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Original Research Article

BIO-DELIGNIFICATION AND PRE-TREATMENT OF OIL PALM FROND (OPF) BY TRAMETES POLYZONA FOR ENHANCED BIOCHEMICAL METHANE POTENTIAL (BMP)

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A b s t r a c t

This study aims to optimize pre-treatment conditions for oil palm fronds (OPF) using white rot fungi (WRF) species, *Trametes Polyzona* for enhancement of biogas production via biochemical methane potential (BMP) test. BMP tests were run under three conditions: OPF alone (control), OPF treated with *Trametes Polyzona*, and OPF treated with *Trametes Polyzona* and supplement of water. Visual structures, FTIR, Klason method and GCMS were used to investigate the effect of pre-treatments conditions on bio-delignification process of OPF. Visual structures results showed preferential degradation on OPF by *Trametes Polyzona* under both dry and moisture conditions compared to that of control conditions. After 30-days of incubation period, 22.5% lignin content in OPF coupled with *Trametes polyzona* pre-treatment (25-35% reduction), underscores biogas generation potential. Notably, the combined *Trametes polyzona* and water pre-treatment achieved a remarkable 35% lignin reduction. Both these conditions showed successful lignin degradation, highlighted by FTIR's carbonyl group reduction.

Keywords: Bio-Delignification, biochemical methane potential (BMP), oil palm fronds (OPF), white rot fungi (WRF)

1. INTRODUCTION

Palm oil trees (*Elaeis guineensis*) are a crucial agricultural asset driving Malaysia's economic growth. Initially hailing from West Africa, Malaysia has strategically leveraged the oil palm sector to establish itself as a global industry leader (Awalludin et al., 2015). In 2020, Malaysia's production of crude palm

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oil (CPO) reached an impressive 19.6 million tons, surpassing the 2019 yield of 18.3 million tons, with Sabah, Sarawak, Johor, Pahang, and Perak being key production hubs. Malaysia's capacity to generate biomass waste is significant, with estimates suggesting that a single hectare of oil palm plantation can yield between 50 to 70 metric tons of waste annually annually (MPOB, 2022). Oil palm cultivation yields a spectrum of by-products, including palm kernel cake (PKC), empty fruit bunches (EFB), oil palm fronds (OPF), and palm oil mill effluent (POME). These by-products, alongside palm press fiber (PPF) and oil palm trunk (OPT), collectively constitute the broader category of oil palm biomass, with OPF contributing significantly to the overall biomass volume.

Massive quantities of OPF are produced through replanting, harvesting, and pruning, with availability year-round. The leaves and petioles from oil palm trees, is referred as OPF. About 24 palm fronds are typically extracted annually from each tree, their weight contingent on the palm's age. Commonly, OPF are left to degrade naturally, burned, or rotted, resulting in greenhouse gas emissions and soil degradation. Different methods, such as gasification, pyrolysis, and microbial fermentation, can transform OPF into biofuels like biogas and bio-oil, offering a sustainable energy solution. OPF can also be utilized as feed for ruminants, contributing to livestock nutrition and supporting farming practices (Pulingam et al., 2022).

The percentage of lignin, cellulose, and hemicellulose in OPF varies according to the kind of oil palm. Lignin is the primary component of oil palm biomass. The structure of the amorphous heteropolymer known as lignin is quite complicated. Because it is stiff, insoluble in water, impermeable, and resistant to microbial attack and oxidative stress, it acts as a barrier for the cell wall (Chieng et al., 2017). The high lignin content of OPF makes it difficult to degrade using traditional methods of anaerobic digestion. The recalcitrant nature of lignin, poses significant barriers to microbial access and enzymatic breakdown. Cellulose, often found in a crystalline form, is resistant to enzymatic attack, and lignin acts as a physical barrier, further impeding the process (Agregán et al., 2022). This complexity necessitates pre-treatment methods to enhance the biodegradability of lignocellulosic biomass. According to Zhang et al. (2023), traditional lignin-biodegradation microorganisms like fungi encounter several issues such as slow reaction processes and stringent requirement for strain culture environment and conditions. The overall degradation efficiency tends to be lower, which limits the effectiveness of lignin degradation.

