2017). The main purpose of pretreatment is to facilitate enzyme access to easily digestible sugars (Amin et al., 2017).
The literature contains detailed studies on various pretreatment methods, including thermal, mechanical, chemical, and biological treatments (Brémond et al., 2018), which assist in hydrolysis, thus enabling the anaerobic consortium to access more readily fermentable saccharides (Sánchez, 2009). From different pretreatment ways, biological pretreatments seem particularly advantageous because they require slight operating conditions, involve relatively low operational investments, and are often eco-friendly (Liu et al., 2017). Biological pretreatment by selective white-rot fungi is one of the most promising processes (Cardona et al., 2010). White-rot fungi are known as ligninolytic enzyme producers (such as lignin peroxidase, laccase, and

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manganese-peroxidase) they are convenient for breaking down the network of lignin (Tanaka et al., 2009). In this paper, considering environmental and economic aspects (Sindhu et al., 2016), we aimed to investigate the effects of biological pretreatment with fungi on the anaerobic digestion of poplar wood substrate. The applied white rot fungus was *Pleurotus* strain HK35. Fungi from the genus *Pleurotus* are cultivated in several countries because of their high adaptability (Synytsya et al., 2009). This genus has more cultivated varieties than any other mushrooms, because of their flexible requirements of temperature and environment (Ragunathan et al., 1996).

This strain has been chosen for the pretreatment process because *Pleurotus* spp. are white rot fungi, making them suitable for initiating the breakdown of lignin, cellulose, and hemicellulose. The use of substrate is out of focus in anaerobic digestion research; most of the papers are interested in agricultural by-products, but the residual biomass from forestry fields can be a remarkable source of biomass utilization for forested areas. This way is more favorable in the aspects of carbon circulation. The conventional use of firewood emits carbon into the atmosphere. In contrast, during anaerobic digestion, a significant part of carbon and nutrition, such as nitrogen, remains in the fermented sludge. Furthermore, due to the application of sludge in land use, a considerable amount of fertilizer can be substituted, thereby promoting soil management and fertilization, which contributes to the goals of sustainability.

2. Material and methods

2.1 Substrate and pretreatment

During the investigation lignocellulose biomass was applied, originating from Nursery Bajti (Hungarian Forest Research Institute) The wood (with bark) of the poplar clone I-214 was totally grounded, then dried at room temperature. After that biomass was treated by *Pleurotus* strain HK35, which is a hybrid of *Pleurotus ostreatus* and *Pleurotus ostreatus (var. Florida)* (Fungal collection of the Institute of Natural Resources and Forest Management, University of Sopron, Hungary).

During the fungal pretreatment, 200 g of air-dry wood material was moistened to a level of approx. 70 % (Shi et al., 2008), then autoclaved at 121 °C and 1.6 atm pressure for 30 min. Following sterilization, the cooled wood samples were inoculated with the HK35 fungus. The colonization process occurred at room temperature (Figure 1). The wood sample was utilized after a colonization period of 21 days (Shirkavand et al., 2017). Air-dry wood without any pretreatment was applied as a control.

Figure 1: Fungal pretreated substrate

2.2 Anaerobic digestion

The laboratory-scale semi-continuous experiment was carried out following the German standard VDI 4630. The anaerobic sludge of 1000 mL in a 2500 mL volume bottle (Merck & Co., Germany) was prepared and fed by fungal pretreated lignocellulose biomass in triplicate. The control fermenters were fed by the untreated substrate in duplicate. The digesters were incubated in a water bath (MemmertWNB 14 Basic, Memmert GmbH & Co.) at a constant mesophilic temperature (38 °C). The anaerobic digester inoculum was obtained from a maize silage fed CSTR full-scale mesophilic (38 °C) biogas plant (Vrbová nad Váhom, Slovak Republic) The reactors were manually mixed three times per day. The biogas was collected in Tedlar® gas sampling bags, and the volume was measured daily with a Hamilton Gas Tight Syringe (Sigma-Aldrich). The biogas and

methane yields were recalculated according to the standard conditions for pressure and temperature. The components of the produced biogas were analyzed using an Ecoprobe 5-IR (RS Dynamics Ltd., Czech Republic).
A previous study (Qiang et al., 2013) defined that optimal methane production from anaerobic digestion can be accomplished by the addition of different trace elements based on the microbial consortia. Therefore, a trace element supplement solution was applied, which contained 1,625 mg of zinc, 13,640 mg of manganese, 93 mg of boron, 20,000 mg of nickel, 600 mg of copper, 50,000 mg of cobalt, 228 mg of molybdenum and 113 mg of selenium, in a special organic complex form, per kg of solution (42.2 % TS). The dosing of microelements was based on our former experiments (Rétfalvi et al., 2016).

