Nutritional improvement of oil palm and sugarcane plantation waste by solid-state fermentation of *Marasmiellus palmivorus*

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**Abstract.** The agricultural sector of Indonesia comprises large oil palm and sugarcane plantations. Nearly 50% of the material resulted in organic waste with high lignin and cellulose content during palm oil and sugarcane production. The lignocellulolytic activity of white-rot fungi has been reported to improve the digestibility and nutrient content of plantation waste into feedstock. This study treated lignocellulose waste, including palm kernel meal, oil palm empty fruit bunch, oil palm frond, sugarcane tops, and sugarcane pressmud with white-rot fungi solid-state fermentation system in a petri dish with two replicates. The growth of the fungus in each waste was observed. The Nutrient content, including water, ash, lipid, protein, crude fiber; and the digestibility, including crude and organic fiber digestion of fermented and unfermented waste, were measured. The molecular identification of white-rot fungi in this study was revealed to be *Marasmiellus palmivorus*. The result indicated that fermentation of *M. palmivorus* increased the protein level of sugarcane pressmud, palm kernel meal, oil palm frond, and empty fruit bunch. Fermentation also improved digestibility up to 12%. It is concluded that *M. palmivorus* has a high potential to enhance the digestibility and nutritive value of lignocellulose waste in oil palm and sugarcane plantations.

**Keywords:** white-rot fungi, lignocellulose waste, feedstock

1. **Introduction**

The plantation industry has proven to be one of the leading agricultural industries in Indonesia, including oil palm and sugarcane plantations. Indonesia recorded in National Statistics of production of 48,296,900 tons crude palm oil (CPO) in 2020. On the other hand, sugarcane plantation has less production nationwide, with 2,130,700 tons of sugar production in 2020 from 420,700 hectares of land. During palm oil and sugarcane production, nearly 50% of the material resulted in organic waste, with high content of lignin and cellulose. Lignocellulosic waste is abundantly available, composed of approximately 20–35% lignin, 20–40% hemicellulose, and 40–50% cellulose [1]. The oil palm industry produces a high quantity of agricultural waste and by-products, including empty fruit bunches (EFB) and oil palm fronds (OPF), which are often burnt and cause air pollution and other environmental problems.

Oil palm by-products are often burnt or used as organic fertilizer, which is costly and produces high greenhouse gases and other environmental problems [2]. Some of oil palm plantation by-products
include palm press fiber (PPF), empty fruit bunch (EFB), palm kernel meal (PKM), decanter cake (DC) of palm oil mill effluent (POME), whereas the other wastes from the plantation comprise of oil palm fronds (OPF) and trunk (OPT) which are produced during harvesting, maintenance, and replanting [3]. In comparison, sugarcane plantation produces molasses, bagasse, sugarcane tops, and pressmud. However, agricultural waste is often low in digestibility because of high fiber and lignin contents which are also low in feeding value due to low protein content [3]. Therefore, different approaches have been developed to enhance nutrient contents and the feeding value of agricultural by-products.

Solid-state fermentation (SSF) system is the preferred method to increase the digestibility of plantation by-products and enhance livestock growth [4,5]. For years SSF has gained interest in the bioconversion of agricultural by-products due to the simple and economical procedures. Different microbes such as bacteria, fungi, and yeast have been utilized in the SSF, but fungi were the preferred agency to degrade the agricultural by-products as being low in water content and the compatibility as a natural habitat to grow [6].

The utilization of white-rot fungi or the enzyme extract to increase the nutrient content of agricultural waste for feedstock production is considered a low-cost and eco-friendly alternative [7-10]. Previous studies reported that white-rot fungi significantly improved the quality and nutrient content of sugarcane bagasse and oil palm fronds [11,12].

Solid-state fermentation (SSF) using white-rot fungi has been utilized frequently. However, this method also faces several challenges, such as high dependency on the substrate content and fungal species or strain. Therefore, this study was conducted to identify the promising white-rot fungal strain to improve the availability of nutrients in palm kernel meal, oil palm empty fruit bunch, oil palm frond, sugarcane tops, and sugarcane pressmud in the solid-state fermentation system.