Thus, this study aims to optimize conditions for white rot fungi (WRF) growth and pre-treatment of OPF. Effective lignin breakdown is crucial, altering lignocellulosic biomass to enhance holocellulose accessibility for bioconversion. Pre-treatment or fractionation is essential for this purpose, with biological approaches like WRF seen as environmentally friendly and energy-efficient alternatives to traditional chemical and physical methods (Rajendran et al., 2018). While the concept of WRF pre-treatment for lignocellulosic biomass is established, this study pioneers the application of WRF pre-treatment to hard wood, specifically the stem of OPF. To solely capture the exact potential of OPF degradation solely by WRF, inoculum from anaerobic bacteria culture normally retrieved from an active digester were not used in the biochemical methane potential (BMP) experiments.

2. METHODOLOGY

2.1. Preparation for WRF culture

Based on our recent study on the degradation of WRF on solid and liquid media supplemented with synthetic dyes namely 2,2’-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), azure B, and phenol red (Nurul-Aliyaa et al. 2023), this study chooses to use *Trametes polyzona* species. The preparation of half strength acid potato dextrose agar (HPDA) as medium for *Trametes polyzona* growth

was carried out with high precision to avoid contamination. The prepared HPDA was kept in 1.5 mL append Dorf tubes. After 3 days, *Trametes polyzona* was transferred to the surface of HPDA and incubated at 28°C for 2 weeks to establish a pure culture. Adherence to sterilization and safety protocols was strictly observed throughout the process.

2.2. Inoculation of OPF and biochemical methane potential (BMP) test

The inoculation process involved cutting fresh OPF into small pieces, and the Vibrating Sieve Shaker Machine was employed to achieve lengths ranging from 2 cm to 3 cm. To ensure contamination prevention, the OPF underwent sterilization via autoclaving for 20-30 minutes at 120°C. The prepared OPF was used as media in BMP test. In the BMP test, three Schotts bottles with volume of 1 L were used. In the first Schotts bottle that acted as control, only 700 g of OPF was put. For second Schotts bottle, *Trametes polyzona* was added to 700 g OPF by using sterilized inoculation loop. Gentle pressure was applied to promote contact between *Trametes polyzona* and OPF. In the third Schotts bottle, 250 mL of distilled water was added to the 700 g OPF + *Trametes polyzona*. All Schotts bottles were tightly sealed or covered with a sealer to prevent biogas from escaping and gas bags were used to collect the produced gas. All Schotts bottles are incubated in water bath at temperature of 28°C for 30 days.

2.3. Bio-delignification process characterization

2.3.1. Visual analysis

Digital camera is used to visualize the growth of *Trametes polyzona* on the surface of OPF during 30 days of incubation period. Changes in colour and texture were recorded each week. To preserve the conditions of sample in BMP test, for morphology analysis, a series of conditions as in Section 2.3 were replicated in petri dish that well sealed.

2.3.2. Modification of lignin absorption peaks via FTIR

The functional groups present in the lignocellulosic material can be analyzed using Fourier Transform Infrared (FTIR) spectroscopy. Based on the absorption and transmission of infrared light, FTIR spectroscopy is a strong analytical technique that offers information about the chemical composition and structure of a material. By comparing the FTIR spectra of untreated OPF with those of delignified OPF, researchers can observe shifts or modifications in the lignin absorption peaks. These spectral changes indicate the extent of lignin removal and the potential modifications in the lignin structure, which can be used to optimize the delignification process and evaluate the efficacy of WRF in degrading OPF under conditions that are wet, fresh, and variable. The collected samples were dried in an oven set at 100°C for 24 hours to facilitate their preparation for analysis.