2.3 Analytical measurements

Titrated volatile fatty acid

Samples of sludges (10 mL) were taken for chemical analysis before daily feeding. Samples were centrifuged for 10 min at 3420×g (EBA 21, A. Hettich, Germany). From the resulting supernatant, 5 mL was used for the determination of titrated volatile fatty acids (tVFA) levels, using a potentiometric pH meter (EuTech PC 510, ThermoFisher Scientific (Rétfalvi et al., 2013). Samples were prepared by adding 45 mL of distilled water into the supernatant sample of 5 mL. The pH value of this solution was decreased by adding 0.1 M HCl with continuous mixing until a pH of 2.0 was reached, followed by 15 min of stirring to eliminate CO_2. The pH value was then raised above 5.0 with 0.1 M NaOH.
The tVFA was determined with the following equation:

$$tVFA\ (mg\ acetic\ acid\ L^{-1}) = \frac{V_{NaOH\ pH\ 5.0} - V_{NaOH\ pH\ 4.0} \cdot f_{NaOH} \cdot 200}{V_{sample}} \cdot 60 \quad (1)$$

where $V_{NaOH\ pH\ 5.0}$ is the volume of the NaOH until pH 5.0 (mL, at 1 atm, 25 °C); $V_{NaOH\ pH\ 4.0}$ is the volume of the NaOH until pH 4.0 (mL, at 1 atm, 25 °C); f_{NaOH} is the ratio of the actual concentration and the nominal (0.1 M) concentration of the NaOH solution; 200 is an empirical coefficient; 60 is the molar weight of the acetic acid (mg mol^{-1}) and V_{sample} is the volume of the sample (mL, at 1 atm, 25 °C).

Total solid and volatile solid

The total solid (TS) contents of substrates were determined by weight loss by drying the samples at 105 °C. VS% (volatile solid) was measured by weight loss ignition of the dried samples at 600 °C.

Cellulose

For the determination of cellulose content of substrates, the Kürschner-Hoffer method was employed. The essence of this procedure involves treating the examined wood material with a mixture of nitric acid and ethanol. The lignin is nitrated and partially oxidized while simultaneously undergoing hydrolysis alongside the hemicellulose, which dissolves. For the cellulose determination, 1 g of samples (dried at 105 °C to a constant mass, drizzled into 0.2-0.63 mm size) were placed in a 100 ml volumetric flask, and an ethanol-nitric acid (4:1 ratio) mixture for dissolution was added. After 1 hour, boiling samples were filtered through a G2 glass filter. The cellulose contents were calculated from the loss, referring to the total solid content.

Lignin

We determined the lignin content according to the T 222 om-98 (Klasson) standard. During this process, the wood material was ground, then sieved, and the particle size between 0.63-0.2 mm. 1g samples (dried at 105 °C to a constant mass) was placed in a 100 mL boiling flask, then 15 ml of 75 % sulfuric acid was added. After 2 hours of standing, 560 mL of distilled water was added, and samples were boiled for four hours in a 1000 mL round-bottom flask. Sedimentation lasted for a night, and then a G4 glass filter was applied.

3. Results and discussion

After 120 days of substrate-adaptation period, an intensive organic loading rate (OLR) was applied. Substrate addition lasted 11 days (Fig. 2.). The OLR was raised to 5.4 $gVSL^{-1}d^{-1}$ for four days, followed by a stable run. On the 8th day, feeding was increased to 6.6 $gVSL^{-1}d^{-1}$ for three days. After that, we reached 7.5 $gVSL^{-1}d^{-1,}$ the maximum OLR of the dosage. From the 12th day, the experiment was closed with an elimination period of 20 days. Methane production followed the substrate addition in pretreated and control trials, as well. A clear influence of fungal pretreatment can be found in Fig. 2. The daily methane yield was significantly higher in the case of treated fermenters during the total digestion. We evaluated the specific methane yield, as well, and results showed 44 and 26 $mLgVS^{-1}L^{-1}$ values in the case of pretreated and control trials, respectively, which strengthens the benefits of treatment.