2. Materials and method

2.1. Materials

The white-rot fungus strain used in this study was isolated from oil palm empty fruit bunch in Indonesia. For maintenance, it was cultured in potato dextrose agar (PDA) medium and sub-cultured to potato dextrose broth (PDB) medium for DNA extraction and molecular identification. Fungal inoculum for solid-state fermentation was grown in autoclaved rice. This study’s oil palm plantation waste includes palm kernel meal, oil palm empty fruit bunch, and oil palm frond. Several pre-treatments for substrates involved separation and chopping. Palm kernel meal was separated from the remained shell, oil palm empty fruit bunch, and oil palm frond was chopped into a fiber. Sugarcane plantation waste was used in this study, including sugarcane tops and pressmud. Sugarcane tops were chopped into pieces. The fermentation took part in the Laboratory of Biochemistry, Indonesian Research Institute for Biotechnology and Bioindustry (IRIBB) in August-September 2020.

2.2. Molecular identification

The fungus was cultured in a PDB medium inoculated with a slice of the same strain grown in a PDA medium. The culture was incubated under sterile conditions at room temperature for 7 days. Genomic DNA source was obtained from the hyphae grown on top of PDB culture medium, washed with sterile aqua dest twice, and extracted using manual DNA extraction method modified from [13]. Around 100 mg fungal hyphae were resuspended in 500 μl pre-heated lysis buffer solution, 2% polyvinylpyrrolidone, 100 mM Tris-HCl pH 8.0, 25 mM Ethylenediaminetetraacetic acid pH 8.0, 2 M NaCl, and aqua dest. β-met was added into the lysis buffer-sample mixture before use and then vortexed for 10 min. The tube was incubated in a water bath at 65°C for 30 min and vortexed every 10 min. A 1xV of phenol:chloroform:isoamylalcohol (25:24:1) was added to the mixture and vortexed. The tube was centrifuged at 10,000 × g for 20 min at 4 °C. The clear phase was transferred into another tube. A 1xV volume of chloroform:isoamylalcohol (24:1) was added, vortexed, and then centrifuged. After centrifugation, the upper layer was transferred into a new tube and then precipitated with 0.1xV of 3 M CH3COONa and 2xV of absolute ethanol. The mixture was gently inverted several times and incubated at -20 °C for 1 hr. The tube was centrifuged at 10,000 × g for 30 min at 4 °C, and the
supernatant was discarded. The DNA pellet was washed with 700 µl of 70% ethanol and centrifuged at 10,000 × g for 30 min at 4 °C. Ethanol was discarded, and the pellet was air-dried. The DNA pellet was resuspended in 30 µl of NFW and kept at -20 °C. PCR mix formula for amplification is similar to the first method, the difference lied on the PCR program which performed by 1 µl was added into 14 µL of PCR Mix using MyTaqTM MIX from Bioline (Meridian Bioscience) following the formula of 5 µl master mix, 0.4 µl forward and reverse each, and 3.2 µl NFW. A set of forward primer ITS1 5’-TCC GTA GGT GAA CCT GCG G-3’ and reverse primer ITS4 5’-TCC TCC GCT TAT TGA TAT GC-3’ was chosen, which amplifies ~700 bp PCR product [14]. The genes were amplified by early denaturation of 95 ºC for 3 min, 30 cycles of denaturation at 95 ºC for 30 s, annealing at 54 ºC for 30 s, and elongation at 72 ºC for 1 min 30 s and final elongation at 72 ºC for 10 min. The PCR products were separated through 0.8% agarose gel electrophoresis and were sent for DNA sequencing (First Base, PT Genetika Science).

DNA sequence analysis was performed in Geneious Prime 2021.1.1 (https://www.geneious.com). Sequences were then assembled into bi-directional contig de novo, and the consensus sequence was created. The consensus sequence was BLAST-ed (Basic Local Alignment Search Tool) in NCBI tools integrated into the software. The percent identity and e-value were observed and compared from both treatments. The phylogenetic tree was obtained using the neighbor-joining method with Tamura-Nei distances and *Ganoderma boninense* ITS1-5.8S-ITS2 [MW866695.1] sequence was selected as the outgroup. Phylogenetic bootstrapping (1000 replicates) was implemented to assess relative support for branches [15].