2.3.3. Percentage of lignin using Klason method

Determination of lignin in OPF followed Klason method which has been described by Abdelrahman and Galiwango (2018). The process begins by collecting random OPF in Schotts bottle, which are then air-dried until their weight stabilizes. These dried samples are finely powdered using tools like a ball mill. The resulting powdered oil palm fronds are placed on a scale within sealed containers, and precisely 0.2 grams of the powder are weighed for each container. The weighed samples in the containers are thoroughly mixed with 25 mL of concentrated sulfuric acid using a Wheaton reagent bottle, with constant stirring for a duration of 20 minutes. This mixture is allowed to react for three hours. After this reaction period, the resulting mixture is transferred to an autoclave and exposed to a temperature of 120°C for one hour, with proper sealing of the containers before the autoclaving process. Following the

autoclave treatment, the mixtures are allowed to cool. The cooled mixtures are then filtered through glass microfiber filter paper with a 70 mm diameter. Hot water is used to wash away debris, and the solution is carefully strained to remove any remaining particles. Prior to this filtration step, the empty crucibles and filters are pre-weighed. The filters containing the particles are subsequently placed in an oven set at 75°C for 24 hours. In the final step, the weight of the crucibles, filters, and particles is recorded using an electron scale. This comprehensive procedure allows for a thorough assessment of the extent of lignin degradation, providing valuable insights into the efficacy of the treatment method. By employing equation (2.1), the percentage of lignin degradation can be calculated. This methodological approach ensures a systematic and accurate evaluation of lignin breakdown, contributing to a deeper understanding of the treatment's effectiveness (Abdelrahman & Galiwango, 2018).

$$\text{Percentage of lignin} = \frac{Crucible\ with\ filter\ and\ particles - crucible\ with\ filter}{weight\ of\ OPF\ powder} \times 100 \quad (2.1)$$

2.3.4 Volatile solid

One way to analyze a sample for the presence of organic material is by the volatile solids procedure, commonly called the loss on ignition method. OPF is carefully weighed to determine the concentration of volatile substances. The next step is to bake the sample at a certain temperature, usually around 105°c to remove any moisture content. The organic matter is isolated during the drying process and then analyzed separately. After being air-dried, the sample is placed in a crucible or combustion boat and heated. The next step involves heating the crucible to a temperature between 550 and 600 °C in a muffle furnace (Letti et al., 2021). The sample is heated for a set amount of time, usually between 2 and 4 hours. This time frame is necessary to ensure OPF organic substance is fully oxidized. The crucible and its contents are placed in a desiccator to cool after the heating procedure is complete. This is a vital step in ensuring that following weighing are accurate and not impacted by moisture absorption. Once the crucible containing the ash has cooled, it may be precisely weighed. Repeat these steps using the opf treated by wrf. Calculate the volatile solid using equation (2.2) (Das et al., 2023):

$$\text{Volatile Solid} = \frac{weight\ of\ dried\ \&\ dish - weight\ of\ residue\ and\ dish\ after\ ignition}{weight\ of\ wet\ sample\ \&\ dish - weight\ of\ dish} \times 100 \quad (2.2)$$

This metric is useful for determining OPF potential energy content or suitability for a number of applications since it reveals the relative amount of organic material present in the fronds. Temperature, time, and sample size are only a few of the variables that can change depending on the criteria and needs of the analysis technique. Therefore, in order to acquire accurate and dependable findings, it is crucial to adhere to the right rules and practices. Calculate the volatile solid using the same formula above.

3. RESULT

3.1. Effect of *Trametes polyzona* on bio-delignification of OPF

The camera imaging technique provided an easily accessible and high-quality method for observing and documenting the physical transformations of OPF caused by *Trametes polyzona* treatment. The images served as visual evidence of the treatment's efficacy in transforming the physical appearance, color, and surface properties of the OPF samples. Figure 1 showed the comparison in OPF bio-delignification process in dry condition without (control) and with *Trametes polyzona*. The treated OPF with *Trametes polyzona* changed color with certain areas displayed a lighter or paler appearance, and some turned white

or greyish, indicating the decomposition of both lignin and other pigments. Control OPF preserved their green color or showed moderate symptoms of natural ageing and browning during the same time range, but less than treated samples. The discoloration marked the lignin degradation process, distinguishing OPF treated with white rot fungi from untreated OPF. The white rot fungi treatment breaks down lignin, changing the OPF samples color. It is noted from this study that OPF's physical decomposition is a slow process and thus requiring extended periods before it breaks down into easily degradable forms. This result is in conjunction with a study by Pulunggono et al. (2019) on decomposition of OPF at different burial depth that showed the average rate of dry matter loss weight for the first month is around 11% and 88.86% after 2 years.