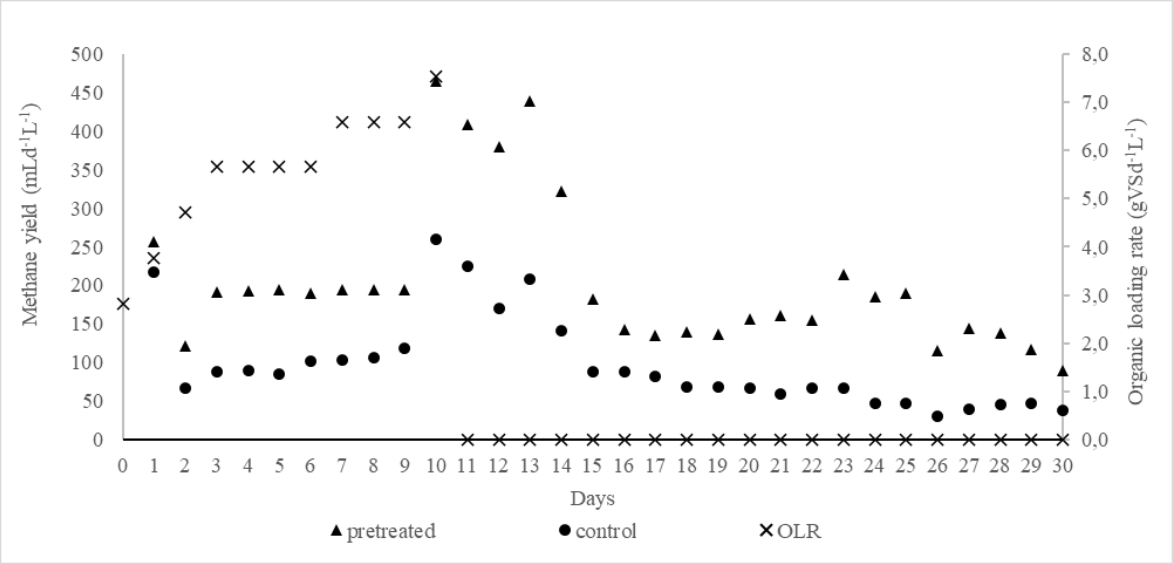

Figure 2: Methane yields of fungal pretreated and control trials

As a result of fungal pretreatment, the cellulose content in the control sample showed a 7.91 % higher value *(Table 1.)*, which can be primarily attributed to the decomposition of more easily degradable components and lignin by the fungus. The higher cellulose content could be correlated with higher methane yield, primarily supporting the operation of a well-adapted consortium that is efficient in cellulose degradation.
Results showed decreased lignin content (Table 1.) in contrast to the cellulose content, demonstrating lignin degradation. This effect is favorable for biogas production since lignin inhibits cellulose biodegradation (Sawatdeenarunat et al., 2015), both by spatially impeding the enzymatic breakdown of the lignocellulosic matrix and through the enzyme-inhibiting properties of lignin components (Shi et al., 2008). The breakdown of phenyl-propanoid compounds that constitute lignin is partially carried out by methanogenic organisms, yet they have been proven to hinder fermentation processes.
Similar findings were made by Mustafa et al.. They also utilized a *Pleurotus* fungal species for pretreatment on rice straw substrate. Their results indicated that pretreatment caused significant degradation of lignin and hemicellulose but had a limited effect on cellulose.
Ubierna et al. (2023) found that pretreatment of the rice straw with an alkaline sodium hydroxide solution resulted in more efficient co-digestion of lignocellulose substrate and sheep manure.

Table 1: Cellulose, lignin, total solid and volatile solid content of substrates

Sample	cellulose content (%)	lignin content (%)	TS% of substrate	VS% of substrate
fungal pretreated	49.37	25.96	90.75	97.77
control	41.46	29.89	90.20	98.02

Table 2: Main operational parameters of anaerobic digestions

Sample	average methane production (mL)	tVFA (mgL^{-1})	methane content (%)
fungal pretreated	205±98.57	1473	49.92
control	98±60.53	1020	47.24

Based on the tVFA content, digestions showed stable operation (Table 2.) Regarding the total experiment, the average methane production was 205 mL in the case of fungal pretreated fermenters and 98 mL in the case of

control trials. which is a major difference and shows the obvious positive effect of pretreatment. In addition, the methane content from the produced biogas of pre-degraded samples exceeds the non-treated control values, 49.92 % and 47.24 %.

4. Conclusions

Fungal pretreatment by *Pleurotus* HK35 was found to be proficient in improving the bio-digestibility of lignocellulosic biomass of I-214 poplar clone due to its cellulose degradation capabilities. Results showed a twofold increase in methane production, with a 2 % higher methane content of the pretreated trial compared to the control, respectively. Titrated volatile fatty acid content indicated a stable run in both cases. By optimizing the operational parameters and selection of appropriate fungal species for various feedstocks, this method has the potential to evolve into an environmentally friendly and cost-effective approach to enhance methane production in the anaerobic digestion process. Further, the selection and mixture of the appropriate fungal species would be also an interesting topic for further research. Further experiments need to be carried out for the full-scale application.

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