2.3. Substrate preparation and fermentation method

Oil palm and sugarcane plantation waste, including palm kernel meal (PKM), oil palm empty fruit bunch (EFB), an oil palm frond (OPF), sugarcane tops (ST), and sugarcane pressmud (SP), were treated with white-rot fungal strain using solid-state fermentation system in a petri dish. For each substrate, three petri dishes were prepared for treatment (inoculated: 2 petri dishes) and control (un-inoculated: 1 petri dish). The substrates were set with water content at around 60% then incubated at room temperature for 30 days. The growth of the obtained fungi in each waste was observed and mixed into one sample for chemical analysis. In addition, the nutrient content, including water, ash, lipid, protein, crude fiber; and the digestibility, including crude and organic fiber digestion of fermented and unfermented waste, were measured. The chemical analysis was performed in Nutrition and Dairy Science Laboratory, Faculty of Animal Science, IPB University-Indonesia.

3. Result and discussion

3.1. Molecular identification

In this study, the fungal strain of white-rot fungus collected from empty fruit bunch in Laboratorium of Biochemistry, IRIBB, was identified using a molecular approach. Around 700 bp portion of the ITS1-5.8S-ITS2 flanked by ITS1/ITS4 primers were amplified, and agarose gel electrophoresis was performed. The visualization of the amplified ITS1-5.8S-ITS2 region as shown in figure 1.
Figure 1. Visualization of PCR amplification of fungal strain using the partial ITS1-5.8S-ITS2 region. DNA marker used is 1 kb DNA ladder, and the target PCR product amplified sequence of ~700 bp (PCR product size is lower than the arrow indicates the size is below 750 bp).

The fragment of the ITS1-5.8S-ITS2 region shown in the agarose gel was extracted and cleaned up for bi-directional DNA sequencing. Electropherograms of DNA sequencing read were trimmed by a 5% and assembled de novo to create an of ±723 bp consensus sequence and then BLAST-ed. The base sequence homology analysis showed that the fungal strain was identified as *Marasmiellus palmivorus* [JQ653438] with query cover of 100%, e-value 0, and a percent identity of 99.6%.

The construction of a phylogenetic tree was performed on ITS1-5.8S-ITS2 region sequences of 13 identified strains with the highest percent identity in BLAST analysis and other species for clade analysis. A total of 13 *M. palmivorus* strains from BLAST with 5 other species were aligned with the consensus sequence using Geneious Alignment Program. Phylogenetic tree construction was built using 19 sequences with Tamura-Nei genetic distance (NJ method), shown in figure 2. *Ganoderma boninense* [MW866695] was used for the outgroup.

Figure 2. Phylogenetic tree of ITS1-5.8S-ITS2 region from the white-rot fungus based on the BLAST result. The tree was constructed by the neighbor-joining (NJ) method. Bootstrap support values of 1000 replicate (%) presented at the nodes.
According to the phylogenetic tree using the ITS1-5.8S-ITS2 region, the fungal strain was grouped into *M. palmivorus* clade, separated with *Omphalina* clade and another species of *M. capilaris*. Based on the Genetic similarity matrix in Table 1, the fungal strain of interest in this study belongs to the same cluster with high genetic similarity with other *M. palmivorus* strains of over 98%. Therefore, the internal Transcribed Spacers region (ITS) became the most common DNA marker used in fungal identification [16]. The entire ITS region in fungi is about 600-800 bp in length and contains ITS1 and ITS2, separated by the highly conserved 5.8S rRNA gene [17]. In addition, the 18S, 5.8S, and 28S rRNA genes were highly conserved, and several sets of universal primer were designed to amplify the ITS1, ITS2, or the entire ITS region in fungi. Therefore, the ITS region was preferred because of the high discrimination ability towards closely related species and also provided high amplification and sequencing success rate in fungal strains. For these advantages, the ITS region has been formally accepted as the primary fungal barcode recommended by the International Fungal Barcoding Consortium [14].