The FTIR analysis revealed significant alterations in functional groups and molecular bonds, indicating substantial structural changes in the OPF after treatment with *Trametes polyzona* in Figure 2. For raw OPF, FTIR spectra showed a prominent peak at approximately 1737.4 cm^{-1}, indicating the presence of carbonyl (C=O) groups in lignin and hemicellulose. Peaks in the range of 1635 cm^{-1} to 1514 cm^{-1} corresponded to the aromatic skeletal vibrations of lignin, while peaks between 1252 cm^{-1} and 1050 cm^{-1} indicated stretching vibrations of C-O and C-C bonds in cellulose and hemicellulose.

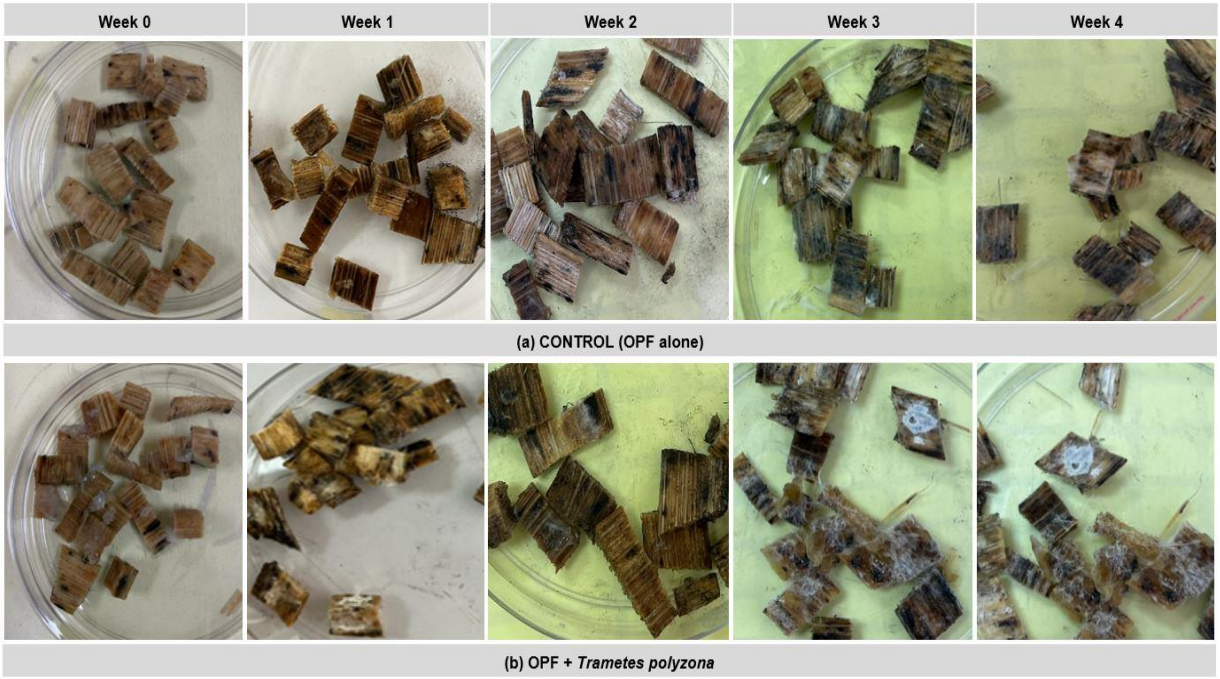

Fig. 1. Comparison of the treated OPF and untreated OPF by *Trametes polyzona* under dry conditions

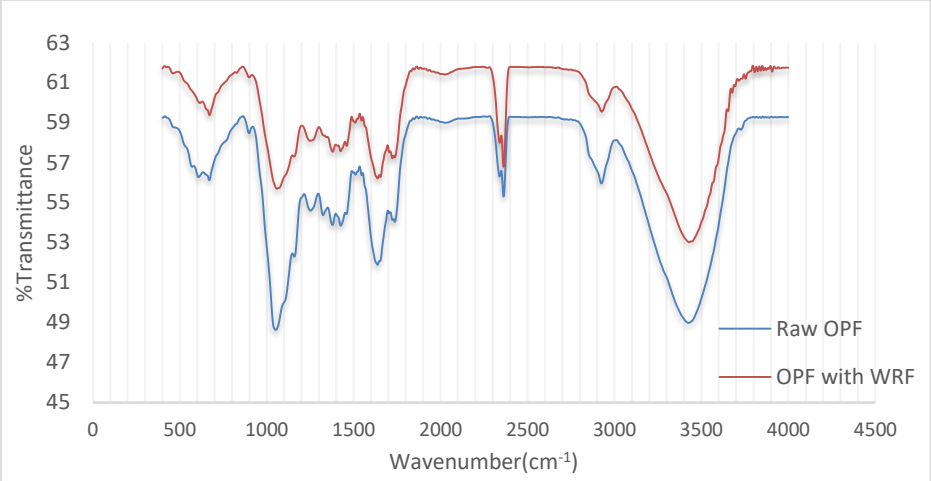

Fig. 2. FTIR spectra obtained from raw OPF and OPF pre-treated with *Trametes polyzona*

Post *Trametes polyzona* treatment, the FTIR spectra exhibited modifications. The peak intensity at 1726 cm^{-1}, associated with carbonyl groups, decreased, suggesting effective lignin degradation. Additionally, a reduction in peak intensity within the aromatic region (1638–1510 cm^{-1}) indicated a decrease in lignin's aromatic content, signifying its decomposition due to WRF treatment. This underscores the efficacy of white rot fungi in delignifying OPF. Comparatively, these findings align with a previous study by (Nordin et al. 2016), which also utilized FTIR spectroscopy to investigate OPF. Both studies (Table 1) found changes in the same parts of the FTIR spectra, especially in carbonyl groups, aromatic vibrations of lignin, and stretching vibrations of cellulose and hemicellulose. But the current study was the only one to look at how pre-treatment with white rot fungi affected these spectral features. This showed how lignin was broken down during the bio-delignification process.

Table 1. Summary of the FTIR spectra analysis for control conditions

Wavelength (cm^{-1})			**Functional groups**	**Assignment (Peak Shape)**
Raw OPF	**OPF with *Trametes polyzona***	**[5]**		
3427.4	3452.2	3415	Alcohol (O-H)	Broad, width
2925.1	2926.8	-	Alkane (C-H)	Broad, sharp
2362.9	2363	-	Alkane(C-H) (stretching)	Asym
2340.1	2040	-	Alkane(C-H) (stretching)	Very tiny peak
2026	2023.6	-	Alkyne (C=C)	Small
1737.4	1726.4	1737	Carbonyl (C=O)	Hemicelluloses/lignin
1637.8	1634.6	1645	H-O-H (absorbed water)	Medium
1513.8-1251.5	1509.61-1249.9	1509-1609	C-C (stretching)	Stretching vibration
1050	1069.6	1058-1060	Carbonyl (C-O)	Tiny shape
900.13-605.37	671.03-460.32	896-900	Asym.out of phase ring stretching	$\beta - Glycosid\ lingkage\ of\ cellulose$

3.2. Effect of moisture condition on bio-delignification of OPF

Treatment of OPF with *Trametes polyzona* under moisture (wet) conditions initiates visible decomposition signs, softening the OPF and rendering it more breakable (Figure 3). The OPF becomes damp and exhibits a mushy, sponge-like texture upon touch. Fiber fracture and disintegration indicate compromised structural integrity. Moisture induces discoloration, with certain areas turning dark brown to black due to fungal growth and lignin breakdown. These changes highlight the impact of wet conditions and *Trametes polyzona* on OPF's physical state. In contrast, under dry conditions (Figure 2), *Trametes polyzona* pre-treatment significantly reduces decay symptoms. The treated OPF maintains structural integrity, retaining firmness and flexibility. Minimal color change occurs, with the original greenish hue mostly intact. Age-related discoloration is possible, but substantial darkening is rare under dry conditions.