The genetic analysis utilized ITS 1, and ITS 4 primers have previously been used for identification of white-rot fungi, including *Pleurotus albidus* (KF280332.1) with 99% identity, *M. palmivorus* (JQ653424.1) with 98% identity, *Trametes hirsuta* (KC414249.1) with 99% identity and *Pycnoporus sanguineus* (AF363759.1) with 99% identity [18]. White-rot fungi are named due to the ligninolytic activity to degrade wood. However, white-rot fungi of *M. palmivorus* were reported to cause bunch rot disease in oil palm trees in Malaysia [19].

### Table 1. Genetic similarity matrix among local the fungal strains

| Strain | M. palmivorus | M. capilaris | Omphalina | P. sanguineus | P. albidus |
|--------|---------------|--------------|-----------|---------------|------------|
| M. palmivorus | 100.00        | 98.85        | 86.42     | 86.42         | 86.42      |
| M. capilaris  | 98.85         | 100.00       | 79.58     | 79.58         | 79.58      |
| Omphalina    | 86.42         | 79.58        | 100.00    | 100.00        | 100.00     |
| P. sanguineus | 86.42         | 79.58        | 100.00    | 100.00        | 100.00     |
| P. albidus    | 86.42         | 79.58        | 100.00    | 100.00        | 100.00     |

### 3.2. Fungal culture and solid-state fermentation system

The fungal strain was originally cultured in a PDA medium for maintenance then sliced and transferred to autoclaved rice medium for inoculum production. The fungal inoculum was incubated for 2 weeks before being used for the solid-state fermentation system. The growth of fungal strain in PDA and rice medium was shown in figure 3.

The growth of *M. palmivorus* isolate was observed after 30 days of solid-state fermentation, and the picture showed in figure 4. The substrates, including OPF, ST, and EFB, were pre-treated by chopping into the size of nearly fiber to increase the surface area. The surface area was one of the important substrate properties which can improve the fungus growth during SSF [20]. Another research by [21] also reported enhanced growth of *Marasmius* sp. on chopped EFB in bio pulping pre-treatment using an SSF system.
Figure 3. Growth of *M. palmivorus* in (A) PDA medium and (B) rice medium

Figure 4. The growth of *M. palmivorus* in PKM (palm kernel meal), OPF (oil palm frond), SP (sugarcane pressmud), ST (sugarcane tops), and EFB (empty fruit bunch)

Based on figure 4, there was no significant difference of growth between replicates based on visual observation, and mycelia covered all the substrates used in this study. The growth of *M. palmivorus* in oil palm frond, empty fruit bunch, palm kernel meal, sugarcane tops, and pressmud indicate that the white-rot fungus is able to degrade the agro-industrial waste. Furthermore, the robust growth in all substrates without sterilization and supplementation of other carbon sources define the potential of *M. palmivorus* to break down lignin and cellulose and increase the digestibility of plantation by-products.

3.3. Changes in chemical composition
Solid-state fermentation using *M. palmivorus* strain was shown in Figure 4, with high fungus growth visible from the hyphae growth in all the substrates used in this study. The obvious chemical change can
be seen in the fat content, in which four out of five substrates showed a decrease of 8.81%. The SNI (Indonesian National Standard) for feedstock under the regulation by the Ministry of Agriculture of Indonesia 46/Permentan/PK.210/8/2015 stated that the fat content of feedstock for cattle is maximum of 7%. The highest fat content among all substrates is palm kernel meal with 10.56%, and solid-state fermentation by \textit{M. palmivorus} may decrease the content to 1.75%, which is approved by the regulation. Protein content showed the highest increment on the fermentation of PKM by 6.9%, followed by EFB with 2.76% increment, OPF and FP increase up to 1.02%, and only SP protein content slightly decrease of 0.21%. The slight changes of protein content on the SSF of OPF and ST may be influenced by a few changes with no replication in the analysis.