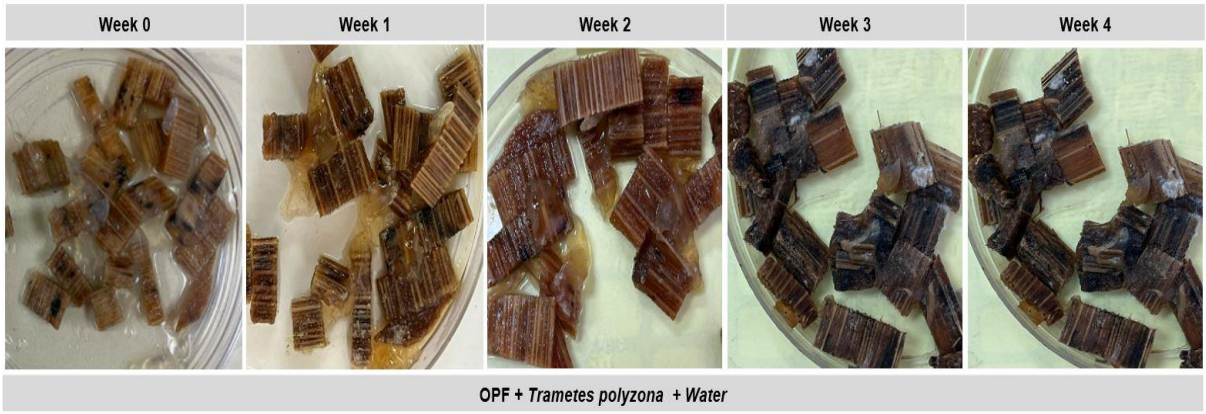

Fig. 3. OPF pre-treated by *Trametes polyzona* in moisture condition

The FTIR spectrum of OPF in moisture conditions in Figure 4 exhibits characteristic peaks: the peak at 1726 cm^{-1} corresponds to carbonyl (C=O) group stretching in lignin and hemicellulose, while the range 1459-10603 cm^{-1} represents aromatic skeletal vibrations of lignin, and 1633-1459 cm^{-1} signifies stretching vibrations of C-O and C-C bonds in cellulose and hemicellulose. Upon bio-delignification pre-treatment with *Trametes polyzona* notable changes occur in the FTIR spectrum of OPF. Compared to water-treated OPF, the peak intensity at 1726 cm^{-1}, associated with carbonyl groups, significantly diminishes. This reduction implies partial removal of carbonyl groups during the bio-delignification process, indicating effective lignin degradation by *Trametes polyzona*.

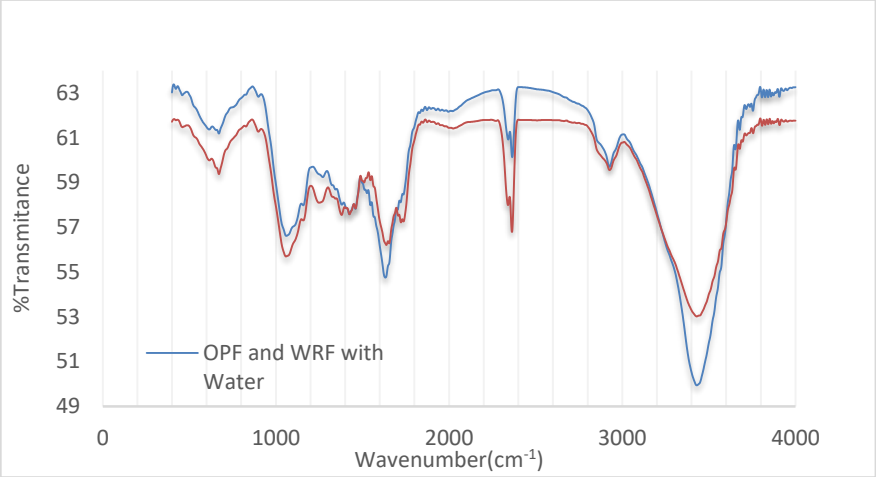

Fig. 4. Fourier transform FTIR spectra obtained for moisture condition (OPF and WRF with Water) against dry condition (OPF with WRF)

Furthermore, the intensity of peaks within the aromatic region (1637-15096 cm^{-1}) decreases in dry condition compared to water-treated OPF (Table 2). This reduction indicates degradation of lignin's aromatic structure, underscoring the efficacy of *Trametes polyzona* in delignification. The FTIR analysis also suggests the possibility of new peaks or shifts in OPF treated with *Trametes polyzona*, indicating alterations in other functional groups or molecular bonds. These changes may result from enzymatic activities of white rot fungi, impacting cellulose and hemicellulose, among other components. It's important to note that previous research hasn't precisely determined FTIR spectra of pre-treated materials or degraded functional groups in humid conditions.

Overall, the FTIR analysis underscores that bio-delignification pre-treatment with *Trametes polyzona* under dry conditions leads to significant chemical composition changes in OPF compared to moisture condition. The observed decrease in carbonyl groups, reduction in aromatic content, and potential modifications in other functional groups collectively highlight the transformation in lignin and cellulose/hemicellulose structures due to *Trametes polyzona* alone.

Table 2. Summary of the FTIR spectra analysis by *Trametes polyzona* under dry and moisture condition

Wavelength (cm^{-1})		**Functional groups**	**Assignment (Peak Shape)**
Dry condition	**Moisture Condition**		
3452.2	3432.2	Alcohol (O-H)	Broad, width
2926.8	2925.6	Alkane (C-H)	Broad, sharp
2363	23643	Alkane(C-H) (stretching)	Asym
2040	2321.4	Alkane(C-H) (stretching)	Very tiny peak
2023.6	2006.7	Alkyne (C=C)	Small
1726.4	2006.7	Carbonyl (C=O)	Hemicelluloses/lignin
1634.6	1633	H-O-H (absorbed water)	Medium

1509.6-1249.9	1409.3-1249.9	C-C (stretching)	Stretching vibration
1069.6	1060.1	Carbonyl (C-O)	Tiny shape
671.03-460.32	999.3- 605.37	Asym.out of phase ring stretching	$\beta - Glycosid\ lingkage\ of\ cellulose$

3.3. Bio-delignification rate of OPF

The Klason method analysis of OPF showed that the acid-insoluble residue weighed 0.045g out of the total OPF powder weight of 0.2g. This led to a calculated lignin content of 22.5% (Table 3), which closely aligns with the established range of 13% to 25% reported by Shinoj et al. (2011) in their study on oil palm fiber and its composites. This consistency highlights that lignin content in this study is in line with existing research. Lignin's presence in OPF affects its structural integrity, making it important for digestibility and potential applications such as bioenergy and chemicals. The Klason method's hydrolysis process involves chemical reactions with cellulose and hemicellulose using an acid mixture (usually sulfuric and hydrochloric acids). This results in their breakdown into soluble products, including glucose and xylose, which are released into the hydrolysis solution. This step plays a crucial role in selectively measuring the lignin fraction, providing insights into biomass composition and potential uses. It aids in breaking down cellulose and hemicellulose, creating soluble products and forming acid-insoluble lignin residue, contributing to our understanding of biomass composition (Abdelrahman & Galiwango, 2018).

Table 3. Summary percentage of volatile solid (VS)

Analysis Method	**Value**
Acid-Insoluble Residue (g)	0.045
OPF Powder Weight (g)	0.2
Calculated Lignin Content (%)	22.5
VS of raw OPF	65%

The VS concentration in raw OPF before processing was calculated to be 65%. This number shows the amount of rapidly decomposable organic matter in the sample that might be used to generate biogas during anaerobic digestion. The VS content of OPF was significantly decreased thanks to the bio-delignification pre-treatment utilizing *Trametes polyzona*. Initial VS concentration in the samples was 65%, however after treatment it dropped by 25% in the treated samples. This decrease is indicative of the *Trametes polyzona* to degrade organic matter, including lignocellulosic components, during the pre-treatment phase of the process. The most significant decrease in VS content was observed in the samples treated with water and *Trametes polyzona*. The amount of VS in these samples was reduced by 35% as compared to the amount in the untreated samples. Decomposition and breakdown of organic waste in OPF were significantly improved by the combination treatment with water and *Trametes polyzona*. *Trametes polyzona* treatment alone resulted in a reduced fall in VS content (20%) compared to the combination treatment.