Organic matter digestibility showed to increase only on OPF, ST, and SP. However, organic matter degradation occurred in all substrates during SSF, indicated by an increment of VFA and NH$_3$ after the process. NH$_3$ concentration was one of the parameters to indicate protein degradation during fermentation. Meanwhile, carbohydrate was digested as an energy source and produced VFA and other organic acids as by-products. Therefore, after SSF, pH decreased into slightly acidic in all substrates. NH$_3$ concentration for \textit{in vitro} protein synthesis was optimum at 5–17.65 mM [22]. The VFA total in this study was sufficient for the fungus growth, indicated by the value is in the range between the normal condition of 60-120 mM [23]. Particularly in EFB, SSF by \textit{M. palmivorus} in this study improves VFA total into the normal condition.

| Table 2. Chemical composition and digestibility of untreated and fermented substrates |
|------------------------------------------|--------|--------|--------|--------|--------|--------|
| No. | Chemical content | PKM 1 | PKM 2 | OPF 1 | OPF 2 | SP 1 | SP 2 | ST 1 | ST 2 | EFB 1 | EFB 2 |
| 1   | Water (%)         | 9.63  | 6.14  | 2.93  | 6.44  | 5.58 | 6.4  | 7.79 | 7.67 | 5.55  | 7.70  |
| 2   | Ash (%)           | 7.51  | 5.62  | 12.38 | 15.28 | 47.12| 42.41| 14.03| 13.81| 9.60  | 7.00  |
| 3   | Fat (%)           | 10.56 | 1.75  | 0.79  | 0.66  | 1.71 | 1.16| 0.74 | 1.37 | 0.41  | 0.39  |
| 4   | Protein (%)       | 11.66 | 18.5  | 7.43  | 7.79  | 10.29| 11.31| 4.86 | 4.65 | 2.99  | 5.75  |
| 5   | Fiber (%)         | 16.16 | 20.85 | 33.83 | 29.78 | 8.8  | 10.76| 30.09| 28.74| 47.40 | 32.25 |
| 6   | DMD (%)           | 75.84 | 59.53 | 34.55 | 47.25 | 34.03| 40.32| 30.94| 40.94| 22.08 | 27.97 |
| 7   | OMD (%)           | 74.26 | 57.59 | 40.69 | 49.84 | 65.38| 66.99| 30.15| 45.23| 31.93 | 28.44 |
| 8   | pH                | 6.87  | 6.76  | 6.92  | 6.87  | 6.92 | 6.95| 6.85 | 6.88 | 6.79  | 6.90  |
| 9   | NH3 (mM)          | 9.88  | 15.15 | 10.68 | 12.66 | 9.62 | 11.52| 7.16 | 8.18 | 6.86  | 7.78  |
| 10  | VFA total (mM)    | 72.2  | 117.05| 75.93 | 93.47 | 79.9 | 95.73| 78.20| 116.81| 58.93 | 88.34 |

Note: DMD (dry matter digestibility), OMD (organic matter digestibility), volatile fatty acid (VFA) total, PKM (palm kernel meal), OPF (oil palm frond), SP (sugarcane pressmud), ST (sugarcane tops), and EFB (empty fruit bunch)

The growth of white-rot fungi \textit{M. palmivorus} caused a chemical change in the composition and digestibility of all substrates. This result indicates that the white-rot fungi used in this study were able to digest the substrate, utilize the materials for the growth, and degrade lignin and release cellulose to improve digestibility. A previous study by [18] reported the high potential of \textit{M. palmivorus} for delignification as it produces a high ligninolytic enzyme complex, including laccases, lignin, and manganese peroxidase [18,24]. Another research reported that the delignification process by the laccase-rich secretome of \textit{M. palmivorus} decreases the lignin content from 23% to 17%, allowing higher sugars yields after enzymatic hydrolysis eucalyptus wastes [25].

4. Conclusion

This research indicated that the \textit{M. palmivorus} strain improved nutrient content and digestibility of PKM, OPF, SP, ST, and EFB during solid-state fermentation systems. Therefore, it is concluded that \textit{M. palmivorus} has a particularly high potential as a pre-treatment of lignocellulose waste in oil palm and sugarcane plantations for feedstock production.
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