In a prior investigation conducted by Acharya (2015), the analysis of VS content revealed significant insights. The initial examination of raw OPF indicated a VS of 69.16%. However, upon subjecting OPF to torrefaction at 300 °C, a noticeable reduction in VS content was observed, with values decreasing to 45.54%. Significant consequences for anaerobic digestion arise from the decrease in VS

content achieved during bio-delignification pre-treatment. The higher digestibility and increased availability of substrate for microbial breakdown during anaerobic digestion is evidenced by the decrease in organic matter content, especially the volatile solids. This, in turn, can increase the methane content of the produced biogas and improve the efficiency of biogas production.

3.4. Gas identification by gas chromatography

Gas analysis using Gas Standard TCD Detection during the bio-delignification pre-treatment of OPF by *Trametes polyzona* alone revealed the presence of nitrogen gas (N_2), but methane (CH_4) was not detected as expected. The gas samples analysed showed nitrogen gas at a retention time of 7.767 minutes, as seen in Figure 5. According to the previous study by Bodelier and Steenbergh (2014), nitrogen is a fundamental nutrient required by methanogens for their growth and metabolic processes. Methanogens have mechanisms to acquire nitrogen either through the fixation of atmospheric nitrogen (N_2) or by actively taking up ammonium (NH_4^+) or nitrate (NO_3^-) from the surrounding environment (Cheng et al., 2022). The intricate interplay between the inhibitory and nutrient aspects of nitrogen in methanogenesis necessitates a delicate balance to ensure optimal anaerobic digestion. The presence of nitrogen in varying forms underscores the importance of managing nitrogen dynamics in anaerobic digestion systems. It is expected, after 30 days of BMP process, the degradation stage for OPF is still at early phase. A study by Suksong et al. (2017) demonstrated the efficiency of thermophilic solid-state anaerobic digestion for converting ground OPF into biogas. By reducing OPF particle size to less than 5 mm, Suksong et al. (2017) achieved a methane yield of 207 m^3 CH_4 $tonne^{-1}$ VS within 45 days of incubation. This highlights the smaller particle size facilitated faster degradation.

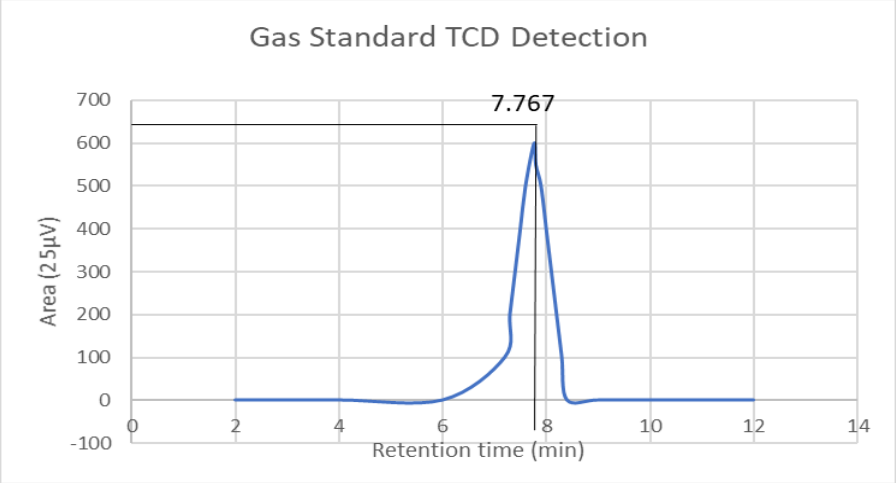

Fig. 5. The reading of Gas Standard TCD Detection

4. CONCLUSION

This study aimed to optimize pre-treatment conditions for OPF using *Trametes polyzona* by assessing the bio-delignification process in different conditions. The most significant bio-delignification were observed with the combination of water and *Trametes polyzona*. A reduced FTIR peak at 1726 cm^{-1} attributed to carbonyl (C=O) groups in lignin and hemicellulose after 30 days showed that bio-

delignification process is happening. This was supported by the reduction of VS content from 65% on day 0 to 35% after 30 days of incubation period. Methane production within 30 days was not achieved, likely due to the limited surface area of the OPF with size of 2 cm – 3 cm, which hindered the effective coverage of inoculation of the *Trametes polyzona.*

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